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SNPs Associated with β-Thalassemia Phenotypes Modification in the Palestinian Population

By
Abeer Mousa Zawahreh

In Partial Fulfillment of the Requirements for the Degree of
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ABSTRACT

Beta-thalassemias are hereditary genetic diseases of blood disorders. In these diseases the production of normal hemoglobin (Hb) is partly or completely suppressed as a result of mutations that lead to the defective synthesis of one or more of β-globin chains. Recent estimates accounts for 1.5% of the global population (80 to 90 million people) are carriers of β-thalassemia, with about 60,000 symptomatic individuals born annually; the great majority is in the developing world. Over 200 mutations of the β-globin gene on chromosome 11 can lead to reduced (β^+) or absent (β^0) synthesis of the β chains of hemoglobin with an autosomal recessive mode of inheritance. The clinical manifestations of β-thalassemias are extremely variable in severity; ranging from blood transfusion-dependent form of thalassemia major to the asymptomatic carrier state. Between the two extremes; thalassemia intermediate patients encompasses a wide spectrum of phenotypes without the need for regular transfusion. In many of these cases, the clinical variability can be explained either by co-existing point mutations or deletions characterized by a residual high β-chain production, coinheritance of α thalassemia, and through the influence of genetic modifiers that increase the production of γ-globin chain increasing HbF that has an ameliorating effect on the phenotype. Recently, genetic variants at the BCL11A locus (SNP rs11886868 C/T) located on chromosome 2p16.1, and the HBS1L-MYB intergenic region (SNP rs9389268 A/G) on chromosome 6q22-23 have been shown to be associated with milder β-thalassemia phenotype by increasing HbF production. The purpose of our study was genotyping of these two variants for thalassemic patients homozygous for the same mutation (IVS 1-6 T/C) by using PCR, RFLP, gel electrophoresis, and DNA sequencing techniques. Genotyping may help to predict the phenotype severity in newborns and accordingly, improve genetic counseling, appropriate clinical management, and effective treatment. Unfortunately, there was no statistical significant correlation between these variants and β-thalassemias

phenotypes, while HbF was high in mild phenotypes indicating that elevated levels of HbF contribute positively in ameliorating the clinical phenotype oβ-thalassemia.

Key words: SNPs, Hemoglobin, β-thalassemia, Mendelian autosomal recessive, β-globin gene, genetic modifiers, HbF, genotyping, PCR, RFLP, DNA sequencing, and gel electrophoresis.

التغيرات على القواعد النيتروجينيه وتأثيرها على الأنماط الشكلية لمرضى البيتا ثلاسيميا في الشعب الفلسطيني

عبير موسى زواهره

بيتا ثلاسيميا هو مرض وراثي من أمراض الدم. في هذا المرض، يكون إنتاج الهيموجلوبين الطبيعي في الدم ناقص، إما جزئيا أو كليا نتيجة وجود طفرات تؤدي إلى خلل في تركيب واحد أو أكثر من سلاسل جلوبين بيتا. بينت الاحصاءات مؤخرا أن ١,٥% من سكان العالم (٨٠ الى ٩٠ مليون شخص) حامل لصفة بيتا ثلاسيميا، مع حوالي ٦٠,٠٠٠ شخص تظهر عليه أعراض المرض، والغالبيه الساحقه في الدول الناميه. أكثر من ٢٠٠ من الطفرات في جين جلوبين بيتا على كروموسوم ١١ تؤدي الى خفض (بيتا$^{+}$) أو غياب (بيتا^{0}) تصنيع سلاسل بيتا في الهيموجلوبين. الأعراض المرضيه للبيتا ثلاسيميا متغيرة من حيث الخطورة; تختلف من الأكثر خطورة، والتي تحتاج إلى نقل متكرر للدم، إلى من لا تظهر عليه أعراض المرض (حامل للمرض)، وأخرى وسط بين النقيضين؛ إذ تظهر تغيير في الأعراض ولكن دون الحاجة المتكررة لنقل الدم. في كثير من هذه الحالات، يمكن تفسير التباين في أعراض المرض إما عن طريق وجود طفرات (تغيير أو حذف) في أحد القواعد النيتروجينيه في جين بيتا والتي تبقي من إنتاج عالي لسلاسل البيتا المتبقية، وجود مشترك لطفرة الفا ثلاسيميا، أو وجود محسنات وراثية والتي تزيد من إنتاج سلاسل جاما مما يؤدي إلى زيادة إنتاج هيموجلوبين F (HbF)، والذي له دور في تحسين أعراض المرض. ثبت مؤخرا أن المتغيرات الجينيه في جين BCL11A على كروموسوم ٢p16.1 (SNP rs11886868) ومنطقة بين الجينات HBS1L-MYB على كروموسوم ٦q22-23 (SNP rs9389268) أنها تترافق مع شدة الأنماط الشكلية لمرض بيتا ثلاسيميا عن طريق زيادة إنتاج هيموجلوبين F. إن الغرض من هذه الدراسه هو تشخيص البنية الوراثية لهذه المتغيرات في جينات مرضى ثلاسيميا مصابين بمتماثلة طفرة IVS1-6 T/C باستخدام PCR, RFLP, Gel electrophoresis, وتقنية DNA sequencing . قد تساعد البنية الوراثيه في التنبؤ بشدة خطورة المرض في حديثي الولاده، ووفقا لذلك، تحسين المشورة الوراثية والعلاج الفعال. ولكن لسوء الحظ، لم يكن هناك علاقة ذات دلالة إحصائية بين هذه المتغيرات وظواهر بيتا ثلاسيميا، في حين أن إرتفاع مستوى HbF تساهم مساهمة إيجابية في تحسين الأنماط الشكلية لمرضى بيتا ثلاسيميا.

DECLARATION

I declare that the Master Thesis entitled " SNPs Associated with β-Thalassemia Phenotypes Modification in the Palestinian Population" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: Abeer Mousa Zawahreh 

Date 4/5/2011

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Dedication

This thesis is dedicated to:

To my family and my husband for their love, support, and believing in me, in addition to their continuous encouragement for me to continue higher education.

To my lovely daughters for their tolerance of my frustration and impatience when I wanted to study and keep them away from me.

To my son who took the responsibility of his sisters when I was in the University, thank you dear.

To my supervisor for his standing by my side through the research steps.

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Abbreviations

β-thalassemia	Beta thalassemia
MCV	Mean Corpuscular Volume
α- ε- γ- δ	Alpha-Epsilon- Gamma- Delta
RBCs	Red Blood Cells
HbF	Fetal Hemoglobin
HbA	Adult Hemoglobin
HbA_2	Minor Adult Component
TM	Thalassemia Major
TI	Thalassemia Intermediate
WHO	World Health Organization
DNA	Deoxyribonucleic acid
IVS	Intervening Sequences
SNPs	Single Nucleotide Polymorphisms
HBA	Hemoglobin Alpha gene
HBB	Hemoglobin Beta gene
LCR	Locus Control Region
QTL	Quantitative Trait Locus
USDA	United States Department of Agriculture
NCBI	National Center for Biotechnology Information
UCSC Genome Browser	University of California, Santa Cruz (UCSC) Genome Browser
GWAS	Genome Wide Association Study
cDNA	Complementary DNA
PCR	Polymerase chain reaction
NTC	Non Template Control
RFLP	Restriction Fragment Length Polymorphism
GTP	Guanosine triphosphate
PGD	Prenatal Genetic Diagnosis
HRL	Hereditary Research Lab
ANOVA	analysis of variance
EtBr	Ethidium Bromide
MboII	*Moraxella bovis* second enzyme isolated
Aci I	*Arthrobacter citreus* first enzyme isolated

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CHAPTER 1

Introduction

β-thalassemia is an inherited, autosomal recessive disease of faulty synthesis of hemoglobin. The name is derived from the Greek word, thalassa meaning "the sea" and hemia meaning "blood" in reference to anemia of the sea, because the condition was first described in populations living near the Mediterranean region, and in portions of Asia, Africa, the South Pacific, and India (Galanello & Origa, 2010).

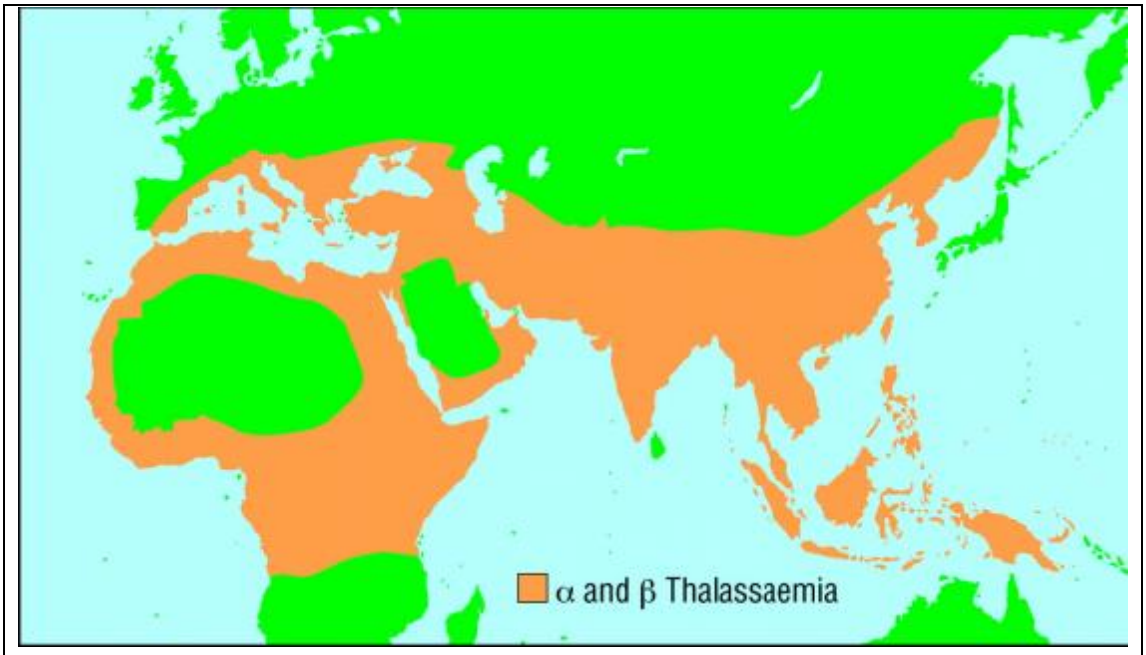


Figure (1): Prevalence of β-thalassemia.
From: Weatherall, (1997)

"β-thalassemia is prevalent in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and the Far East as well as countries along the north coast of Africa and in South America. The highest carrier frequency is reported in Cyprus (14%), Sardinia (10.3%), and Southeast Asia. The high gene frequency of β-thalassemia in these regions is most likely related to the selective pressure from *Plasmodium falciparum* malaria". Recent estimates accounts for 1.5% of the global population (80 to 90 million people) are carriers of β-thalassemia, with about 60,000 symptomatic individuals born annually, the great majority in the developing world (Galanello & Origa, 2010).

β-thalassemia is widespread in the West Bank and Gaza areas, a study conducted by Younis, *et al*. (1994) showed 23.6% carrier frequency among the west bank students of higher education. While 9734 of the total screened students, 304 were having elevated level of HbA2 (3.0%) with low MCV (< 79fL); hence the frequency of β-thalassemia trait was 3.13%. According to the Society of Thalassemia Patients Friends in Ramallah (n.d.), the carrier frequency in the Palestinian Population is 4.3% that equals to 100-120 thousand persons.

Hemoglobin is the substance that carries oxygen and gives blood its red color. It is made up of heme (i.e the porpherin ring) and globin (i.e the protein chains that surround the heme complex).

There are four types of hemoglobin (Bank, *et al*., 1980):

1) Embryonic hemoglobins; Gower 1 $\zeta_2\varepsilon_2$, Gower 2 $\alpha_2\varepsilon_2$, and Portland $\zeta_2\gamma_2$ those are detectable from the 10th to the 12th week of gestation.
2) Fetal hemoglobin (HbF, $\alpha_2\gamma_2$); predominant oxygen carrier during pregnancy.
3) Adult hemoglobin (HbA, $\alpha_2\beta_2$); that replaces HbF shortly after birth.
4) A minor adult component (HbA2, $\alpha_2\delta_2$).

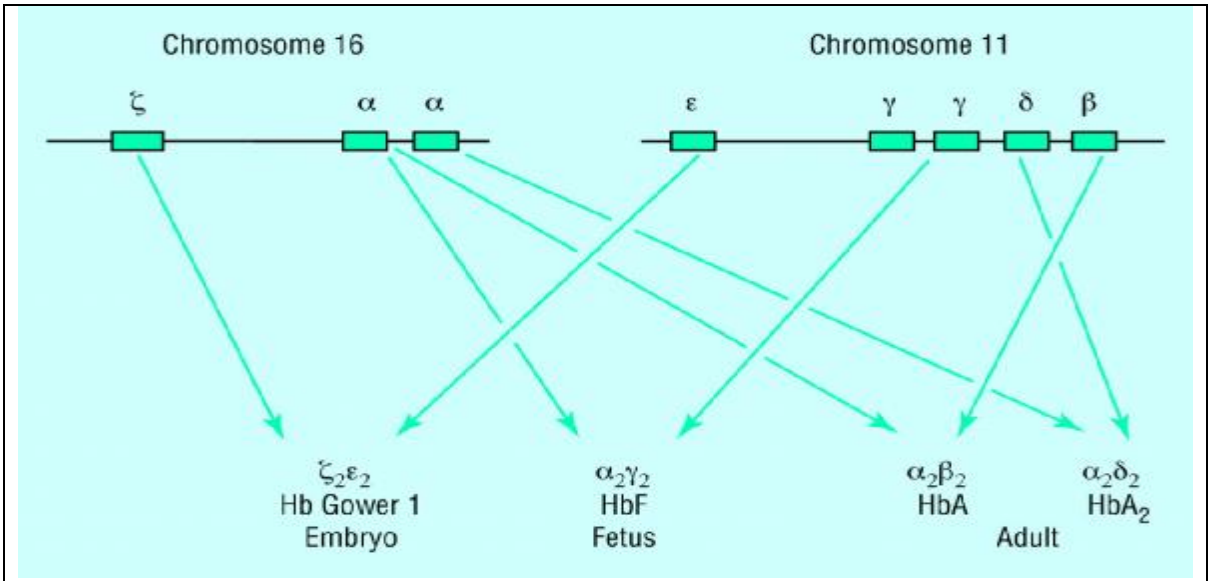


Figure (2): Types of Hemoglobin.
From: Weatherall, *et al*., (1997)

In normal conditions, RBCs contain 98% HbA, 2% HbA2, and traces of HbF (< 0.6%) (Aessopos, *et al*., 2007, Menzel, *et al*., 2007).

1.1 Classification

Clinical presentation ofβ-thalassemia has three main forms according to severity:

1.1.1 Thalassemia Major (TM): referred to as Cooley`s anemia and Mediterranean anemia (the severe disease). observed between 6 to 24 months of age (Galanello & Origa, 2010), a baby looks normal, but toward the end of the first year, he or she experiences slower growth, loss of appetite, pale skin, certain bones of the face may become prominent, energy diminish, anemia is found and iron treatment fails, a lifelong blood transfusions are required figure (3), and are generally continued throughout life. Organ failure plus endocrine complications appears when iron-overload is not treated with chelating therapy. Although there is a treatment, it is not satisfactory and there is no cure. It does not cause mental retardation, but is a severe physical handicap. On the overall, life span is reduced (division of genetics, 2009,Martinez, *et al*., 2007, Uda, *et al*., 2007a).

Figure (3): β-thalassemia transfusion dependent child.

From: Cooleys anemia foundation, (n.d.)

1.1.2 Thalassemia intermedia (TI): does not or occasionally require blood transfusion, the severe form presents late at age 2 and 6 years, capable of surviving without regular blood transfusion but their growth and development are retarded. The other spectrum of patients is asymptomatic until adult life with only moderate anemia. They suffer from the complications of reduced availability of β chains in hemoglobin including bone deformities and splenomegaly (Galanello & Origa, 2010).

1.1.3 Thalassemia minor, also named β-thalassemia carrier, β-thalassemia trait, or heterozygous β-thalassemia: clinically are asymptomatic; usually people are unaware of having the condition, but sometimes have a mild anemia, some cases, heterozygous β-thalassemia may lead to the thalassemia intermedia phenotype instead of the asymptomatic carrier state due to excess functional alpha globin genes (triplication or quadruplication) which increases the imbalance in the ratio of alpha/non-alpha globin chain synthesis (Galanello & Origa, 2010).

Classification between thalassemia intermedia and thalassemia major can be confusing; the borderline between them is blood transfusions; regular transfusions are likely to be classified as thalassemia major (Cooley`s Anemia Foundation, n.d.). Known genetic modifiers were identified and are able to modify the severity of the homozygous β^0thalassemia such as polymorphism in BCL11A gene located on chromosome 2p16.1, and in the HBS1L-MYB (intergenic region) located on chromosome 6q22-q23.3 unlinked to β-globin gene cluster (Galanello & Origa, 2010).

1.2 Inheritance of β-thalassemia:

The disease is inherited in a Mendelian recessive manner, and when a normal individual marries a carrier, half their children will be wildtype and half carriers figure (4.a).

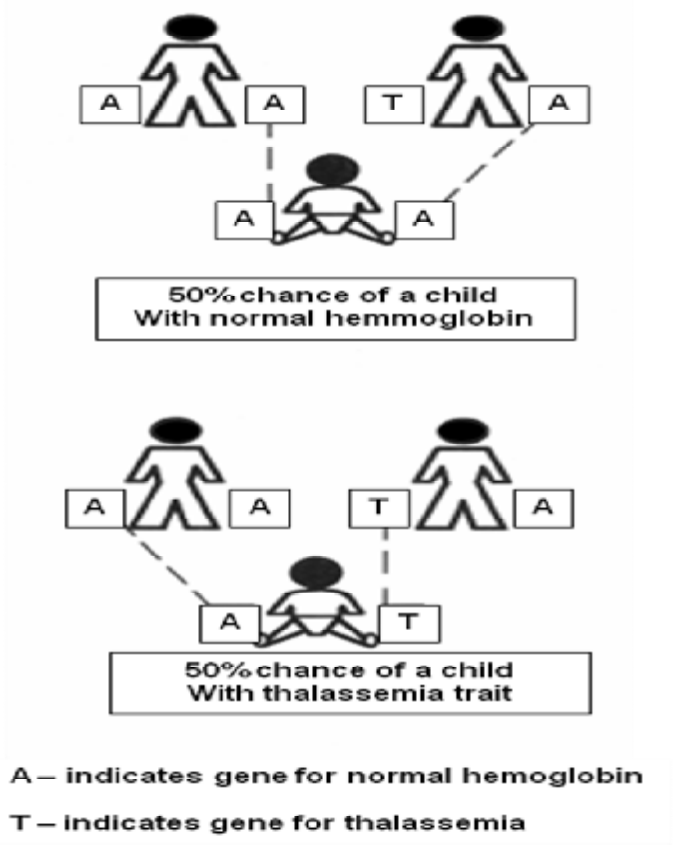


Figure (4.a): Mode of inheritance of β-thalassemia of a carrier and a normal parents. From: Division of genetics, (2009).

If two carriers marry, there is a 50% chance to pass thalassemia alleles in each pregnancy to their child, 25% chance of thalassemia major, and 25% completely normal child. Male or female get the disease equally (division of genetics, 2009) figure (4.b).

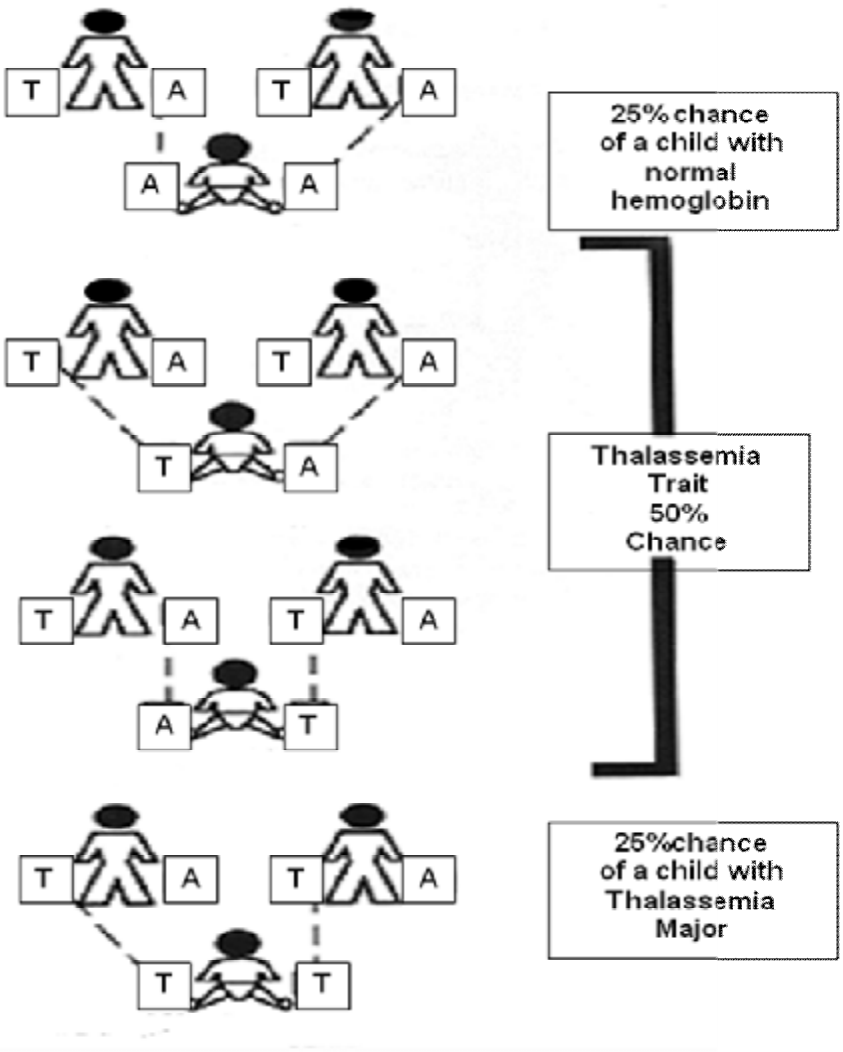


Figure (4.b): Mode of inheritance of β-thalassemia of two carrier parents

From: Division of genetics, (2009).

1.3 Genetics of β-thalassemia:

Normal hemoglobin vs. thalassemia: HbA (normal adult hemoglobin), is composed of two alpha (α) and two beta (β) polypeptide chains, there are two copies of the hemoglobin alpha gene (HBA1 and HBA2), each encode α-chain, and both genes are located on chromosome 16. The hemoglobin beta gene (HBB) encodes the β-chain and is located on chromosome 11.

Normally, α and β globin chains are made in roughly equal amounts figure (5).

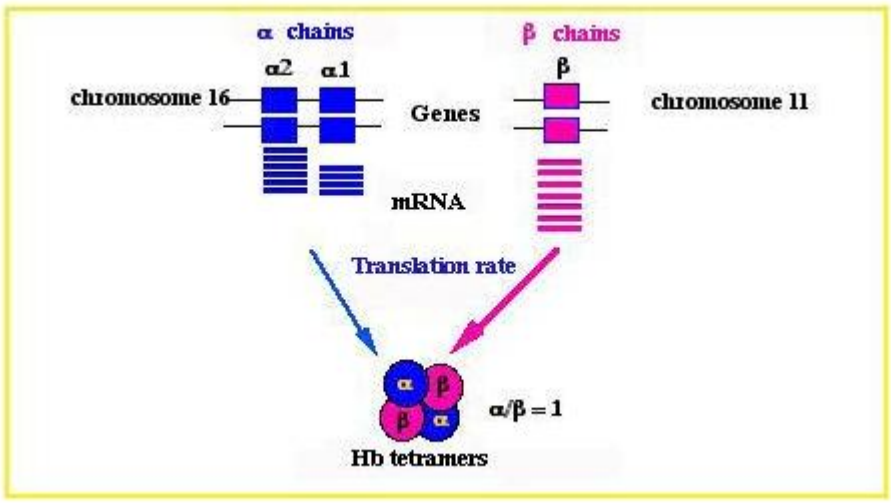


Figure (5): Globin chains components of normal adult hemoglobin HbA ($\alpha_2\beta_2$)
From: Wajcman, (n.d.).

When β-globin chains are in short supply or absent, this lead to imbalance of globin chains available for hemoglobin dimmer construction, andα-chains are in excess. The excess α-chains are highly unstable;"they precipitate in the bone marrow erythroid precursors causing membrane damage and cell death. This ineffective erythropoiesis is the principal determinant of anemia" (Galanello & Cao, ١٩٩٨) figure (6).

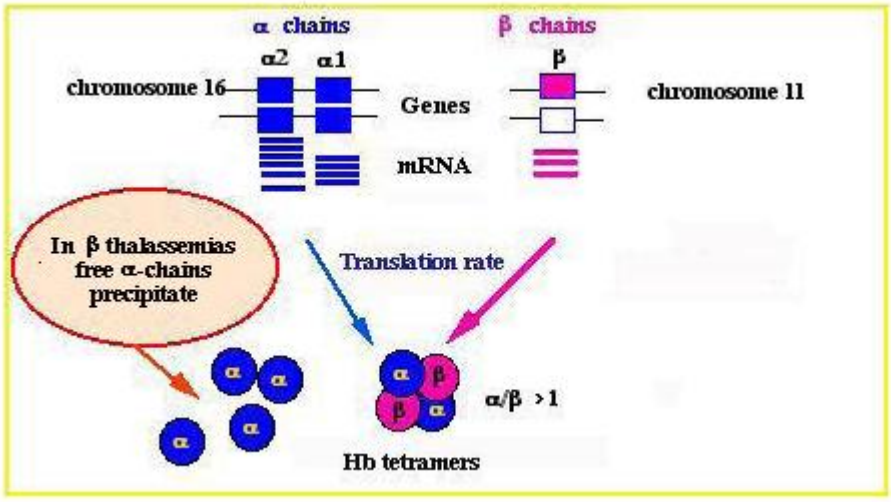


Figure (6): β-thalassemia genetics.
From: Wajcman, (n.d.).

1.4 Normal Beta Gene:

The order of the genes in the β-globin cluster is: 5'-epsilon -- gamma-G -- gamma-A -- delta -- beta--3'. The cluster is located on chromosome 11p15.5, and contains 5 functional genes presented in the same order in which they are expressed during development. The β–locus control region (β–LCR) is a major regulatory element located 6-20bp far upstream from epsilon gene. It is necessary for the high level of expression of those genes figure (7.a) (Strachan & Read, 2004, p.300).

During fetal life, Hb F (α2γ2) is the predominant type of hemoglobin. Fetal switching refers to the developmental process that "leads to the silencing of γ-globin gene expression and the reciprocal activation of adult β-globin gene expression. This results in the replacement of Hb F by Hb A (α2β2) as the predominant type of hemoglobin in adult life" figure (7.c) (Frenette & Atweh, 2007).

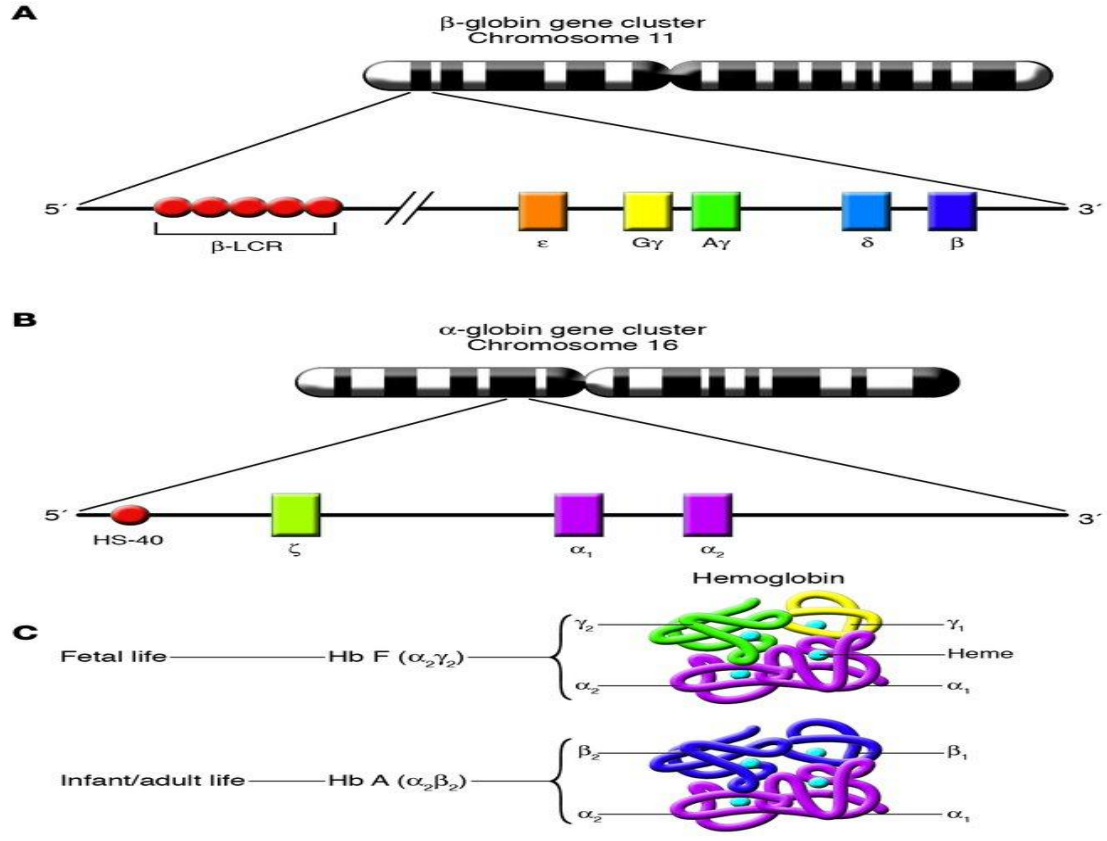


Figure (7): beta-globin gene cluster.

From: Frenette & Atweh, (2007).

1.5 β-Thalassemia mutations:

Over 200 mutations affect one or both of the β-globin genes. Deletion mutations are rare; the majority of mutations are point mutations in important functional regions of the β globin gene (Galanello & Origa, 2010).

Splice mutations and those that occur in the promoter region tend to cause a reduction, rather than a complete absence of β-globin chains and thus result in milder disease. Nonsense mutations and frameshift mutations tend not to produce any β-globin chains leading to severe disease (loss of function) figure (8).

The severity of anemia caused by β-thalassemia depends on which mutations are present and on whether they decrease beta globin production (called $beta^{+}$ thalassemia) or complete elimination (called $beta^{0}$ thalassemia) figure (9).

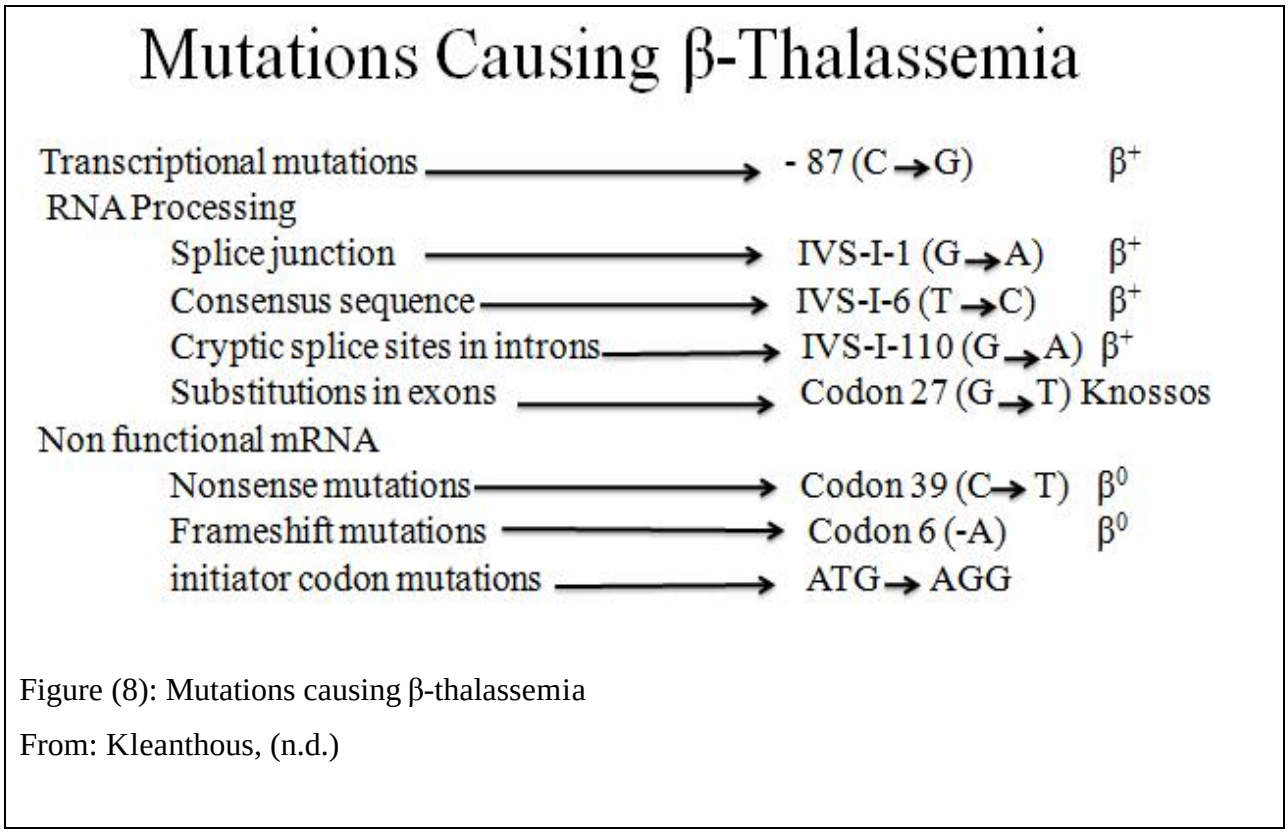


Figure (8): Mutations causing β-thalassemia

From: Kleanthous, (n.d.)

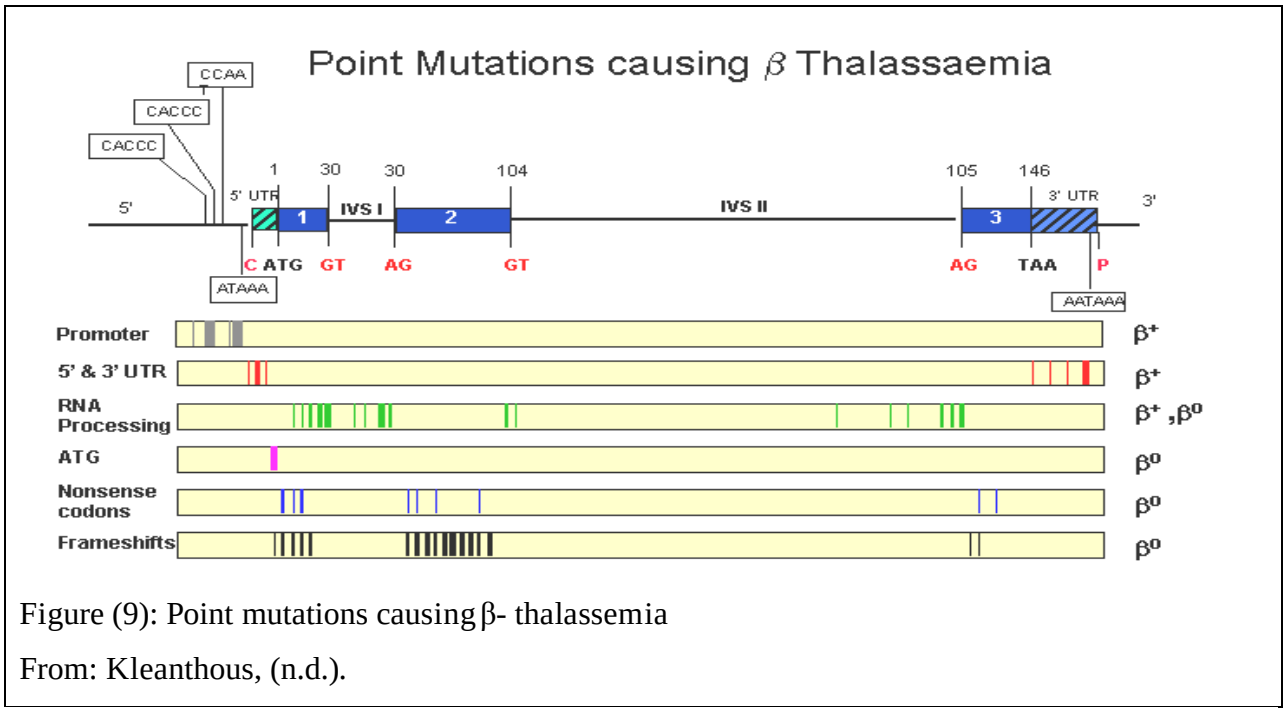


Figure (9): Point mutations causing β- thalassemia

From: Kleanthous, (n.d.).

Table (1): Common mutations reported according to severity and ethnic groups

Population	β-gene mutation	Severity
Indian	-619 del	β^0
Mediterranean	-101 C→T	β^{++}
Mediterranean; African	-87 C→G	β^{++}
Japanese	-31 A→G	β^{++}
African	-29 A→G	β^{++}
Southeast Asian	-28 A→C	β^{++}
Mediterranean; Asian Indian	IVS1-nt1 G→A	β^0
East Asian; Asian Indian	IVS1-nt5 G→C	β^0
Mediterranean	IVS1-nt6 T→C	$\beta^{+/++}$
Mediterranean	IVS1-nt110 G→A	β^+
Chinese	IVS2-nt654 C→T	β^+
Mediterranean	IVS2-nt745 C→G	β^+
Mediterranean	codon 5 -CT	β^0
Mediterranean; African-American	codon 6 -A	β^0
Southeast Asian	codon 41/42 -TTCT	β^0
African-American	AATAAA to AACAAA	β^{++}
Mediterranean	AATAAA to AATGAA	β^{++}
Mediterranean	codon 27 G→T Hb (Hb Knossos)	β^{++}
Southeast Asian	codon 79 G→A (Hb E)	β^{++}
Mediterranean	codon 39 CTT	β^0

β^0:complete absence of beta globin on the affected allele

β^+:residual production of beta globin (around 10%)

β^{++}:very mild reduction in beta globin production

From: Galanello & Origa, (2010).

1.6 IVS 1-6 mutation:

IVS1-6 (T → C) mutation is one of the most common causes of β-thalassemia in the Mediterranean and the Middle East descent. This mutation interferes with mRNA splicing and results in reduced expression of β-globin chains (β^+-thalassemia) (Waye, *et al*. 1995, Galanello & Origa, 2010). Rosatelli, *et al*. (1992) found that IVS1-6 mutation is a mild mutation, because a less marked decrease of the red cell volume and Hb content per cell as compared to the other β-thalassemia mutations investigated indicating that mild mutations may produce a mild phenotype. Also, they found that mild hematological characteristics of heterozygotes for mild mutations (IVS1-6 or β -87) as compared to carrier of β^0-thalassemia or severe β^+ thalassemia are certainly related to the residual relatively high output of β-globin chains from the affected locus. "The IVS-I-6 mutation is of moderate severity, allowing normal splicing to occur in a portion of the β-globin transcripts" (Abd El-Latif, *et al*. 2002).

1.7 Genetic modifiers

Genetic modifiers are defined as: "genetic variants that lead to differences in disease phenotype affecting the clinical severity of the phenotype, by reducing the globin chain imbalance, resulting in a milder form of thalassemia" (Waye, et al. 1995, Galanello & Origa, 2010).

They include:

1. The coinheritance of α thalassemia.
2. Co-existing point mutations or deletions characterized by a residual high β-chain production.
3. Recently, the genome wide association study revealed genetic elements that increase the production of HbF; such as polymorphism in BCL11A gene (rs11886868 C to T) and in the HBS1L-MYB intergenic region (rs9389268 A to G) unlinked to beta globin gene cluster, that are able to modify the severity of β-thalassemia.

1.8 Literature Review

Geneticists and hematologists have been interested in β-thalassemia for decades. Several studies have been recognized the importance of such disease for the increased widespread throughout the world, and not confined to a particular region.

Being a global public health problem, this is due to the high rate of migration from Mediterranean basin to "USA, Canada, Australia, South America, the United Kingdom and France, where migration occurred up to a century ago and where large ethnic minority groups are now entering their fourth and even fifth generation" (Atweh, *et al*.,1987 & Aessopos, *et al*., 2007).

The disease causes anemia of variable severity which becomes manifest in early childhood. Most patients have the severe form referred to as thalassemia major, characterized by lifelong transfusion dependency. However, in about 10-20% of homozygous patients, the clinical phenotype is less severe and the anemia does not require (regular) transfusion treatment (thalassemia intermedia) (Colah,*et al*., 2004).

Waye, *et al*. (1995) found that the co-inheriance of α-thalassemia was correlated with some mild-transfusion independent phenotype, while in the remaining families there was no correlation, suggesting the presence of other genetic determinants that ameliorate the phenotype.

By genome-wide association scan, it has been found inβ-thalassemia patients with elevated levels of HbF, there were Quantitative trait loci (QTL) contributed in ameliorating the clinical phenotype of these patients. QTL"are loci, or locations, on chromosomes for genes that govern a measurable trait with continuous variation, such as height, weight, or color intensity" (United States Department of Agriculture [USDA], 2008).

By genome-wide association mapping strategy applied by Menzel,*et al*. (2007a) to individuals with elevated levels of HbF, they mapped an F cell QTL to a region on chromosome 2p15 included the BCL11A gene. The 2p15 QTL accounted for 15.1% of HbF variance. The strongest associations were in a region spanning 14 kb in the second intron of the BCL11A gene. The second association cluster spanned

67 kb in the 3-prime region of the gene downstream of exon 5 of the BCL11A. The molecular mechanism of HbF reactivation is not fully understood, but when HbF elevated levels interact with β-thalassemia, it shows a milder phenotype in severity of these patients.

BCL11A gene refers to "B-cell CLL/lymphoma 11A" encodes a C_2H_2 type zinc finger protein, located on chromosome 2p16.1 figure (10). During hematopoietic cell differentiation, this gene is down-regulated (National Center for Biotechnology Information [NCBI], 2010), implicated previously in myeloid leukemia and lymphoma pathogenesis (Menzel, *et al*. 2007a). This gene is required for B-cell formation in fetal liver. Genetic variation of BCL11A is associated with quantitative variation in the production of F cells (erythrocytes containing measurable amounts of fetal hemoglobin HbF) (UCSC, 2009).

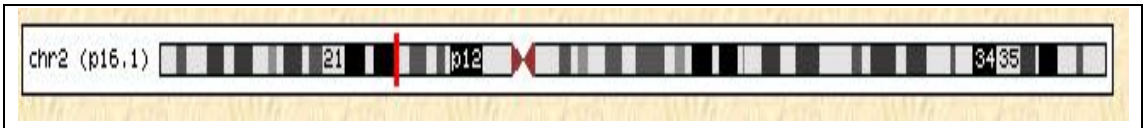


Figure (10): BCL11A gene location on chromosome 2p16.1.
From: UCSC Genome Browser on Human (2009).

According to Menzel, *et al*. (2007a), BCL11A found to be expressed in a variety of tissues, including erythroid cells and shown that BCl11A is essential for early lineage commitment in the development of T and B cells.

DNA analysis of SNP rs11886868 (C to T) in BCL11A gene was tested by Restriction Fragment Length Polymorphism (RFLP) digest of a PCR fragment, discussed later in the materials and methods.

Uda, *et al*, (2007) found by genome-wide analysis conducted on 4,305 Sardinian individuals, a significant association between the BCL11A gene and hepatocellular persistent fetal hemoglobin. The strongest association was with the C allele of a SNP rs11886868 in intron 2 of the BCL11A gene. The C allele was associated with an ameliorated phenotype in patients with β-thalassemia and sickle cell anemia, indicating that SNPs in the BCL11A gene may modify these phenotypes by increasing HbF levels.

Sankaran, *et al*. (2008) tested the expression of the BCL11A locus in erythroid cells, a multi–zinc finger transcription factor, which encodes a stage-specific regulator of HbF expression through its involvement in control of the γ-globin genes. They observed that "cells homozygous for the "high-HbF" allele expressed a lower level of BCL11A transcripts than those homozygous for the "low-HbF" allele or heterozygous for both alleles. The difference in expression between the "high" and "low" HbF-associated BCL11A alleles is 3.5 fold. Hence, relatively modest differences in BCL11A expression appear to be associated with changes in HbF expression".

HBS1L gene encodes a member of the GTP-binding elongation factor family. Located on chromosome 6q23.3 figure (11), and found to be expressed in various tissues with the highest expression in heart and skeletal muscle. DNA polymorphisms at this region associated with fetal hemoglobin levels and pain crises in sickle cell disease (Lettre, *et al*., 2008). A single nucleotide polymorphism in exon 1 of this gene is significantly associated with severity inβ-thalassemia/Hemoglobin E. Multiple alternatively spliced transcript variants encoding different protein isoforms have been found for this gene (NCBI, 2010).

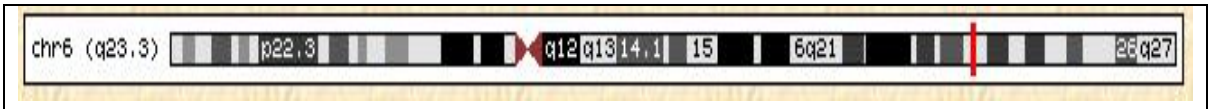


Figure (11): HBS1L gene location on chromosome 6q23.3.
From: UCSC Genome Browser on Human (2009).

MYB gene encodes a transcription factor that is important in the regulation of hematopoiesis (NCBI, 2010). This gene is located on chromosome 6q22-q23 figure (12). Major site of expression include: bone marrow, colon, leukocyte, and hematopoietic stem cells (Human Protein Reference Database, n.d.).

The intergenic region of HBS1L gene and the MYB genes have been identified to be QTL, and have a role in controlling fetal hemoglobin level (Menzel, *et al*., 2007b , Nancy, 2009).

DNA analysis of SNP rs9389268 (A to G) in this intergenic region in our population was tested by RFLP digest of a PCR fragment.

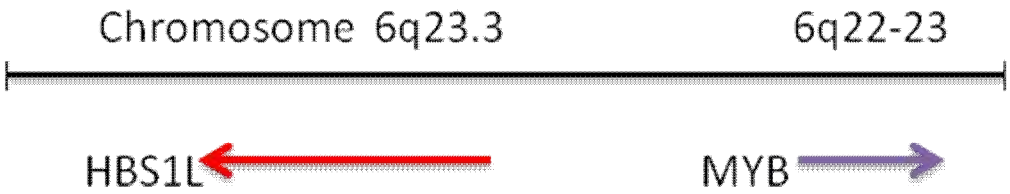


Figure (12): The location of HBS1L-MYB intergenic region

From: National Center for Biotechnology Information [NCBI], 2010.

CHAPTER 2

Objectives

2.1 Overall objectives

Even though the clinical severity of β-thalassemia is variable, most patients are β-thalassemia major depending on transfusion and will suffer from related transfusion complications of iron overload and viral infection. On the other hand, β-thalassemia major is considered a socioeconomic burden, due to the pain and suffering of the affected families and the society. The WHO calculated the annual cost of US $10,000 per patient (Younis, et al., 1994).

β-thalassemia severity is determined by the degree of imbalance in globin-chain synthesis; the absence or reduced production of β-globin leads to excess of α-chains, any factor able to reduce the degree of globin imbalance may produce milder phenotype (Galanello & Cao, 1998).

This study was designed to investigate the variable severity of anemia caused by β-thalassemia patients who have been previously tested and found to be homozygous for (IVS 1-6 T ⟶ C) mutation. This mutation affects position 6 of the splice consensus sequence of intron 1 of the β-globin gene.

The clinical variability can be explained either by a high residual activity of the β-globin gene itself due to the inheritance of mild β-thalassemia alleles, by coinheritance of α-thalassemia, or by genetic modifiers that increase the activity of the γ-globin genes (HbF) (Galanello & Cao, 1998).

2.2 Specific objectives

Screening for these modifiers to correlate between the genotype and the phenotype in our population, which is important to help proper diagnosis in early infancy before onset of symptoms appear, and to offer the parents genetic counseling for the complications associated with severe anemia if not treated properly.

The Palestinian population has a high rate of consanguinity of 50% (Gayth, 2004), so it's important to have genetic tests demanded by law before marriage for carriers to help them manage for the disease to decrease the number of thalassemia major patients.

This study tried to find answers to the following questions:

1. Is there a significant correlation between genotype and phenotype for the people homozygous with IVS 1-6 mutation when genotyped at:

    a. SNP rs11886868
    b. SNP rs9389268

2. Dose elevated levels of HbF ameliorate the clinical phenotype?

CHAPTER 3

Methodology/Materials and Methods

Molecular genetics studies are commonly reasonable to confirm the diagnosis, prognosis, and to be able to offer comprehensive genetic counseling to family members. It is also necessary when prenatal diagnosis is required by the family to avoid the birth of another affected baby in future pregnancy.

The molecular investigation usually confirms the phenotypic findings, but in some cases, phenotypes can be misleading. The classification of genotype- phenotype correlation of subjects is particularly important in the diagnostic steps of β-thalassemia, as well as familystudies.

3.1 Design

The design was based on biotechnological PCR based assays such as RFLP and DNA sequencing because of its availability.

3.2 Setting

This study was conducted at Bethlehem University Hereditary Research Laboratory (HRL), Science Faculty.

3.3 Sampling

The sample included extracted DNA from 75 patients homozygous for the same mutation IVS1-6 previously genotyped in an earlier study conducted at HRL.

Clinical phenotype classification of the patients was evaluated using a set of criteria for grading system. The severity of the disease is usually measured by the age of diagnosis, age of first regular transfusion, transfusion frequency, splenectomy and age of splenectomy.

Table (2): Grading criteria.

Group I - Severe:

1. Age at diagnosis < 3years
2. Received first transfusion at age 2 years or under.
3. Receiving regular transfusions at a rate of one every 1-3 month or more frequently (shorter interval).

Group II – Mild:

1. Age at diagnosis 3years
2. Received first (if any) transfusion at age 5 year or over.
3. Never or rarely transfused (<1/yr) until adulthood (for women, during pregnancy which may precipitate lifelong need for transfusion).
4. Baseline Hb without transfusion: minimum 7.
5. Splenectomy, if required, was performed in adulthood (over age 15).

Group III – Intermediate:

All the patients milder than Group I and more severe than Group II.

3.4 Instrument:

In this study, these instruments were used:

1. Gene Amp thermal cycler (PCR system 9700).
2. Agarose Gel Electrophoresis (Mini SubCell GT®) from Bio-Rad Laboratories.
3. Gel Documentation System (Syngene Corporation).
4. Sanger Sequencer Machine ABI PRISM® 3130 DNA analyzer.
5. NanoDrop ® instrument.
6. Analytical balance.
7. Microwave.
8. Centrifuge
9. Vortex
10. Multichannel pipettes

3.5 Data Collection:

In this study all β-thalassemia patients were selected from National Center for Blood Diseases Hippocrates in Ramallah, from districts in the West Bank.

The files which included the past history of the cases were used to include laboratory parameters such as: Hb level, HbA2 level, HbF level, and MCV in the statistical analysis (Appendix 1).

3.6 Ethical Considerations:

To ensure the subjects confidentiality, each subject was assigned a code number. The data collection tools were identified only by the code number. A subject list that included the patient code number, subject name, date of birth, age at diagnosis, age of first transfusion, transfusion requirements, age at splenectomy, and hematological laboratory tests were kept in a locked filing cabinet in the lab office.

3.7 Method of Data Analysis:

The data analysis procedure of this study included both descriptive and inferential statistics. The descriptive statistics included mean, and standard deviation. The statistical test used was one way ANOVA, LSD for multiple comparison, independent sample t-test, and Chi-Square. The program that was used for the analysis was the IBM SPSS statistics version 19.

3.8 DNA analysis:

2µl of DNA was measured by NanoDrop® instrument, working concentration 100 ng/µl was used for each sample in the PCR reaction (see Appendix 8.1)

3.9 Primer design:

1. SNP rs9389268 (A**à** G)

The sequence below for the SNP was taken from NCBI data base for SNPs:

AGTGCTTCTG GCAGTGAATT AACCTTGTAA CAATAAATAG TATTACTGAA AACAATCTTA
GTCCATTTGT TATTGTTTTA CTTTAAATGG TATCTTGCCA GGTTTTTAAA ACTCTGTATT
TCTTTTGTTA CTAATGATAC TCaaatttta aaatatattt atcttttatt ttaCTTTCTT
TTGTGAAACA CCTTTTCATA TACTTCATCA TTTTTATACT GGGCATTTTT CATTATTCAT
TTCTGTGGAT TGTTATGTAG TACTCCTTTG TTATACATGT AGCAATGTCT TTATCAGTCT
TTTTTCTTTT GATTTTTATT TTTTACTAAT TTAtttttgt ttgtctttgc ttttttttt
ttggagacag agtcttgctg tgtcacccag gctggagtgc agtggcacaa tctcatctca
ctgcaacctc cgcttccagg gttcaagcaa ttctcgtgcc tcagcctcct gggtagctga
gattacaggc gcatgcaacc
A
cacccgacta atttttgtg ttttagtaga tacgatgttt caccgtgttg ctcaggttgc
tctcgaactc ctgagctcag gcaatccacc tgcctcagtc tcctaaagca tgattacagg
tgtgagctac tacacATGGG CAGATTCTTA ATTTTTCATG TTTTAACATA AAACAGCTCT
CAAAGTTTTA AGCTTTATGT AAACTAATCT GCTACCTTTG GCACCAAACT TAGAAATTCT
CACTCTACCT GAATATTGTA AAAATATTAA CAATTATTCT CTGGTACTCA AAGGTTTTAG
TTTTAACATT TGAGAACATG ATATCTCTGG AATCTGTTTT GGGGCATTGT TTGAGTTAGA
GAGACACCTG TATAGGatat aaataatata ttttatattt tGCCCTCAAA TGATTAGCCA
TTCATCAAAA CACTATCGAA TAACTATCCT TTCCCCATTG ATTAGAAATG CCATTTTGTT
CATATACTTC ATTCTTCTTT

The primers were designed by MacVector software:

Rs 9389268-F 450-469 5`-ATTCTCGTGCCTCAGCCTCC-3`
20 nt forward primer
Pct G+C: 60.0 Tm: 55.6

Rs9389268-R 726-700 5`- GGTGCCAAAGGTAGCAGATTAGTTAC -3`
27 nt backward primer
Pct G+C: 44.4 Tm: 57.0

The primers will be flanking this sequence that includes 277 nucleotides where a represents the SNP in the wildtype.

ATTCTCGTGCCTCAGCCTCCTGGGTAGCTGAGATTACAGGCGCATGCAACC
aCACCCGACTAATTTTTTGTGTTTTAGTAGATACGATGTTTCACCGTGTTGC
TCAGGTTGCTCTCGAACTCCTGAGCTCAGGCAATCCACCTGCCTCAGTCTC
CTAAAGCATGATTACAGGTGTGAGCTACTACACATGGGCAGATTCTTAAT
TTTTCATGTTTTAACATAAAACAGCTCTCAAAGTTTTAAGCTTTATGTAAA
CTAATCTGCTACCTTTGGCACC

2. SNP rs11886868 (C à T)

The sequence below for the SNP was taken from NCBI data base for SNPs:

Cctccctctc tctctctctc tctctctctc tctGTCATTA CCAACTTCTA TAAAATCTCA
GAATACAAAG GGCATTCTCA TTTCCCTGAA ATGTACTTTG GTGCTACCCT GAAAGACGGC
TAGAGTCTTG AGGAGACCCA AACAGTTAAA GGTTACAGAC AGACTTGGTT CCACTCCAGT
GGTGGGTGTT TTGTTTTGTT TCGCTTTAGC tttattaagg tatactttac atacaacaaa
agtcattcat tttaagtgta cagttggatt caatttgcca gttgtataca gctgtgtggc
ctccaccaaa atcaagatac agaacacgtc caccagtcta gaaagccccc tcatgcccct
ttgctgtcaa tccccttccc taaccctctg acccccttcct tctaaccact gaacccccac
ctaccaccac agtgttgaga attctagaat ttgttacaaa tggactcata caggatatcg
tcttttgtgt ttaatttctt
Y
cacagagcag aatgattctg ggattcatcc atggtgtgga gtgtgttact ctttcattct
tctactgttg agttgtattc caccataggg cgtttatcca ttcaacctat tggttgccat
gtgggttgtg accacttttt gaccactatg aacagtgcta caatggacac tgtgtagatg
tgtttatagg aatgtatgtc tacatttctc ttaggtaaac atctaagGCA TTTCGAGAAC
ACAGAAAAGG TTTTGAGTTT GAGTCCAGGG AGCCCTCATT TTGTCATCCT CCAGGCTGCC
CCCCTCCCAC CCCATTCCCT GGAGAGTTCA ACTCCAAACC CTATATCCCA GCTATCTGTT
TGGCTCAGCT ACCAAATGTC ATCCTATTCC GAATAGAAAC CTCTCCACTA CACTAAGGAA
ATGATGG

Y represents where the single nucleotide polymorphism has changed.

The primers were designed by MacVector software:

rs11886868-F 323-346 5'- ACCCTCTGACCCCTTCCTTCTAAC -3'
24 nt forward primer
pct G+C: 54.2 Tm: 57.0

rs11886868-R 545-524 5'- TGAATGGATAAACGCCCTATGG -3'
22 nt backward primer
pct G+C: 45.5 Tm: 55.5

The primers will be flanking this sequence that includes 223 nucleotides where c represents the SNP in the wild type.

ACCCTCTGACCCCTTCCTTCTAACCACTGAACCCCCACCTACCACCACAGT
GTTGAGAATTCTAGAATTTGTTACAAATGGACTCATACAGGATATCGTCTT
TTGTGTTTAATTTCTTcCACAGAGCAGAATGATTCTGGGATTCATCCATGGT
GTGGAGTGTGTTACTCTTTCATTCTTCTACTGTTGAGTTGTATTCCACCATA
GGGCGTTTATCCATTCA

The primers where ordered from hy-labs, lyophilized and diluted by nuclease free water to reach 100pmol/µl, the working concentration is 10pmol/µl.

3.10 PCR reaction:

The reaction volume prepared was 25µl that included all components of PCR reaction: 12.5µl ReddyMix (see Appendix 2 for contents), 0.5µl Forward primer, 0.5µl reveres primer, 1µl DNA, and 10.5µl nuclease free water. A negative control was prepared with all the components except the DNA.

The PCR was run in an Applied Biosystem`s Thermal cycler, the cycler was programmed touchdown 55-45 as follows:

95°C (5:00)……..1 cycle

The annealing temperature starts at 63°C and it goes down 2 degrees till it reaches 55°C. Each time the PCR machine does 2 cycles.

94°C (0:30), 55°C (0:30), 72°C (0:45)…… 30 cycles

72°C (4:00)…….....1 cycle

10°C (10:00)……...…∞ (overnight).

3.11 Agarose Gel Electrophoresis:

1% agarose was prepared: 1gm powder per 100ml 1XTris-acetate-EDTA electrolyte buffer solution in a 250ml flask. The resulting media became foggy. Then using the microwave, the media is allowed to boil for 3 minutes or more until it become clear, cooled by running tap water, in order to prepare for the addition of Ethidium Bromide (EtBr), finally poured in the gel castor until solidified with combs in place.

3µl PCR product was loaded in the well of each sample and 5µl of DNA ladder (fragments: 100, 300, 500, 700, 900, and 1200 base pair/bp).

The power supply was set at 120V for 20 minutes. The gel was observed on a gel documentation system for detection of PCR products.

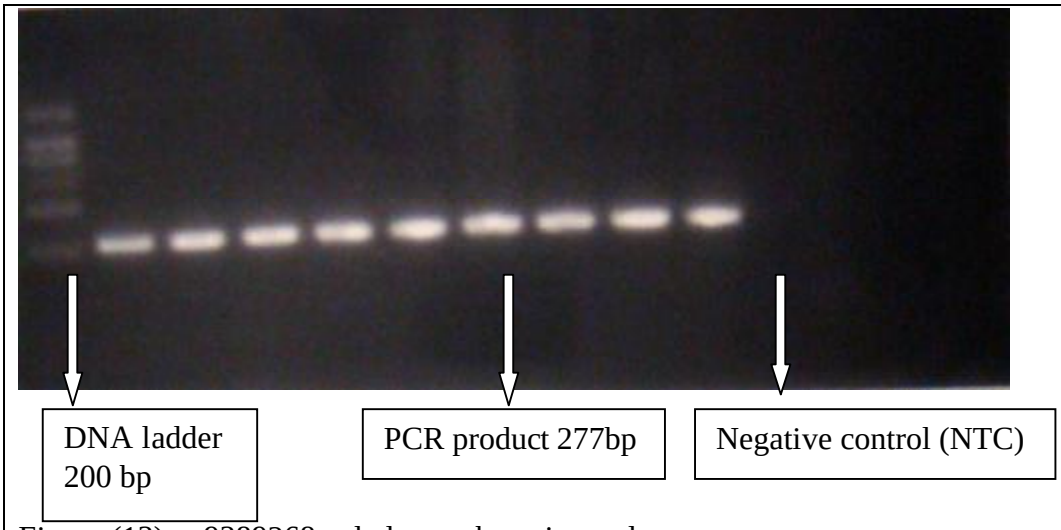


Figure (13): rs9389268 gel electrophoresis result.

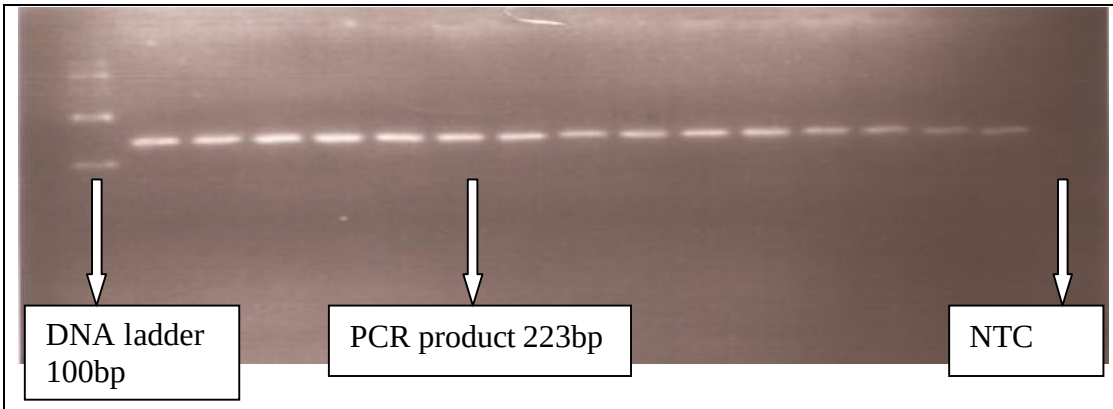


Figure (14): rs11886868 gel electrophoresis result

3.12 Restriction Fragment Length Polymorphism (RFLP):

In this procedure, the genotypes of the two SNPs were tested by restriction digest to differentiate between the wildtype allele, homozygous mutant, and heterozygous polymorphic status.

First, the Webcutter version 2.0 was used, to choose the most suitable restriction enzyme.

1. For rs11886868, there is 1 cut in the mutant allele at position 181 by the enzyme MboII (recognition site gaaga) which also makes 2 cuts in the wildtype allele at position 119 and 181.

MboII

5'...G A A G A(N)$_8$▼... 3'

3'...C T T C T(N)$_7$▲... 5'

From: Nebcutter online http://tools.neb.com/NEBcutter2/enz.php?name=76430814-&enzname=MboII&recpos=176 Retrieved August 26,2010.

Table (3): the pattern of cuts in the wildtype allele and the mutant for rs11886868

Enzyme	Allele Type	No./ Position Cut of sites	Recognition Sequence	Length of bases after cuts
MboII	Wildtype	2 / 119 181	gaaga	119, 62, 42
	Mutant	1 / 181		181, 42

2. For rs9389268, there is 1 cut in the mutant allele at position 53 by the enzyme AciI (recognition site ccgc) and no cuts in the wildtype allele.

AciI

5'... C▼C G C... 3'

3'... G G C▲G... 5'

From: Nebcutter online http://tools.neb.com/NEBcutter2/enz.php?name=76430814-&enzname=AciI&recpos=49 Retrieved August 27, 2010

Table (4): the pattern of cuts in the wildtype allele and the mutant for rs9389268

Enzyme	Allele Type	No./ Position cut of sites	Recognition sequence	Length of bases after cuts
AciI	Wildtype	No cuts	CCGC	277
	Mutant	1 / 53		224, 53

Table (5): RFLP reaction recipe (total volume 20µls):

1st SNP	Reagents	1X reaction	Source
Rs11886868	10x buffer 2	2µl	TAKARA SHUZO (DALIAN) CO., LTD Biomedical Group, Japan http://www.takara.com
	MboII	1 µl	TAKARA Biomedicals
	Substrate DNA	5 µl	PCR product from previous step
	Nuclease free water	12 µl	
Rs9389268	10x buffer 3	2 µl	New England BioLabs www.neb.com
	AciI	0.5	New England BioLabs www.neb.com
	Substrate DNA	5µl	PCR product from previous step
	Nuclease free water	12.5	

Uncut: was used as control that only contains the PCR volume.

The incubation period used for the reactions was at 37°C for 16 hours in a thermal cycler and then loaded on 3% gel electrophoresis at 80Volts for ~45 minutes to1:15 hour.

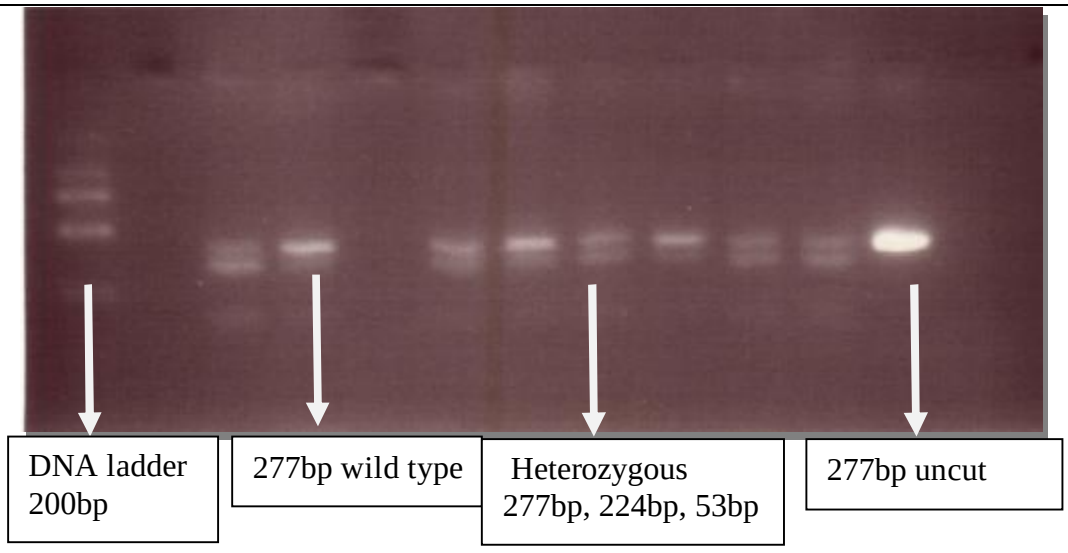


Figure (15): rs9389268 RFLP gel electrophoresis result.

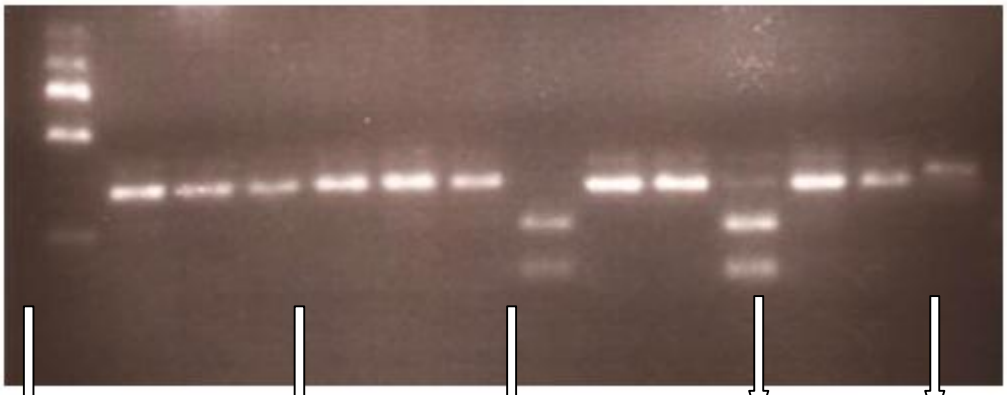


Figure (16): rs11886868 RFLP gel electrophoresis result.

3.13 Cleaning PCR for sequencing:

Promega kit (Cat # A9282) Wizard® SV Gel and PCR Clean-Up System was used to remove excess primers and nucleotides.

1. A membrane based system in which 25µl of membrane binding solution were added to the PCR to clean it up from fragments and primers.
2. The solution was transferred to a filter column then centrifuged for 1 min. at 14.000 rpm.
3. The residue was thrown and 750 µl of membrane washing solution (MWS) were added to the filter, centrifuged again for 1 min. at 14.000 rpm.
4. Step 3 was repeated but 500 µl MWS were added, centrifuged for 5 min. at 16.000 rpm.
5. Finally, the filter column was transferred to an empty eppendorf tube and 20µl sterile water was added. This should be our cleaned PCR.

3.14 PCR for Sequencing:

Table (6): The reaction volume used for sequencing PCR

Reagents	1X reaction
5x reaction buffer	4 µl
Big Dye Terminator version 1.1	1 µl
Clean PCR product	0.3 µl
Water	13.7 µl
Primer (forward or reverse) 10 pm/µl	1 µl
Total volume	20 µl

Note: the amount of PCR product used in sequencing PCR depends on the length and purity of the PCR product (for each 100bp of sequence, 10ng of PCR product is needed).

1. The PCR reaction was run in a PCR machine programmed as follow: 96ºC for 1 min.denaturation, [96 ºC for 10 sec, 50ºC for 5 sec, 60ºC for 4 min]×25 cycle. Then 4ºC overnight or as needed. This takes about 2.30 hr.
2. The PCR product was transferred to gel tube, centrifuged at 750rpm for 2 min.
3. The tube was transferred to speed vacuum with heat 60ºC for 30 min to dry the sequence PCR product.
4. 16 µl of Hi-Di™ Formamide (from applied biosystem, UK) were added to the tube to denature the DNA, vortex, heated at 95 ºC for 2 min, then directly put it on ice for 5 minutes.
5. 16µl from the sample was transferred to a 96 well tray and run it in a sequencer machine ABI 3130.

3.15 Sequencing output:

The sequences were tested for each SNP to see the polymorphism; they were analyzed by software called Finch TV, in which the SNP was detected by the number of peaks representing the polymorphism; wildtype, heterozygous, or homozygous mutant.

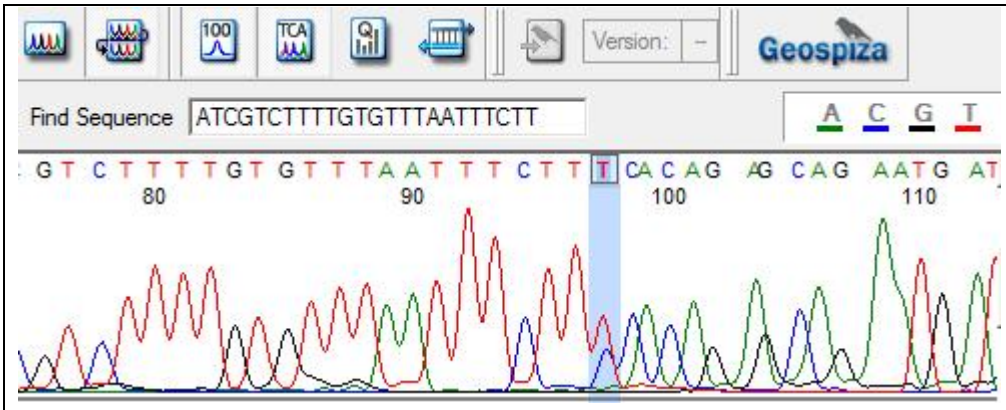


Figure (17.a): Finch TV view displays the sequence with the SNP rs11886868 (C to T) heterozygous

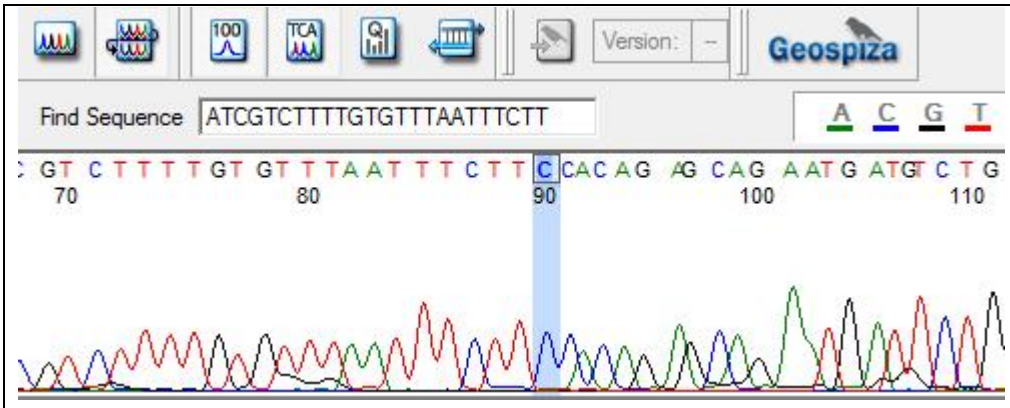


Figure (17.b): Wildtype sequence for SNP rs11886868

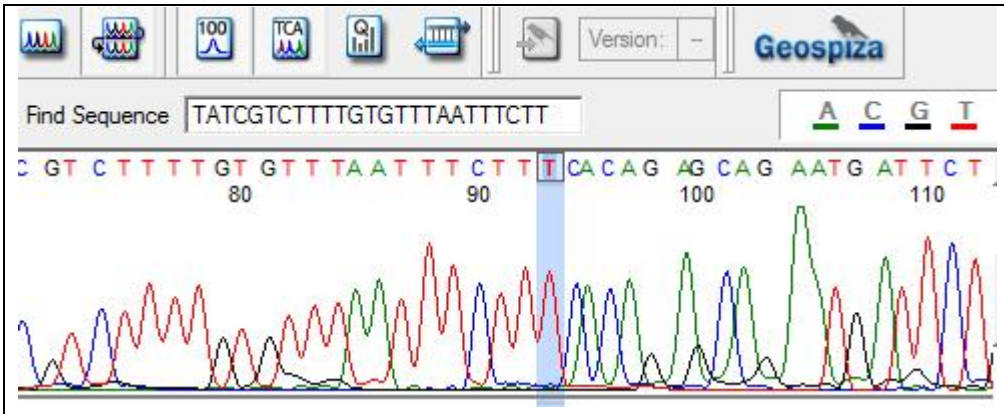


Figure (17.c): homozygous mutant for SNP rs11886868

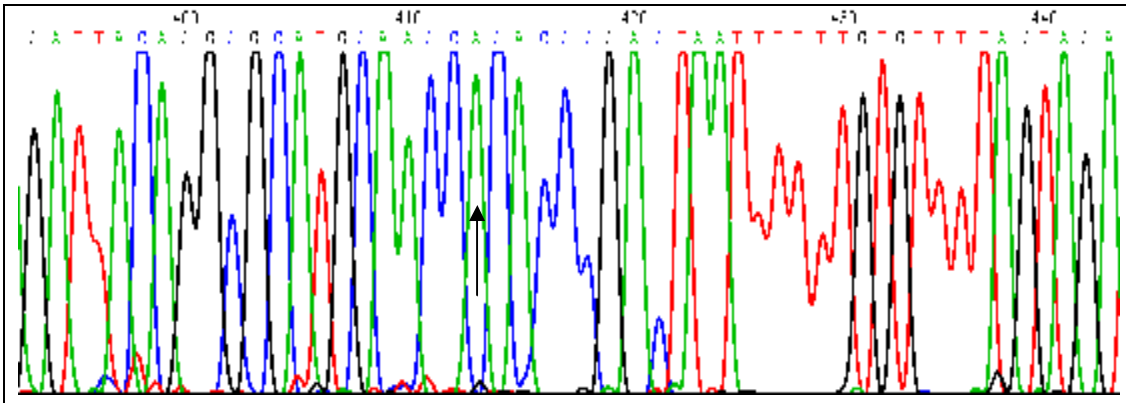


Figure (18.a): Wildtype sequence for SNP rs9389268 (A to G)

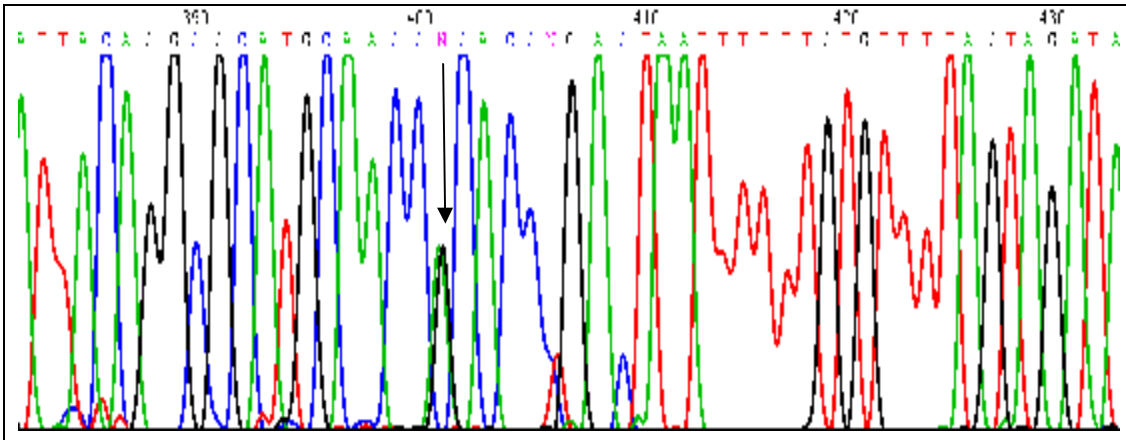


Figure (18.b): Heterozygous for SNP rs9389268 (A to G)

CHAPTER 4

Results

Hypothesis 1:

Does the means of HbF differ with respect to phenotype at the level of significant $\alpha = 0.05$?

To answer the question, one way ANOVA was used to compare the means of HbF with respect to phenotype.

Table (7): One way ANOVA results to compare the means of HbF with respect to phenotype.

HbF					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	386.178	2	193.089	2.880	.063
Within Groups	4827.945	72	67.055		
Total	5214.123	74			

The table shows that there is no differences at the level $\alpha = 0.05$ in the means of HbF with respect to phenotype since the calculated significant is 0.063 which is greater than 0.05.

For multiple comparisons the LSD test is calculated and the results are shown in the following Table.

Table (8): Results of Multiple Comparisons.

HbF LSD						
		Mean Difference			95% Confidence Interval	
(I) phenotype1	(J) phenotype1	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Mild	Intermediate	3.6147	2.6658	.179	-1.699	8.929
	Severe	5.8792*	2.4604	.019	.974	10.784
intermediate	Mild	-3.6147	2.6658	.179	-8.929	1.699
	Severe	2.2645	2.1859	.304	-2.093	6.622
Severe	Mild	-5.8792*	2.4604	.019	-10.784	-.974
	Intermediate	-2.2645	2.1859	.304	-6.622	2.093

*. The mean difference is significant at the 0.05 level.

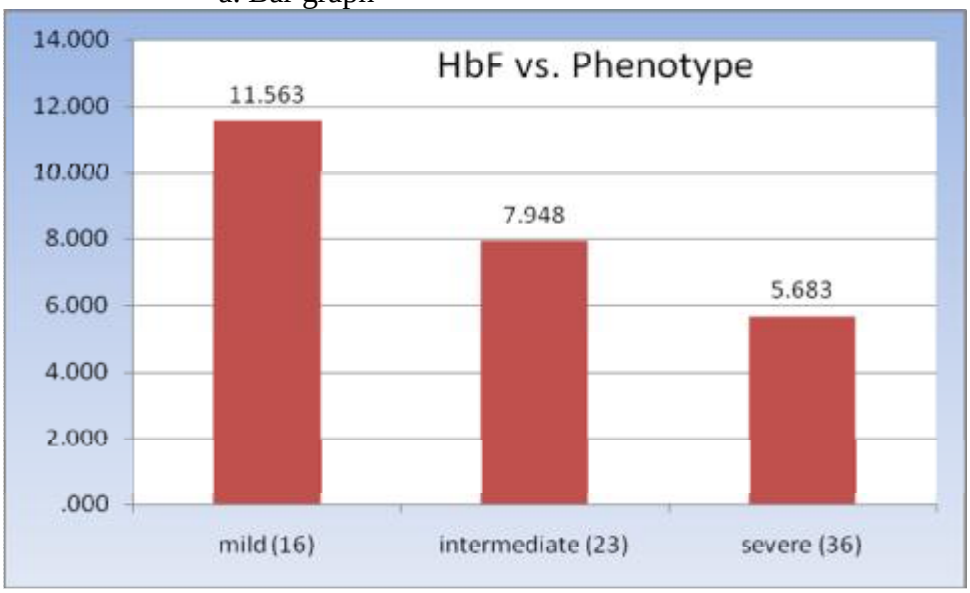
Table (9): Means of HbF level with respect to phenotype

	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Mild	16	11.563	10.8168	2.7042	1.5	45.0
intermediate	23	7.948	7.4253	1.5483	.5	30.0
Severe	36	5.683	7.2898	1.2150	.5	30.0
Total	75	7.632	8.3941	.9693	.5	45.0

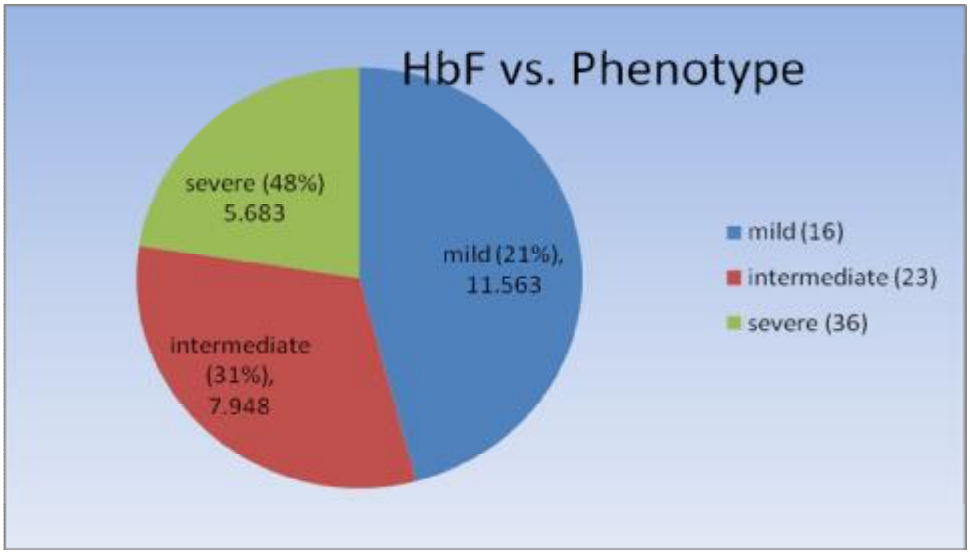
The table shows that there is a difference between the mild and severe phenotype in HbF level to the mild. The mean for HbF in mild patients is 11.563 while it is 5.683 for severe ones.

Graph 1: Means of HbF level with respect to phenotype by:

a. Bar graph



b. Pie chart.



Hypothesis 2:

Does the means of HbF differ with respect to gender at the level of significant α = 0.05?

To answer the question t-test was used to compare the means of HbF with respect to gender.

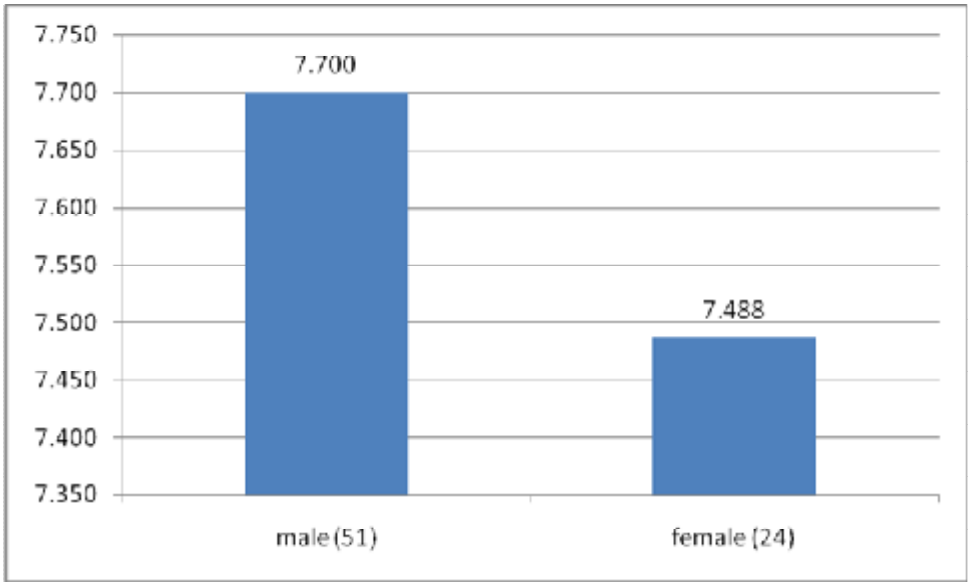
Table (10): T-test results to compare the means of HbF with respect to gender

Group Statistics

	gender	N	Mean	Std. Deviation	Std. Error Mean	df	T	sig
HbF	**male**	51	7.700	9.1758	1.2849	73	0.102	0.919
	female	24	7.488	6.6059	1.3484			

The table shows that there is no significant differences at the level of α = 0.05 in the means of HbF with respect to gender since the calculated significant is 0.919 which is greater than 0.05

Graph2: Means of HbF level with respect to gender



Hypothesis 3.a:

Does the phenotype correlate to genotype for SNP rs11886868 at the level of significant $\alpha = 0.05$?

To answer the question chi-square test was used as a test of independence to check the correlation between the phenotype and genotype.

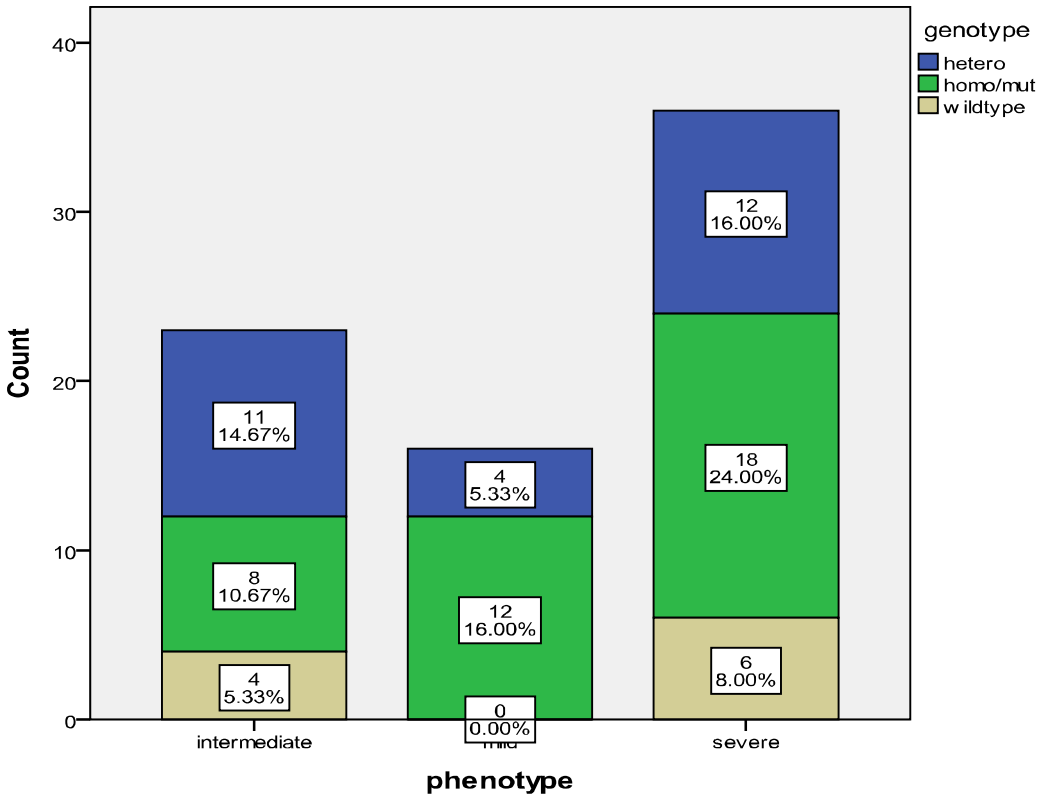
Table (11): Genotype * Phenotype Crosstabulation for rs11886868

			Phenotype			
			intermediate	**mild**	**severe**	**Total**
SNP genotype	**hetero**	Count	11	4	12	27
		Expected Count	8.3	5.8	13.0	27.0
		% of Total	14.7%	5.3%	16.0%	36.0%
	homo/mut	Count	8	12	18	38
		Expected Count	11.7	8.1	18.2	38.0
		% of Total	10.7%	16.0%	24.0%	50.7%
	wildtype	Count	4	0	6	10
		Expected Count	3.1	2.1	4.8	10.0
		% of Total	5.3%	.0%	8.0%	13.3%
Total		Count	23	16	36	75
		Expected Count	23.0	16.0	36.0	75.0
		% of Total	30.7%	21.3%	48.0%	100.0%

Chi-square =7.238 df= 4 sig. = 0.124

There is no relationship between the phenotype and genotype at level of significant $\alpha = 0.05$ since 0.124 is greater than 0.05

Graph (3.a): Genotype-phenotype correlation for SNP rs11886868.



Hypothesis 3.b:

Does the phenotype correlate to genotype for SNP rs9389268 at the level of significant $\alpha = 0.05$?

To answer the question chi-square test used as a test of independence to check the correlation between the phenotype and genotype.

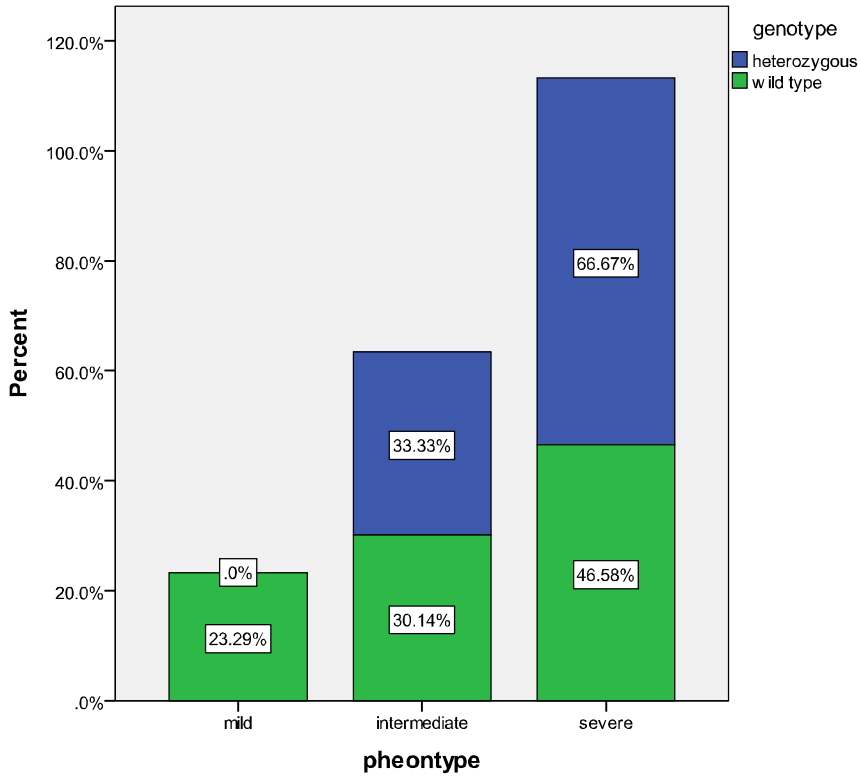
Table (12): Genotype * Phenotype Crosstabulation for rs9389268

			Phenotype			
			mild	intermediate	severe	Total
genotype	heterozygous	Count	0	1	2	3
		Expected Count	.7	.9	1.4	3.0
		% of Total	.0%	1.3%	2.6%	3.9%
	wildtype	Count	17	22	34	73
		Expected Count	16.3	22.1	34.6	73.0
		% of Total	22.4%	28.9%	44.7%	96.1%
Total		Count	17	23	36	76
		Expected Count	17.0	23.0	36.0	76.0
		% of Total	22.4%	30.3%	47.4%	100.0%

Chi-square =0.945 df=2 sig. = 0.621

There is no relationship between the phenotype and genotype at level of significant α =0.05 since 0.621 is greater than 0.05

Graph (3.b): Genotype-phenotype correlation for SNP rs9389268.



Hypothesis 4.a:

Does the means of HbF differ with respect to genotype for SNP rs11886868 at the level of significantα = 0.05?

To answer the question, one way ANOVA was used to compare the means of HbF with respect to phenotype

Table (13): One way ANOVA results to compare the means of HbF with respect to genotype.

ANOVA

HbF

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	119.384	2	59.692	.844	.434
Within Groups	5094.740	72	70.760		
Total	5214.123	74			

The table shows that there is no significant differences at the levelα = 0.05 in the means of HbF with respect to genotype since the calculated significant is 0.434 which is greater than 0.05

For multiple comparisons the LSD test is calculated and the results are shown in the following table.

Table (14): Results of Multiple Comparisons.

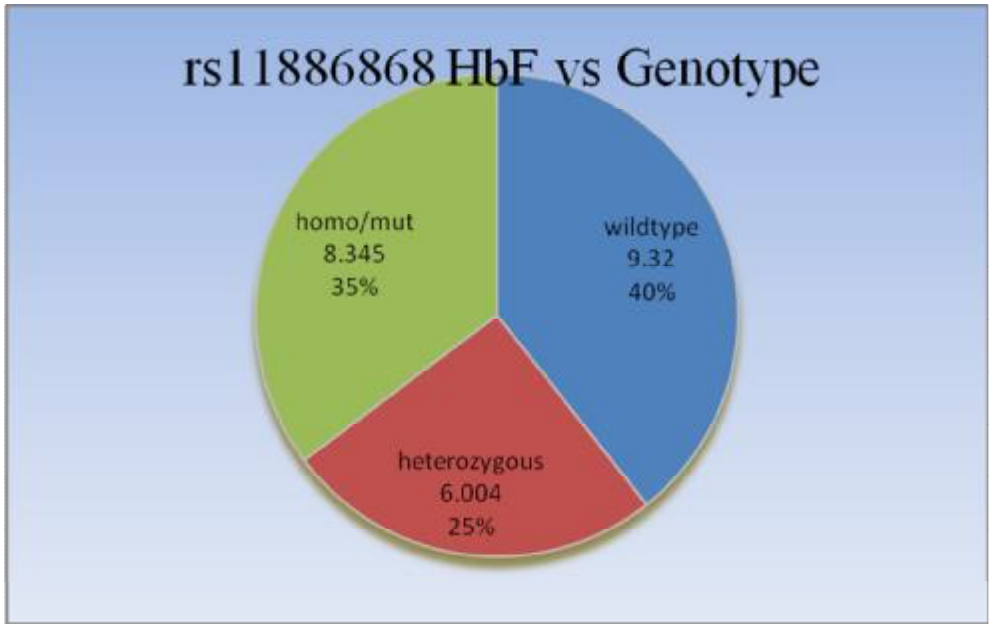
HbF LSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Wildtype	heterozygous	3.3163	3.1140	.290	-2.891	9.524
	homo/mut	.9753	2.9897	.745	-4.985	6.935
heterozygous	wildtype	-3.3163	3.1140	.290	-9.524	2.891
	homo/mut	-2.3410	2.1173	.273	-6.562	1.880
homo/mut	wildtype	-.9753	2.9897	.745	-6.935	4.985
	heterozygous	2.3410	2.1173	.273	-1.880	6.562

Table (15): Means of HbF level with respect to genotype.

	HbF				
	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
wildtype	10	9.320	10.3387	3.2694	1.5	30.0
heterozygous	27	6.004	6.5809	1.2665	.5	30.0
homo/mut	38	8.345	9.0146	1.4624	.5	45.0
Total	75	7.632	8.3941	.9693	.5	45.0

Graph (4.a): Means of HbF level with respect to genotype for SNP rs11886868.



Hypothesis 4.b:

Does the means of HbF differ with respect to genotype for SNP rs9389268 at the level of significant $\alpha = 0.05$?

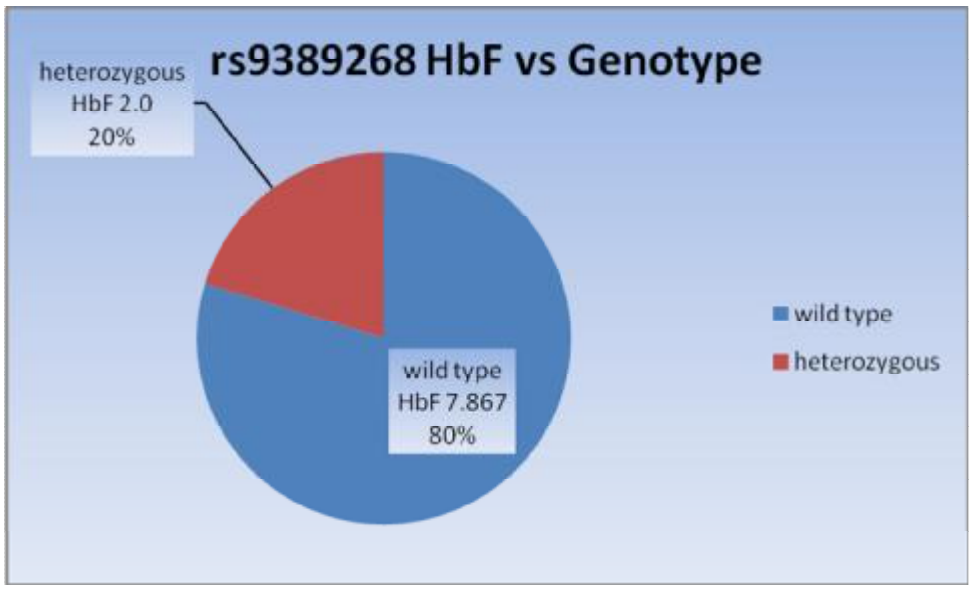
To answer the question independent sample t-test was used to compare the means of HbF with respect to genotype.

Table (16): T-test results to compare the means of HbF with respect to genotype.

Group Statistics							
genotype	N	HbF Mean	Std. Deviation	Std. Error Mean	df	T	Sig.
wild type	72	7.867	8.4865	1.0001	73	1.189	0.238
heterozygous	3	2.000	.8660	.5000			

The table shows that there is no significant differences at the level of $\alpha = 0.05$ in the means of HbF with respect to genotype, since the calculated significant is 0.238 which is greater than 0.05.

Graph (4.b): Means of HbF level with respect to genotype for SNP rs9389268.



Finally, the frequencies of Hb level, HbA2 level, and MCV level were calculated for our samples as shown in table below.

Table (17): Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Hb	75	5.4	10.6	8.056	1.1165
HbA2	75	1.8	25.0	3.504	2.6344
MCV	75	45.7	88.1	63.863	9.6170

The mean of hemoglobin level (Hb) forβ-thalassemia patients in our sample is 8.05g/dl.

The mean of hemoglobin A_2 level (HbA_2) for β-thalassemia patients in our sample is 3.5%

The mean of MCV level forβ-thalassemia patients in our sample is 63.8 fL.

CHAPTER 5

Discussion

Recently, Genome-wide association progress has identified many loci associated with common diseases such as β-thalassemia. The two SNPs (rs11886868, and rs9389268) were previously reported to be associated with fetal hemoglobin (HbF) levels. We analyzed the presence of these two SNPs in patients showing variable phenotypic heterogeneity, and previously genotyped to be homozygous with IVS1-6 (T→C) mutation. The technique used is PCR, RFLP and direct DNA sequencing, the aim of the study was to find positive correlation between the two SNPs and the clinical severity in phenotype (genotype-phenotype correlation).

5.1 Discussion of the Study Finding

One of the important findings which contribute in ameliorating the severity of β-thalassemia phenotype is elevated levels of HbF. After testing the correlation between HbF levels and patients phenotype at the level of significantα=0.05, we found that HbF =11.563% for mild cases and 5.683% for severe patients (see table 9, graph 1). But there was no correlation between HbF level and polymorphism at these SNPs (see tables 15, 16 & graphs 4.a, 4.b) which indicates that these particular SNPs may have a minor influence on HbF level in our population.

Genetic disease such as β-thalassemia (autosomal recessive disorder) is transmitted equally to both females and males. In this study it was found that from the total subjects, 68% were males and 32% were females (see table 10). The sample of this study was selected by studying the IVS1-6 homozygous patients from total population of 266 β-thalassemia patients.

Chi square test was performed to find out the correlation between genotype of the two SNPs and the patients phenotype. Unfortunately, no correlation between these polymorphisms and disease severity was found. Furthermore, 24% of the patients were homozygous mutant for rs11886868 and showed sever phenotype (see table 11, graph 3.a). On the other hand, all mild patients did not have SNP rs9389268, and the more heterozyosity percentage was found in severe cases (66.6%). This is contradictory to what was found in previous studies of GWAS, but is compatible with other study investigated by Nguyen, *et al*., (2010) who did not find statistically

significant correlation between the two SNPs and HbF level, suggesting that the BCL11A and HBS1L-MYB loci have a minor effect on HbF level.

β-thalassemia trait (heterozygosity) is suspected when MCV <79 fL and elevated HbA2 (3.0%), our samples were having MCV = 63.8 fL, and mean HbA2 3.5%, genetically it was confirmed by PCR test that all 75 patients were homozygous for IVS1-6 mutation. Applying PCR tests along with hematological tests for β-thalassemia patients can help in establishing more accurate hematological parameters identifyingβ-thalassemia traits.

5.2 Limitations

When performing gel electrophoresis for RFLP PCR digests, it needs long run at 3% agarose gel and the ladder lowest band is 100 bp. One of the cuts of 42 bp length was missed. So, direct DNA sequencing is a good validation tool to confirm the results of gel electrophoresis directly. Also, ethidium bromide is carcinogenic and need much care when handling the gel into the gel documentation system.

Sometimes, incomplete digests were misleading to read the pattern of cuts on the gel due to the volume of enzyme used (0.5 µl of MboII per PCR reaction), after using 1µl the results got better cuts.

CHAPTER 6

Conclusion & Recommendations

IVS1-6 mutation is one of the mild β+ thalassemia mutations, due to the high residual activity of β-globin chains from the affected locus. Nevertheless, homozygous patients of this mutation exhibit a variable clinical presentation of phenotype; mild, intermediate, and severe forms.

The genetic modifiers that were previously found by GWAS to contribute in ameliorating the disease such as rs11886868 at BCL11A gene and rs9389268 at HBSlL-MYB intergenic region had no correlation with the phenotype heterogeneity of 75 homozygous IVS patients from our population.

Since elevated levels of HbF was found in mild cases, molecular determination of fetal hemoglobin switch in early childhood will help to identify candidates for pharmacological HbF switching such as hydroxyurea. However, in most cases, the genetic basis for the observed clinical variability has remained unknown.

Recommendations for further research steps, genotyping for other SNPs located in BCL11A region (rs4671393) and three SNPs located in the HBS1L-MYB region (rs28384513, rs9399137 and rs4895441).

Finally, the application of molecular methods of DNA analysis can elucidate the relationship between genotype and phenotype, furthermore predict the phenotype in genetic counseling and prenatal diagnosis and this is important for planning the proper treatment in homozygous β^0 thalassemia. Hopefully, in the coming future, the HRL at Bethlehem University will find novel SNPs that are significant to our Palestinian population (West Bank and Gaza Strip) by applying the microarray chip technique,

CHAPTER 7

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CHAPTER 8

Appendixes

Appendix (8.1)

Table containing: Sample number, Patients code, hematological parameters (HbF, Hb, HbA2, and MCV) and phenotype.

Sample Number	Patients code	HbF %	Hb g/dl	HbA_2 %	MCV fL	Phenotype
1.	001	٣,٠	٦,٣	٤,٠	٤٥,٧	mild
2.	009	١,٠	٩,٥	٤,٠	٧٤,٠	intermediate
3.	011	٤٥,٠	٨,٨	٢,٠	٧٤,٢	mild
4.	012	٨,٦	٨,٣	٣,٦	٥٢,٤	mild
5.	018	٥.	٧,٤	٣,٥	٥٩,٠	severe
6.	019	٢,٠	٨,٢	٣,٠	٥٧,٩	intermediate
7.	020	٢,٥	٨,١	٣,٥	٦٥,٦	intermediate
8.	023	٣,٠	٩,٢	٣,٠	٦٥,٧	severe
9.	026	٢,٠	٨,٣	٣,٠	٧٢,٦	severe
10.	035	٢,٠	٨,٨	٣,٠	٧٠,٤	severe
11.	040	٢,٥	١٠,٠	٣,٥	٦٦,٥	severe
12.	057	٣,٠	٩,٥	٣,٥	٦٤,٦	severe
13.	059	٣,١	٦,٢	٣,٩	٥٤,٣	mild
14.	064	٢,٥	٧,١	٢,٥	٦٠,٤	intermediate
15.	068	٦,٠	٧,٨	٣,٠	٥٦,١	severe
16.	071	١٠,٠	٩,٢	٢,٠	٦٨,٧	mild
17.	072	١,٠	٧,٩	٣,٥	٦٠,٣	severe
18.	073	٥,٠	٨,٠	٢,٠	٦٥,٢	intermediate
19.	074	١,٠	٩,٣	٢,٠	٨٦,٦	severe
20.	075	٢,٥	٧,٧	٣,٥	٦٢,٣	severe
21.	077	١,٠	٨,٨	٢,٥	٦٣,٨	severe
22.	078	١,٩	٨,٧	٢,١	٦٤,٨	intermediate
23.	080	٩.	٨,٦	٣,١	٥٦,٨	severe
24.	083	٣٠,٠	٨,١	٣,٠	٦٠,٥	intermediate
25.	089	٣,٥	٧,٢	٣,٥	٥٧,٨	severe

26.	090	٥,٠	٧,٠	٤,٠	٥٦,٠	severe
27.	108	٦,٠	٩,٠	٣,٠	٦٤,٨	severe
28.	117	٢,٠	٨,٩	٣,٥	٧٧,٣	Severe
29.	125	١,٩	٨,٦	٢,١	٨٨,١	Severe
30.	126	١,٢	١٠,٢	١,٨	٨٧,٢	Severe
31.	132	٢,١	٨,٦	٢,١	٦٩,٠	Severe
32.	143	٢,٠	٨,٨	٣,٠	٦٢,٢	Severe
33.	170	٣٠,٠	٨,٧	٢,٠	٧٩,٤	Severe
34.	179	١٣,٤	٦,٦	٣,٦	٥٢,٣	Mild
35.	180	١٠,٠	٧,٤	٤,٠	٤٦,٦	Intermediate
36.	187	٢٠,٠	٨,٣	٤,٠	٥٠,٦	Intermediate
37.	189	٣,٤	٧,٦	٤,٦	٥٦,٠	Mild
38.	190	٢٦,١	١٠,٤	٤,٢	٧٠,٧	Severe
39.	191	١٠,٠	٨,٠	٤,٠	٥٠,٤	Mild
40.	192	١٥,٠	٨,٢	٤,٠	٥٦,٢	Intermediate
41.	194	٣,٥	٩,٣	٢٥,٠	٦٦,٤	Severe
42.	196	٢,٣	٨,١	٣,٧	٦٢,٣	Mild
43.	198	٤,٢	٨,٠	٦,١	٥٠,٤	Mild
44.	199	٢,٤	٥,٤	٣,٦	٦٨,٨	Severe
45.	200	٣,٥	٩,١	٣,٥	٦١,١	Intermediate
46.	203	١,٥	١٠,٤	٢,٥	٦٩,٨	Severe
47.	207	١٥,٠	٨,٩	٣,٠	٥٩,٩	Intermediate
48.	208	٢,٠	٩,٠	٢,٧	٦١,٢	Severe
49.	209	٥,٠	٨,٥	٥,٠	٥٥,٧	Intermediate
50.	211	٥,٠	٨,٩	٤,٠	٨٤,٩	Severe
51.	212	٥.	٥,٩	٣,٨	٥٥,٦	Intermediate
52.	215	٦,٥	٧,٤	٣,٥	٥٣,٥	Severe
53.	217	٣,٠	٧,٧	٣,٥	٦٦,٧	Intermediate
54.	219	٣,٩	٧,١	٣,١	٦٤,٢	Intermediate
55.	220	١٠,٥	١٠,٦	٢,٥	٦٨,١	Severe
56.	225	١٥,٠	٨,٥	٤,٠	٦٤,٦	Mild
57.	228	١٢,٥	٦,٦	٣,٥	٦٤,٥	Severe
58.	229	١,٥	٦,٩	٢,٥	٨٠,٩	Severe

59.	231	١,٥	٨,١	٣,٧	٥٦,٨	mild
60.	232	٣,٠	٨,٥	٣,٠	٧٧,٦	Severe
61.	242	٣,٠	٨,١	٢,٠	٨١,٣	Severe
62.	244	١٥,٠	٨,٢	٣,٠	٦٩,٨	Intermediate
63.	245	١٣,٠	٧,٨	٣,٠	٧٣,٨	Severe
64.	247	٢٠,٠	٧,٧	٤,٠	٥١,٨	Mild
65.	248	١٠,٥	٧,٢	٣,٥	٥٨,٥	Mild
66.	249	١٢,٠	٦,٩	٤,٠	٦٠,٤	Intermediate
67.	250	٨,٠	٦,٦	٢,٠	٥٣,٨	Intermediate
68.	251	٤,٠	٦,٨	٣,٠	٥٨,٠	Intermediate
69.	255	١٥,٠	٧,٨	٣,٠	٦٨,٥	Mild
70.	256	٢٠,٠	٧,٦	٢,٠	٥٧,٤	Mild
71.	257	٢٥,٠	٧,٤	٣,٠	٥٤,٤	Severe
72.	258	١٠,٠	٦,١	٣,٠	٥٩,٥	Severe
73.	259	٣,٠	٦,٤	٣,٠	٧٢,٣	Intermediate
74.	262	٥,٠	٧,٢	٣,٠	٦٥,٩	Intermediate
75.	264	١٥,٠	٦,٢	٣,٠	٥٨,٣	Intermediate

Appendix (8.2)

1. Components of 2x ReddyMix™PCR Master Mix from ABGene, UK
2. Concentration of lyophilized primers (forward and reverse).

Component	**Content per 50µl PCR**
Thermoprime Plus DNA Polymerase	1.25 Units
Tris-HCL (pH 8.8 at 25°C)	75mM
$(NH_4)_2SO_4$	20mM
Tween 20®	0.01% (v/v)
dATP , dCTP, dGTP, dTTP	200µM of each
$MgCl_2$	1.5mM

SNP	**Concentration of lyophilized primers**	
	Forward	**Reverse**
rs9389268	29.2 nmol	16.8 nmol
rs1188268	26.0 nmol	17.3 nmol