T.R.C.N

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

# GENOTYPING OF THE *BCL11A* rs11886868 T>C (*Mbd*I) AND rs766432 A>C POLYMORPHISMS AND THE *HBS1L–MYB* rs9399137 T>C POLYMORPHISM IN BETA-THALASSEMIA PATIENTS OF TURKISH - CYPRIOT ORIGIN

Kefas Konyan JAMES

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MASTER OF SCIENCE THESIS

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SUPERVISOR

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2018

The Directorate of Graduate School of Health Sciences,

This study has been accepted by the Thesis Committee in Medical Biochemistry programme as a Master of Science Thesis.

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# DECLARATION

I hereby declare that the work in this thesis entitled **"GENOTYPING OF THE *BCL11A* rs11886868 T>C *(Mbo*II) AND rs766432 A>C POLYMORPHISMS AND THE *HBS1L−MYB* rs9399137 T>C POLYMORPHISM IN BETA-THALASSEMIA PATIENTS OF TURKISH-CYPRIOT ORIGIN"** is the product of my own research efforts under the supervision of Assist. Prof. Kerem TERALI. No part of this thesis was previously presented for another degree or diploma in any University elsewhere, and all information in this document has been obtained and presented in accordance with academic ethical conduct and rules. All materials and results that are not original to this work have been duly acknowledged, fully cited and referenced.

Name, Last Name:

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## ABSTRACT

**James, K. K. Genotyping of the *BCL11A* rs11886868 T>C *(Mbo*II) and rs766432 A>C Polymorphisms and the *HBS1L−MYB* rs9399137 T>C Polymorphism in Beta-Thalassemia Patients of Turkish-Cypriot Origin. Near East University, Graduate School of Health Sciences, M.Sc. Thesis in Medical Biochemistry Program, Nicosia, 2018**.

Beta-thalassemia (β-thalassemia) is caused by a reduction or absence in the synthesis of the beta-globin chains that constitute the hemoglobin molecule. Three clinical phenotypes of β-thalassemia have been identified. Only two are symptomatic, thus clinically relevant which are; β-thalassemia intermedia (TI) and β-thalassemia major (TM). β-thalassemia is prevalent in the Mediterranean region, the Middle East, North America and South East Asia, with Cyprus having the highest prevalence. Some background genes such as *BCL11A* and other loci epistatically interact with the β-globin gene to influence the disease severity in patients with β-thalassemia. In the present study, 67 β-thalassemia patients ($N_{TM}$ = 42 and $N_{TI}$ = 25) were enrolled after obtaining due consent. Genomic DNA from each of these patients was isolated from whole blood. The genotype of the rs11886868 T>C polymorphism was determined using PCR-RFLP with *Mbo*II restriction enzyme digestion, and the rs766432 A>C and rs9399137 T>C polymorphisms were genotyped using tetra-primer ARMS analysis. To visualize the DNA amplicons, agarose gel electrophoresis was employed. The rs11886868 T→C (*Mbo*II) variation was detected in 76.1% of the patients with genotype distributions of 0.239, 0.239 and 0.522 for the TT, CC and TC genotypes, respectively. The rs766432 A→C variation was detected in 56.8% of the patients with genotype distributions of 0.433, 0.09 and 0.478 for the AA, CC and AC genotypes, respectively. The rs9399137 T→C variation was detected in 41.8% of the patients with genotype distributions of 0.582, 0.09 and 0.328 for the TT, CC and TC genotypes, respectively. Allele frequencies for the three polymorphisms were found to comply with the Hardy–Weinberg equilibrium and no significant association was established between the genotypes carrying the supposedly protective alleles and the two groups of β-thalassemia patients for any of the three polymorphisms.

**Keywords**: Beta-thalassemia; Polymorphism; Genotyping; PCR-RFLP; Tetra-primer ARMS

# ÖZET

**James, K. K. Kıbrıslı Türk Beta-Talasemi Hastalarında *BCL11A* rs11886868 T>C *(Mbo*II) ve rs766432 A>C Polimorfizmleri ile *HBS1L−MYB* rs9399137 T>C Polimorfizminin Genotiplemesi. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyokimya Programı Yüksek Lisans Tezi, Lefkoşa, 2018.**

Beta-talasemi (β-talasemi), hemoglobin molekülünü oluşturan beta-globin zincirlerinin sentezindeki bir azalma veya eksiklik sebebiyle ortaya çıkmaktadır. β-talaseminin üç klinik fenotipi tanımlanmıştır. Bunlardan yalnızca ikisi semptomatiktir yani klinikle alakalıdır: β-talasemi intermedia (TI) ve β-talasemi majör (TM). β-talasemi Akdeniz bölgesinde, Orta Doğu'da, Kuzey Amerika'da ve Güneydoğu Asya'da yaygın olup en sık görüldüğü yer Kıbrıs'tır. *BCL11A* gibi bazı arka plan genler ile diğer lokuslar β-globin geniyle epistatik olarak etkileşime girerek β-talasemi hastalarında hastalığın ciddiyetini etkileyebilmektedir. Bu çalışmaya onam formu veren 67 β-talasemi hastası ($N_{TM}$ = 42 ve $N_{TI}$ = 25) kaydolmuştur. Her bir hastaya ait genomik DNA tam kandan saflaştırılmıştır. rs11886868 T>C polimorfizminin genotipi *Mbo*II restriksiyon enzim kesimini içeren PCR-RFLP yöntemiyle belirlenmiş, rs766432 A>C ve rs9399137 T>C polimorfizmleri ise tetra-primer ARMS analizi ile genotiplenmiştir. DNA amplikonlarının görüntülenmesi agaroz jel elektroforezi ile gerçekleştirilmiştir. rs11886868 T→C (*Mbo*II) varyasyonu hastaların %76.1'inde saptanmış ve TT, CC ve TC genotip dağılımlarının sırasıyla 0.239, 0.239 ve 0.522 şeklinde olduğu bulunmuştur. rs766432 A→C varyasyonu hastaların %56.8'inde saptanmış ve AA, CC ve AC genotip dağılımlarının sırasıyla 0.433, 0.09 ve 0.478 şeklinde olduğu bulunmuştur. rs9399137 T→C varyasyonu hastaların %41.8'inde saptanmış ve TT, CC ve TC genotip dağılımlarının sırasıyla 0.582, 0.09 ve 0.328 şeklinde olduğu bulunmuştur. Her üç polimorfizm için de allel frekanslarının Hardy–Weinberg dengesiyle uyumlu olduğu gösterilmiş ve koruyucu olduğu varsayılan alleli taşıyan genotiplerle iki β-talasemi grubu arasında herhangi bir anlamlı ilişki olmadığı ortaya konulmuştur.

**Anahtar kelimeler:** Beta-talasemi; Polimorfizm; Genotipleme; PCR-RFLP; Tetra-primers ARMS

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## ABBREVIATIONS

ARMS : Amplification refractory mutation system.

Da : Dalton.

DNA : Deoxyribonucleic acid.

EDTA : Ethylenediaminetetraacetic acid.

Fwd : Forward.

Hb : Hemoglobin.

kDa : kilo Dalton.

MCH : Mean corpuscular hemoglobin.

MCV : Mean corpuscular volume.

PCR : Polymerase chain reaction.

RBCs : Red blood cells.

Rev : Reverse.

RFLP : Restriction fragment length polymorphism.

SNPs : Single nucleotide polymorphisms.

TE : Tris(hydroxymethyl)aminomethane Ethylenediaminetetraacetic acid.

TI : Thalassemia intermedia.

TM : Thalassemia major.

Tris : Tris(hydroxymethyl)aminomethane.

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# Chapter one

## 1.0 Introduction

Thalassemias are a group of heterogeneous genetic disorders of hemoglobin synthesis which are described by the reduction of the synthesis of one or more of the globin chains to cause an imbalance in the synthesis of globin chains, defective hemoglobin synthesis and subsequently causing anemia (Tangvarasittichai, 2011; Thein, 2013). Depending on the type of globin chain being synthesized in reduced amounts, thalassemia can be classified as either alpha-thalassemia (α-thalassemia) or beta-thalassemia (β-thalassemia).

α-thalassemia is caused by the reduced synthesis of the alpha globin chains that constitute the hemoglobin molecule. This reduced synthesis of the alpha globin chains is caused by the deletion or other mutation in one or more of the four alpha globin genes which are located on chromosome 16. Four genes synthesize the alpha globin chains; two pairs being inherited from each parent (Hillman & Ault, 1995). Two phenotypes of the α-thalassemia have been identified. They are; α-thalassemia 1, which is characterized by thalassemia minor in the heterozygous state and α-thalassemia 2, which is characterized with no clinical or hematological abnormality in the heterozygous state. These two variants of α-thalassemia are now known as $\alpha^{o}$-thalassemia and $\alpha^{+}$-thalassemia respectively (Thein, 2013).

β-thalassemia also is a genetic disorder which is caused by the reduction or absent synthesis of the beta globin chains that constitute the hemoglobin molecule. This reduction or absent synthesis of the β-globin chains is due to mutations on chromosome 11 which affect the transcription, the translation and also the integrity of the β-globin chains being synthesized (Tangvarasittichai, 2011). The two genes located on chromosome 11, one gene inherited from each parent, synthesize the β-globin chains. Two phenotypes of β-thalassemia have been identified. They are; $\beta^{0}$-thalassemia and $\beta^{+}$-thalassemia. In $\beta^{0}$-thalassemia, there is complete absence of the synthesis of beta globin chains, whereas, in $\beta^{+}$-thalassemia, there is reduction in the synthesis of the beta globin chains (Thein, 2013). Most of the β-thalassemias are inherited as Mendelian recessives with a rare subgroup of the β-thalassemia alleles being inherited as Mendelian dominant negatives (Thein, 2013).

An estimated over 40,000 people all over the world are born affected with β-thalassemia every year (Weatherall, 2010), with the highest incidence in the Mediterranean region, the Middle East, North America and South East Asia (Maria, Vip & Ali, 2014). In Cyprus, thalassemia used to be a serious public health problem with one in every 230 Cypriot children below 10 years diagnosed to be thalassemic until 30 years after the Thalassemia Prevention and Control Programme of 1980 was implemented. Currently, no baby has been born with thalassemia since 2002 due to the effectiveness of the Thalassemia Prevention and Control Programme. The screening programme showed that the Turkish Cypriot population has the highest prevalence of β-thalassemia in the whole world (Gulsen, 2007).

β-thalassemia patients vary from those dependent on regular blood transfusions to those who are not dependent on blood transfusions regularly depending on the severity of the clinical features of the disease. β-thalassemia major patients regularly depend on blood transfusions for their survival (T.I.F., 2008), β-thalassemia intermedia patients only require occasional blood transfusion for the prevention and management of complications or when there is need due to specific clinical symptoms (T.I.F., 2013; Olivieri, 2012; Taher *et al.*, 2012), while β-thalassemia minor patients are non-transfusion-dependent.

The hemoglobin molecule is a tetramer of globin chains made up of two pairs of different polypeptide subunits (globin chains). Each globin subunit forms a stable linkage with heme molecule situated at the external surface of the protein. The primary role of hemoglobin is to transport molecular oxygen ($O_2$) from the lungs to the peripheral tissues. Besides that, it also functions in the transport of carbon dioxide ($CO_2$) and protons ($H^+$) from the peripheral tissues back to the lungs when it unloads the $O_2$ molecule.

Three principal hemoglobin molecules have been identified. They are; the fetal hemoglobin (HbF), composed of two alpha and two gamma globin subunits ($\alpha_2\gamma_2$), the normal adult hemoglobin (HbA), comprised of two alpha and two beta globin subunits ($\alpha_2\beta_2$) and the minor adult hemoglobin ($HbA_2$), composed of two alpha and two delta globin subunits ($\alpha_2\delta_2$) (Peter & Victor, 2009; Bernard & Franklin, 2016).

In the normal healthy condition, when there is a balance in the ratio of α-globin to non-α-globin subunits, hemoglobin functions in the optimal oxygen homeostasis and

the buffering of acidic metabolic wastes such as $CO_2$. Whereas, in the disease condition, when there is imbalance in the ratio of α-globin to non-α-globin subunits, free α-globin subunits become toxic particularly to the red blood cells giving rise to the pathophysiology of thalassemias (Nienhuis & Nathan, 2012; Bernard & Franklin, 2016).

HbF is an important contributor to the severity of the clinical features of β-thalassemia. High levels of HbF are associated with milder clinical features in β-thalassemia intermedia patients (Khaled *et al.*, 2012). Lower levels of HbF production have been shown to be related with β-thalassemia major in β-thalassemia patients with the same thalassemia mutations (Galanello *et al.,* 2009). In the case of sickle cell disease patients, HbF has also been shown to be a significant modifier of disease and mortality in adults with the disease (Platt *et al.,* 1991; Platt *et al.,* 1994).

A better balance of the ratio between the α-globin chains and the non-α-globin chains of hemoglobin have been shown to be the cause of milder clinical symptoms observed among thalassemia patients (Akhtar *et al.,* 2013). Increased levels of HbF, for example, have been shown in many studies to affect the clinical outcome of patients with β-thalassemia and even sickle-cell disease (Haj Khelil *et al.,* 2011; Mtatiro *et al.,* 2015).

Some background genes or other loci epistatically interact with the β-globin gene and are thereby able to influence the disease severity in patients with β-thalassemia intermedia and β-thalassemia major. These background genes or other loci are called genetic modifiers of β-thalassemia (Thein, 2008).

At the primary level, these genetic modifiers of β-thalassemia are the $\beta^0$ and $\beta^+$ alleles and the co-inheritance of α-thalassemia determinants. At the secondary level, they are the variations otherwise called polymorphisms at HbF-associated quantitative trait loci (QTL). These QTL include; *Xmn*I-Gγ (HBG2c.-211 C>T) which is mapped inside the β-globin gene cluster (Thein *et al*., 1987), *BCL11A* which is mapped outside the β-globin gene cluster and *HBS1L-MYB* which is also mapped outside the β-globin gene cluster (Thein, 2008).

The presence of the *Xmn*I-Gγ (*HBG2*c.-211 C>T) polymorphism in the homozygous state has been reported to be related with the increased production of HbF in normal adults at a minimal level and at a beneficial level in patients with homozygous

mutations or compound heterozygous mutations of the β-globin gene (Papai *et al.*, 2012).

The B-cell CLL/lymphoma 11A gene (*BCL11A*) is a 91,197-Da protein coding gene located on chromosome 2 with 835 amino acids which functions molecularly as a direct repressor of HbF production and a major regulator of developmental γ-to-β globin gene switch (Sankaran *et al*., 2008). It is a myeloid and B-cell proto-oncogene and also plays an important role in leukemogenesis and hematopoiesis (Nakamura *et al*., 2000).

Currently, more than 800 different types of mutations and structural variations have been well characterized in the β-globin gene using the existing genomic protocols for the characterization of genes (Giardine *et al.*, 2014).

In β-thalassemia, more than 200 point mutations have been identified and are believed to be responsible for the various clinical states due to the varying arrangements of compound heterozygous alleles (Cao, Moi & Galanello, 2011). Among the Turkish-Cypriot population, there are basically four distinct β-thalassemia alleles that account for 96% of the β-thalassemia mutations in this population. These four distinct β-thalassemia alleles are; IVS-I-1(G-A), IVS-I-110 (G-A), IVS-I-6(T-C) and IVS-II-745(C-G) alleles (Tuzmen, Basak & Baysal, 1997).

Three major loci have been reported in some genome-wide association studies (GWAS) to play important roles in increasing the levels of HbF in β-thalassemia patients and healthy individuals. These loci include; *BCL11A* gene located on chromosome 2p16.1, *HBG2*, and intergenic regions *HBS1L-MYB* in the 6q23.3 chromosomal regions, and they collectively are accountable for, and influence the variation of HbF in β-thalassemia patients and healthy individuals by up to 20–50% (Danjou *et al*., 2012; Thein, 2008; Bauer *et al.*, 2013).

The presence of the polymorphism has been shown to be promising in predicting the response of β-thalassemia patients to treatment. Saqib *et al.* (2013) reported that the presence of *Xmn*I-Gγ (*HBG2*c.-211 C>T) polymorphism in β-thalassemia patients is a predictor of the response of β-thalassemia patients to hydroxyurea treatment and hence the possibility that β-thalassemia patients with this polymorphism can be managed with less dependence on blood transfusion.

### 1.1 Aim of the Research

The aim of this research is to genotoype and subsequently determine the frequency of the *BCL11A* rs11886868 C>T (*Mbo*II*)* and rs766432 A>C polymorphisms and the *HBS1L-MYB* rs9399137 C>T polymorphism in beta-thalassemia patients of Turkish-Cypriot origin.

# Chapter two

## 2.0 Review of literatures

### 2.1 Hemoglobin

The red blood cells (RBCs) have an important function of transporting molecular oxygen ($O_2$) from the lungs into the tissues and transporting carbon dioxide ($CO_2$) from the tissues back to the lungs. Molecular oxygen has a poor solubility in water hence, to achieve this important function of the RBCs in higher organisms, there is need for a special transport system. In the plasma, the solubility of $O_2$ is barely 3.2 ml $l^{-1}$ of blood plasma whereas, in the RBCs, the protein; hemoglobin can bind a maximum of 220 ml $l^{-1}$ of RBCs (Koolman & Roehm, 2005).

Hemoglobin is contained in the blood at concentrations between 140–180 g $l^{-1}$ in men and between 120–160 g $l^{-1}$ in women which is about two times the concentration of plasma proteins at a range between 50–80 g $l^{-1}$ (Koolman & Roehm, 2005).

#### 2.1.1 Hemoglobin structure

Hemoglobin (Hb) is a heterotetrameric globular protein which consists of two α-chains and two non-α-chains (usually β-chains) of globin subunits each with masses of 16 kDa. The entire Hb molecule is made up of nearly 600 amino acid residues in the four globin subunits folded into globular shapes and connected to form a structure which is 5.5 nm in diameter (Nelson & Cox, 2008). The four globin sub-units are held together by noncovalent interactions. The α-globin and non-α-globin subunits have different sequences of amino acids but are both folded in the same manner (Koolman & Roehm, 2005; Nelson & Cox, 2008). On the α-globin subunits, there are 141 residues of amino acids. On the β-globin subunits, there are 146 residues of amino acids each. On each of the four globin sub-units, a heme (ferroprotoporphyrin IX) prosthetic group is attached which contains an iron atom which is in the ferrous state ($Fe^{2+}$). It therefore consists of four heme prosthetic groups buried in hydrophobic pockets of the four globin chains and depends on the four iron atoms on the of the heme group in the ferrous state. The $Fe^{2+}$ ions constitute only 0.3% of its mass. Hemoglobin molecule is soluble in water, has an isoelectric point of 6.8 and a molecular weight of 64,500Da. In normal hemoglobin, each of the α-globin sub-unit of hemoglobin is paired with a β-globin subunit in an identical symmetric manner. Therefore, Hb molecule can also be considered to be a dimer of αβ-protomers. Each

of the globin sub-unit of hemoglobin is structurally different, has a different affinity for $O_2$, has a different electrical charge and hence, different electrophoretic mobility (Tangvarasittichai, 2011; Koolman & Roehm, 2005).

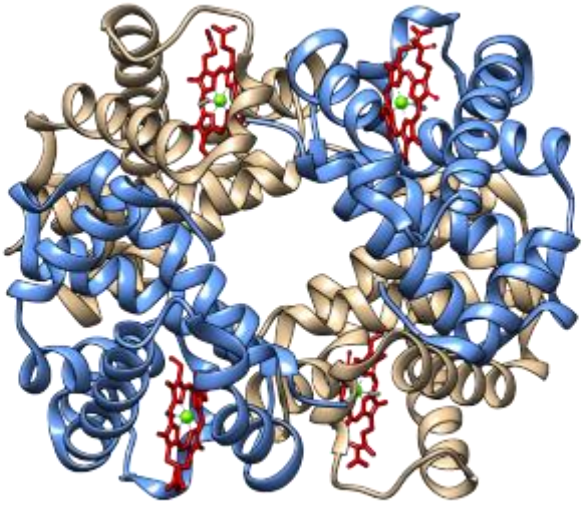


**Figure 2.1**: Tetrameric structure of globular hemoglobin molecule showing the α-subunits in brown, β-subunits in blue, heme groups in red and $Fe^{2+}$ ions in green (Sebia, 2015).

Two alternative forms of Hb exist in equilibrium in the mammalian RBCs. These two alternative forms are the tensed form (T-form) and the relaxed form (R-from) which correspond to the deoxyhemoglobin and the oxyhemoglobin forms, respectively. In the absence of a ligand, Hb molecule is preferably in the T-form due to the existence of additional salt bridges and other noncovalent interactions in the interface between the two αβ-dimers. In the presence of a ligand, there is a modification in the tertiary structure of the Hb molecule due to the progressive loosening of the noncovalent bonds holding the Hb tetramer together in the T-form giving rise to the switch to the R-form which has a high affinity for $O_2$ (Perutz, 1970; Jensen, Fago & Weber, 1998).

### 2.1.2 Hemoglobin function

Hemoglobin molecule plays transport, metabolic, homeostatic and buffering roles in the red blood cells of the blood.

In mammalian tissues, oxygen ($O_2$) is needed for the metabolic needs of oxidation and the products of oxidation in these mammalian tissues; carbon dioxide ($CO_2$), needs to be excreted in order to maintain optimum homeostasis. To supply these tissues with $O_2$ and get rid of $CO_2$, hemoglobin is needed.

To transport $O_2$ molecular oxygen ($O_2$) binds reversibly with the hemoglobin molecule at the heme group. This reversible binding of $O_2$ ensures that the heme iron is maintained in the ferrous ($Fe^{2+}$) state and that the binding of $O_2$ is more preferred compared to the binding of other potential heme ligands.

The affinity hemoglobin to $O_2$ varies significantly with the globin structure and it is allosterically modulated by the binding of allosteric co-factors such as organic phosphates, protons ($H^+$), and chlorides to specific binding sites on the hemoglobin molecule hence lowering the $O_2$ affinity of the heme groups of hemoglobin. These allosteric effectors preferentially bind to the T-form and stabilize them by the formation of additional bonds. The binding of $O_2$ is cooperative, meaning that, the binding of $O_2$ to one subunit of the hemoglobin molecule facilitates the binding of $O_2$ to the other deoxygenated subunits by increasing their $O_2$ affinity. This interaction is revealed by the sigmoid shape characteristic of the $O_2$ equilibrium curve. The $O_2$ cooperativity of the mammalian hemoglobin is independent of the pH values whereas, in the lower animals such as pishes, it is dependent on the pH value (Antonini & Brunori, 1971; Perutz, 1990; Riggs, 1988).

To transport metabolically produced $CO_2$ out of the peripheral tissues into the lungs for excretion, $CO_2$ is taken up by hemoglobin in a reaction facilitated by the binding of $H^+$ to the hemoglobin at its allosteric sites. This reaction ($H^+$ binding), drives the hydration of $CO_2$ in the red blood cells towards bicarbonate formation in a reaction catalyzed by the enzyme carbonic anhydrase. The bicarbonate produced is then transported to the plasma by $HCO_3^-/Cl^-$ exchange through the membranes of the red blood cells and diffuses. In this way, both $H^+$ and $HCO_3^-$ produced are excreted and the equilibrium moves further to the right favoring the binding of $CO_2$ as blood flows through the capillaries in the tissues as shown in the equation below;

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

Alternatively, $CO_2$ also reacts with the uncharged α-amino groups of globin subunits of hemoglobin to form carbamic acids. At physiological pH, the α-amino groups may be charged and the carbamic acid formed dissociates to carbamate as shown below;

$$Hb\text{-}NH_3^+ \rightleftharpoons Hb\text{-}NH_2 + H^+$$

$$Hb\text{-}NH_2 + CO_2 \rightleftharpoons Hb\text{-}NHCOOH$$

$$Hb\text{-}NHCOOH \rightleftharpoons Hb\text{-}NHCOO^- + H^+$$

The formation of carbamate is more prominent in the T-form of hemoglobin than in the R-form and this has a physiological importance in mammals. In humans for example, the binding of $CO_2$ to the T-form of hemoglobin accounts for 87% of $CO_2$ exchange while the binding of $CO_2$ to the R-form only accounts for 13% of $CO_2$ exchange (Klocke, 1988).

The structure and function of hemoglobin of hemoglobin molecule fundamentally depends on its equilibrium. To ensure the binding/release of $H^+$ by hemoglobin in the red blood cells necessary for the hydration–dehydration of $CO_2$, exchange of $H^+$ between hemoglobin and its solvent (plasma) is essential. This exchange of $H^+$ makes hemoglobin an effective non-bicarbonate buffer which functions by limiting the slight changes in blood pH upon the changes in the blood acid or base concentration. The net charge on the hemoglobin molecule determines the pH of the red blood cells by distributing the $H^+$ across the membranes of the red blood cells. This distribution of $H^+$ is vital in forming intrasubunit and intersubunit salt bridges in hemoglobin molecule and is also vital for the binding of ligands like chlorides and organic phosphates to hemoglobin (Jensen, Fago & Weber, 1998).

An imbalance in the α-globin and non-α-globin subunits of hemoglobin causes the free α-globin subunits to precipitate leading to loss of normal function and subsequently giving rise to the pathophysiology of the thalassemias (Nienhuis & Nathan, 2012).

### 2.1.3 Hemoglobin variants

Currently, more than 1000 disorders of hemoglobin synthesis and/or structure have been identified and well-studied, giving an insight on how these mutant genotypes alter the functions of the hemoglobin molecule synthesized and its clinical phenotype. The correlation between the genotype and phenotype of these mutant hemoglobins has elucidated the pathophysiological mechanisms of the associated hemoglobinopathies (Forget & Bunn, 2016).

These mutant hemoglobins, otherwise called hemoglobin variants, are caused by genetic variations. Some hemoglobin variants cause diseases and are referred to as pathological hemoglobin variants while others have no detectable pathology and are referred to as non-pathological variants (Forget & Bunn, 2016).

Some non-pathological hemoglobin variants include; hemoglobinA (HbA) which constitutes 95–98% of adult hemoglobin, hemoglobin$A_2$ ($HbA_2$) a minor hemoglobin constituting 2–3% of adult hemoglobin and hemoglobin F (HbF) the fetal hemoglobin produced by the fetus during pregnancy and is adapted for efficient $O_2$ transport in low $O_2$ environment constituting about 2.5% of adult hemoglobin (Peter & Victor, 2009).

Some hemoglobinopathological hemoglobin variants include; sickle hemoglobin (HbS) in which there is a substitution of glutamine with valine at position six of the β-globin subunit (B-Gln6 $\longrightarrow$ Val6). The heterogeneous form of this variant (HbAS) confers survival advantage against complications of *Falciparum malaria* due to the fact that HbAS has 40% HbS and 56–68% Hb A. Hemoglobin H (HbH) is usually synthesized in response to severe shortage of α-globin subunits, it has an unusual high affinity for $O_2$. It occurs in α-thalassemia patients and is composed of four β-globin sub-units ($B_4$). HemoglobinM (HbM) is characterized by the substitution of histidines to tyrosines in either the α-globin, β-globin or γ-globin subunits within the heme pockets resulting in the stabilization of the iron ion in the heme pocket in the ferric ($Fe^{3+}$) state (Forget & Bunn, 2016).

### 2.1.4 Post translational alteration of hemoglobin molecule

Hemoglobin molecules are subject to post-translational alterations that modify their structure. Some of these modifications significantly alter their functions. Glycation of the hemoglobin molecules at the amino terminals of α-globin and β-globin subunits with relatively low p$K_a$ by the open aldehyde molecules of glucose form an aldimine abduct which is unstable and rearranges to form ketoamine linkage that is more stable. Amino-terminal acetylation occurs at the amino terminal of almost 20% of γ-globin subunits. Rare variants of human hemoglobin have been identified with amino acid replacement at the amino terminal region giving rise to the retention of the initiator methionine and amino terminal acetylation. The amino terminals of α and β-globin subunits of hemoglobin in uremia patients have been found to be carbamylated by cynate to form adducts. Deamination of the asparagine residues adjacent to the histidine residues on the globin subunits of hemoglobin have been identified in seven variants of human hemoglobin without impacting function to these variants (Forget & Bunn, 2016).

### 2.1.5 Acquired alteration of hemoglobin function

The $Fe^{2+}$ on the heme group of hemoglobin molecules can be oxidized to $Fe^{3+}$ by certain drugs and toxins to form methemoglobin. The $Fe^{3+}$ of methemoglobin does not bind $O_2$ and the presence of one or two $Fe^{3+}$ per tetramer causes stabilization of the relaxed quaternary structure thereby increasing the $O_2$ affinity of the remaining heme groups. Carbon monoxide (CO) binds readily to with the heme iron ($Fe^{2+}$) to form carboxyhemoglobin since the affinity of hemoglobin to CO is 210 times greater than that of $O_2$. Carboxyhemoglobin impairs the transport of $O_2$ to cells and tissues leading to stupor, coma and death. The binding of allosteric modulators such as $H^+$ and 2,3-bisphosphoglycerate to the deoxyhemoglobin shifts the oxyhemoglobin binding curve to the right thereby altering the $O_2$ and $CO_2$ transport function of hemoglobin (Perutz & Brunori, 1982; Forget & Bunn, 2016).

### 2.1.6 Thalassemia

Thalassemias are disorders of the autosomal recessive genes which cause impairment in the production of one or more of the four globin subunits that constitute the hemoglobin molecule. They are inherited disorders of autosomal recessive gene that cause different degrees of anemia ranging from the significant to the life threatening anemia (Ridolfi *et al.*, 2002).

Clinically, it was first discovered by Dr. Thomas Cooley in the year 1925 and he characterized it as an anemic syndrome with microcytic erythrocytes. Hence, it was referred to as "Cooley's anemia". The name was later changed from Cooley's anemia to 'thalassemia' because it was found in regions around the Mediterranean Sea. The name 'thalassemia' originates from the old Greek words; 'thalassa' meaning 'sea' and 'haima' meaning 'blood' (Cooley, 1946). Weatherall (1997) reported a higher risk of having the thalassemia genes among individuals of the Mediterranean, South East Asian, Middle Eastern and African descent.

Thalassemia was previously reported to be exclusively distributed in areas around the Mediterranean basin stretching to the Middle East, the Indian subcontinent up to the South East of Asia known as the 'thalassemia belt' but people's migration round the world has spread the thalassemia genes to all parts of the world (Tangvarassittichai, 2011). The thalassemias are caused by the decreased or absent synthesis of either the α-globin or β-globin subunits of hemoglobin. Two types of thalassemias have been characterized viz; alpha-thalassemia (α-thalassemia) and beta-thalassemia (β-thalassemia) (Thein, 2013).

In α-thalassemia, there is absence or decreased synthesis of normal α-globin subunits to pair with the non α-globin subunits in order to form hemoglobin molecules. This gives rise to relative excess of γ-globin subunits in the red blood cells of the fetus and new born babies, and a relative excess of β-globin subunits in the red blood cells of children and adults. In α-thalassemia patients, the excess β-globin subunits form soluble tetramers known as hemoglobin H which are unstable and also precipitate within the red blood cells to form insoluble inclusions called "Heinz bodies" which damage the membranes of the red blood cells. In general, α-thalassemia has less severe clinical symptoms due to the excess β-globin subunits which have a less

damaging effect on the red blood cells (Tangvarasittichai, 2011; Forget & Bunn, 2016).

In a diploid human cell, there are four copies of the α-globin gene (two pairs), one pair each inherited from the maternal and paternal chromosome 16. Deletion or loss of function of one or more of these four α-globin genes results in α-thalassemia (Higgs *et al.,* 1989).

In the β-thalassemias, there is absence or decreased synthesis of normal β-globin subunits to pair with the α-globin subunits in order to form the hemoglobin molecule. This gives rise to a comparative surplus of α-globin subunits in the red blood cells. These excess α-globin subunits accumulate in the red blood cells and bone marrow, and because they are highly insoluble, they precipitate causing the destruction of the red blood cells consequently leading to ineffective erythropoiesis, an increase in erythropoietin and bone marrow proliferation which results in the characteristic bone deformation observed in β-thalassemia patients if left untreated (Tangvarasittichai, 2011).

In a diploid human cell, there are two copies of the β-globin gene (one pair), one copy each inherited from the maternal and paternal chromosome 11. Deletion or loss of function of one or both β-globin genes results in β-thalassemia (Lee *et al.,* 1999).

### 2.1.7 Beta thalassemia

β-thalassemia is a recessive autosomal disorder of the β-globin gene caused by the absence ($\beta^0$) or reduced synthesis ($\beta^+$) of the β-globin subunits that constitutes the hemoglobin tetramer (Cao & Galanello, 2010).

Three distinct conditions of β-thalassemia have been identified each with varying severity of clinical conditions viz; β-thalassemia minor, β-thalassemia intermedia and β-thalassemia major in order of increasing severity of clinical symptoms (Weatherall, 1994). The severity of the clinical conditions depends on the level of imbalance between α-globin and non-α-globin subunits (Cao & Galanello, 2010). At the molecular level, the β-thalassemias are heterogeneous with over 200 disease causing mutations being identified (Cao & Galanello, 2010; Weatherall, 1994; Tangvarasittichai, 2011).

β-thalassemia minor is the heterozygous form ($\beta^+/\beta^0$) of β-thalassemia. Patients affected with this form of thalassemia have heterozygous mutation on the β-globin

gene and are termed β-thalassemia carriers (Kazazian Jr., 1990). β-thalassemia intermedia is a complex form of β-thalassemia. Patients affected with this form of thalassemia are either homozygous or heterozygous for the mutation on the β-globin gene. In homozygous individuals ($\beta^0/\beta^0$ or $\beta^+/\beta^+$), both genes are affected and the disease is inherited in recessive manner (Galanello & Cao, 1998). In a few individuals, the compound heterozygous ($\beta/\beta^0$), only one of the genes is affected while the other is normal and the disease is inherited in a dominant manner (Weatherall & Clegg, 2011). β-thalassemia major is the homozygous form of the disease ($\beta^0/\beta^0$ or $\beta^+/\beta^+$). Individuals with this form of thalassemia are homozygous for the mutation on the β-globin gene and usually require medical attention within the first two years for survival (Cao & Galanello, 2010).

### 2.1.8 Prevalence of β-thalassemia

It is estimated that over 40,000 people are born affected with β-thalassemia worldwide annually. The highest incidences are recorded in the Mediterranean region, the Middle East, North America and South East Asia (Maria, Vip & Ali, 2014). The average trait prevalence of the β-thalassemia syndrome is 4.8% Thailand, 7% in Greece and 15% in Cyprus (Wheatherall, 1998; Weatheral & Clegg, 2001). Before the implementation of the thalassemia prevention program in 1980, Gulsen (2007) reported that one child out of every 230 children below the age of 10 in Cyprus was thalassemic. In African and Asian populations, α-thalassemia is most prevalent (Weatherall, 1987).

High prevalence of the thalassemias and that of sickle cell disease observed in some of the tropical and the subtropical regions of the world have been attributed to the resistance of these patients against *Plasmodium falciparum* caused by the heterozygous carriers of the thalassemias and sickle cell disease. Carriers of sickle cell disease and also carriers of the thalassemias thus have selective advantage and are resistant to malaria compared with their homozygotes. This makes the abnormal genes to be persistent in the population at polymorphic frequencies so long as the malaria parasite exists and is active in the population (Allison, 1954; Allen *et al.*, 1997).

### 2.1.9 Molecular basis of β-thalassemia

A few common mutations as well as rare mutations are found within populations that are at risk for β-thalassemia. These common and rare mutations are in convincing linkage disequilibrium with some specific polymorphisms existing on the β-globin gene cluster located on chromosome 11 (Flint *et al.,* 1993).

The β-globin gene cluster is mapped on the short arm of chromosome 11 in a region that also contains the delta globin gene, a pseudo gene (γβ1), fetal A-gamma gene, fetal G-gamma gene and epsilon gene. It is a 1.6 kb gene with three exons. This gene is controlled by an adjacent 5' promoter containing a CAAT, TATA and a duplicated CACCC nucleotide sequences. It also has a major regulatory region mapped 50 kb from the β-globin gene (Cao & Galanello, 2010).

Unlike the α-globin gene cluster which is duplicated, the β-globin gene cluster is not duplicated; each haploid cell has only two β-globin genes (Kazazain Jr., 1990). Mutations on the β-globin genes have been described that have effect on the process of gene expression starting from transcription to translation and post-translational stability of the synthesized β-globin subunits (Weatherall, 1994). The extent of the severity of the clinical symptoms of the β-thalassemias depends on the significance of the mutations on the β-globin gene and the effect on β-globin synthesis (Kazazain Jr., 1990; Weatherall, 1994).

The mutations of the β-globin gene either result to lack of β-globin subunits synthesis ($\beta^0$-thalassemia) or reduced synthesis of β-globin subunits ($\beta^+$-thalassemia). Mutations that occur close to or in the conserved promoter regions and in the 5' untranslated regions of the β-globin gene down-regulate the transcription of β-globin subunits usually causing mild $\beta^+$-thalassemia (Orkin *et al*., 1982). Transcription of the β-globin gene is completely deactivated by deletions in the 5' regions of the β-globin gene consequently causing $\beta^0$-thalassemia. The splicing of mRNA precursor and the ineffectual cleavage of mRNA transcript leads to β-thalassemia as well. Some other mutations produce normal mRNA while others significantly decrease the quantity of normally spliced mRNA. When the mutations occur on the same dinucleotide sequences at the exon–intron junctions necessary for the elimination of interfering sequences and exon splicing to yield a useful mRNA, it results to $\beta^0$-thalassemia (Treisman *et al*., 1982). Alternatively, when the mutations occur on the extremely

preserved nucleotides that flank these nucleotide sequences or occur in hidden splice sites that are similar to donor or receptor splice sites, it causes severe as well as mild $\beta^{+}$-thalassemia (Orkin *et al*., 1982). Small deletions or substitutions that occur at the conserved AATAAA sequence of the 5' untranslated regions leads to abnormal cleavage of the mRNA transcript causing mild $\beta^{+}$-thalassemia (Wong *et al*., 1987). Other mutations that interfere with the translation of β-globin subunits at the initiation, elongation or termination steps cause $\beta^{0}$-thalassemia. Nearly half of all the mutations of the β-globin gene affect translation. These mutations include nonsense or frame-shift mutations that introduce early stop codons and terminate the translation prematurely causing $\beta^{0}$-thalassemia (Orkin & Goff, 1981).

In the dominantly inherited β-thalassemia ($\beta^{+}$), mutations on exon 3 causes the synthesis of unstable β-globin subunits with varying lengths which precipitates alongside the excess α-globin subunits in red blood cells precursors causing erythropoiesis even in the heterozygous state (Tangvarasittichai , 2011).

**2.2.0 Clinical features of β-thalassemia**

Individuals affected with β-thalassemia major have serious anemia and require regular blood transfusion because they become progressively pale and fail to thrive after birth. Clinically, they are characterized with feeding disorders, irritability, diarrhea, recurring bouts of fever and enlarged abdomen. Initiation of a regular blood transfusion program at the onset of the disease symptoms to help maintain hemoglobin concentration at a minimum of 95–105 g $l^{-1}$ will allow normal growth and development until the age of 10–11 years when these individuals become at risk of developing problems due to post-transfusion iron overload which depends on the effectiveness of iron chelation therapy. These complications include; retarded growth, failure of sexual maturation, immune decline, hypersplenism and osteoporosis among others (Borgna-Pignatti & Galanello, 2004; Voskaridou & Terpos, 2004).

Untreated individuals affected with β-thalassemia intermedia are clinically between the extremes of the β-thalassemia major and β-thalassemia minor. They have milder anemia, hence, they do not require regular blood transfusions (Nathan & Oski, 1993).

Individuals who are at the milder end of this spectrum are totally asymptomatic until their adult life when they develop slight anemia. Individuals at the severe end of the spectrum are characterized by retarded growth and development, require blood

transfusion between the age of 2–6 years and are able to survive without consistent blood transfusion at birth (Tangvarasittichai, 2011). The common clinical symptoms of β-thalassemia intermedia include; jaundice, spleen and liver enlargement, moderate to severe bone deformation, pallor, leg ulcers, propensity to develop osteoporosis and thrombotic complications (Cao & Galanello, 2010).

Individuals affected with β-thalassemia minor (carriers) have no serious clinical symptoms. They are mildly anemic and do not require blood transfusion. Such individuals require serious genetic counseling because they are at risk of producing children with β-thalassemia major or a combination of sickle cell disease and β-thalassemia trait (Sβ-thalassemia) if both parents are β-thalassemia carriers (Tangvarasittichai , 2011).

### 2.2.1 Hematological features of β-thalassemia

Individuals affected with β-thalassemia major have severely reduced red blood cell volume (severe microcytic anemia), severely reduced red blood cell hemoglobin content (severe hypochromic anemia) due to increased red blood cells and low mean corpuscular volume (MCV) and low mean corpuscular hemoglobin (MCH). Peripheral blood smears of β-thalassemia major patients shows nucleated red blood cells. Individuals affected with β-thalassemia minor have reduced red blood cell volume (microcytosis), reduced red blood cell hemoglobin content (hypochromia) and increased $HbA_2$ level. Individuals with β-thalassemia intermedia have hematological features ranging in severity from those of β-thalassemia major to those of β-thalassemia minor (Cao & Galanello, 2010).

### 2.2.2 Molecular diagnosis of β-thalassemia

The molecular diagnosis of β-thalassemia begins with screening to detect defects in hemoglobin synthesis then a further analysis is carried out to determine the type of thalassemia. Negatively screened samples do not need to undergo further analysis while positively screened samples undergo further analyses which are often complicated and expensive in order to determine the type of thalassemia (Tangvarasittichai, 2010).

Full blood count is the primary screening for the diagnosis of thalassemia. This test employs an automated hematology analyzer to accurately measure erythrocyte indices of the blood such as MCV, MCH and red blood cell count (RBC). MCH and MCV

are the most essential indices that reveal whether an individual is thalassemic or not. Individuals with hypochromic microcytosis (MCH < 27 pg and MCV < 80 fl) are likely to be thalassemic, hence, should be subjected to further analysis. The Mentzer index which is a ratio of MCV to RBC (MCV/RBC) is important for distinguishing thalassemia from iron deficiency. Individuals with Mentzer index less than 13 (<13) are likely to be thalassemic while individuals with Mentzer index greater than 13 (>13) are iron deficient (Klee *et al.,* 2000; Tangvarasittichai, 2010).

### 2.2.3 Prenatal diagnosis of thalassemia

Pregnancies in which both parents are known carriers of thalassemia are at high risk for thalassemia. Such pregnancies could be diagnosed by analyzing fetal DNA collected via chronic villi sampling (CVS) between 9–12 weeks of gestation (Tuzmen *et al*., 1996) or by amniocentesis between 15–18 weeks of gestation (Tangvarasittichai, 2010).

Analysis of globin chain synthesis can also be used for prenatal diagnosis of thalassemia using a sample fetal blood collected via percutaneous blood sampling between 18–21 weeks of gestation (Tangvarasittichai, 2010).

### 2.2.4 Treatment of β-thalassemia

The treatment of β-thalassemia solely depends on the severity of the phenotype (Taher *et al*., 2012). β-thalassemia major patients are transfusion dependent, β-thalassemia intermedia patients are generally non-transfusion dependent except for those patients who are at the most severe end of the β-thalassemia intermedia spectrum (Taher *et al.*, 2011). β-thalassemia carriers do not show any clinical symptoms hence, do not require medical treatment but adequate genetic counseling.

Available treatment options for the β-thalassemias include; blood transfusion therapy, iron chelation therapy, splenectomy, bone marrow transplantation and stem cell transplantation (Walters *et al.,* 2005; Thomas *et al.,* 1982; Taher *et al*., 2012).

Some other promising treatment options such as hydroxyurea administration, gene therapy and sildenafil citrate administration are still under investigation (Sankaran & Nathan 2010).

### 2.2.5 Genotype–phenotype correlation in β-thalassemia

The extent of the α-globin/non-α-globin subunits imbalance in the hemoglobin molecule is the main determinant of the β-thalassemia phenotypes (Cao *et al*., 1990). Factors that can reduce this α-globin/non-α-globin subunits imbalance may have improving effect on the phenotype of the β-thalassemias. For example, β-thalassemia major which is transfusion dependent results from the homozygous or compound heterozygous forms of the β-thalassemia but a consistent proportion of the homozygotes results in the milder form of thalassemia known as β-thalassemia intermedia (Wainscoat, Thien & Weatherall, 1987; Cao *et al.*, 1990; Ho *et al.*, 1998).

In the homozygous β-thalassemia, three mechanisms have been proposed to have a genotype-phenotype correlation with ameliorating effect on the phenotype of the β-thalassemia. These mechanisms include;

**i.** The coinheritance of compound heterozygous or homozygous alleles for the mild β-thalassemia associated with reduced synthesis of β-globin subunits. The coinheritance of the homozygous alleles for the mild β-thalassemia ($\beta^+$) results in a mild phenotype whereas, coinheritance of compound heterozygous alleles for the mild and the severe β-thalassemia leads to a spectrum of phenotypes that range from mild to severe forms of β-thalassemia. In the presence of severe/mild β-thalassemia (compound heterozygous alleles), it is impossible to predict a mild phenotype (Cao & Galanello, 2010).

**ii.** The coinheritance of homozygous β-thalassemia and an α-thalassemia alleles that are able to reduce the synthesis of α-globin subunits thereby decreasing the α-globin/non-α-globin subunits imbalance. In the homozygous $\beta^+$-thalassemia genotype, a single deletion of an α-globin gene or inactivation of the $\alpha_2$-globin is required to improve the phenotype (Cao, Galanello & Rosatelli, 1994).

**iii.** The coinheritance of genetic factors that are able to sustain the constant synthesis of γ-globin subunits in adult life so as to reduce the extent of the α-globin/non-α-globin subunits imbalance. Such genetic determinants include; point mutations at Gγ-promoter or Aγ-promoter regions of the β-globin gene that results in hereditary persistence of fetal hemoglobin

(HPFH), the *BCL11A* gene mapped on chromosome 2 and the *HBS1L-MYB* intergenic regions mapped on chromosome 6 (Galanello *et al*., 2011). The point mutation on the Aγ-promoter region of the β-globin gene identified in some Sardinian β-thalassemia population is believed to compensate for the absence of the β-globin subunit synthesis in Sardinian $\delta\beta^{o}$-thalassemia patients (Pirastu *et al.,* 1984).

In the heterozygous β-thalassemia which is completely asymptomatic, two mechanisms have been identified to be responsible for modifying the phenotype to that of β-thalassemia intermedia. These mechanisms include;

i. Coinheritance of both heterozygous β-thalassemia and triple or quadruple α-globin genes that are able to increase the imbalance of α-globin/non-α-globin subunits. β-thalassemia minor individuals having the heterozygous forms of triplicate α-globin genes may develop a severe phenotype of β-thalassemia intermedia (Galanello *et al.,* 1983; Thien, Al-Hakin & Hoffbrand, 1984). β-thalassemia minor individuals with inherited chromosome containing the quadruple α-globin gene have been seen with the β-thalassemia intermedia phenotype (Sollaino *et al*., 2009).

ii. The presence of β-globin gene mutations capable of causing the synthesis of extremely unstable β-globin subunits. Heterozygous individuals with this condition are severely affected more than individuals with the β-thalassemia minor genes due to the synthesis of unstable β-globin subunits in addition to the synthesis of excess α-globin subunits causing an increase in the severity of the clinical phenotype (Thien, 1992).

### 2.2.6 Genetic modifiers of β-thalassemia

Genetic modifiers of β-thalassemia are those genes or loci that epistatically interact with the β-globin gene to cause variations in the severity of the β-thalassemia phenotypes (Thein, 2008). Basically, these genetic modifiers are broadly categorized into two groups, viz;

i. Primary modifiers: these are genetic variations or factors mapped on the β-globin gene and α-globin gene clusters that directly affect the synthesis of β-globin subunits or the severity of the clinical phenotypes of the β-thalassemia. They are the coinheritance of homozygous or compound

heterozygous alleles of the β-thalassemia, or the coinheritance of homozygous β-thalassemia alleles with α-thalassemia determinants capable of reducing the synthesis of α-globin subunits, or the coinheritance of genetic factors that are able to sustain the constant synthesis of γ-globin subunits in adults, or the coinheritance of heterozygous β-thalassemia alleles with the triple or quadruple α-globin genes, or the heterozygous mutations of the β-globin gene that results in the synthesis of unstable β-globin subunits (Galanello *et al.*, 2011).

ii. Secondary modifiers: these are genetic variations or factors mapped outside the β-globin cluster that affect some of the disease complications of the β-thalassemia phenotypes. These include; variations at the HbF associated quantitative trait loci (QTL) such as *BCL11A* gene (Menzel *et al*., 2007), the *HBS1L-MYB* intergenic region (Thein *et al*., 2007) and polymorphic motif in the promoter region of the gene that codes for bilirubin UDP-glucuronosyltransferase (Borna-Pignatti *et al*., 2003).

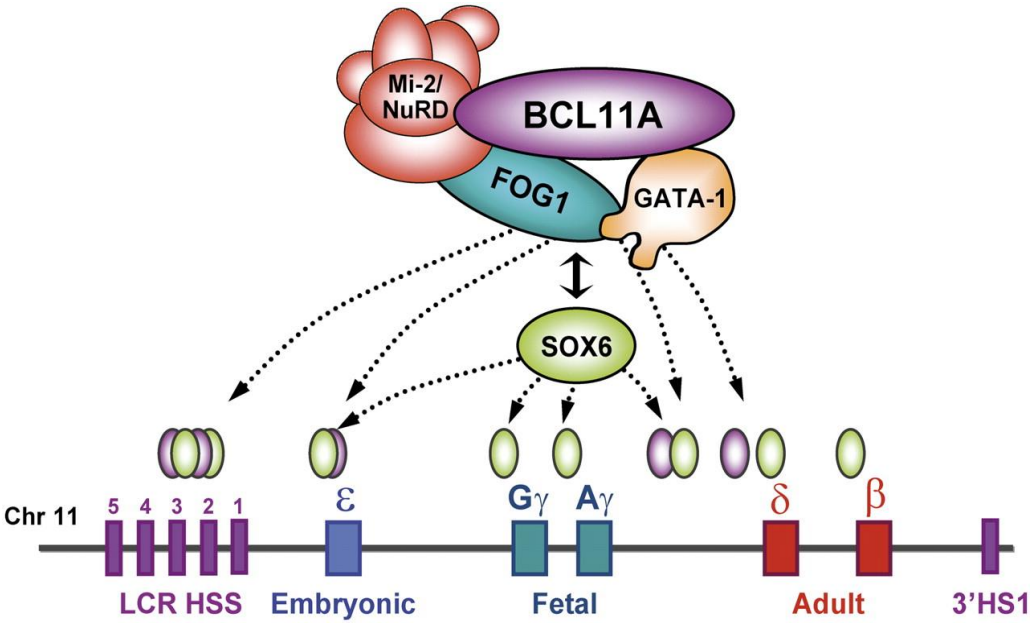



**Figure 2.2:** Molecular interaction of *BCL11A* gene with erythroid Transcription Factor (*GATA1*), Transcription Regulator (*FOG1*) and Mi-2/Nucleosome Remodeling and Deacetylase complex (*Mi-2/NuRD*) to activate the β-globin gene. *BCL11A* gene interacts with the upstream LCR and γδ-intergenic regions on the β-globin gene cluster to silence the γ-globin genes and activate the β-globin gene (Xu *et al.*, 2010).

### 2.2.7 Methods for determination of genetic polymorphism

Genetic polymorphisms on the β-globin gene are commonly determined using a number of polymerase chain reaction (PCR) methods.

Among these PCR methods are; amplification refractory mutation system (ARMS) analysis and restriction fragment length polymorphism (RFLP) (Rujito *et al.,* 2016). Other commonly used methods include the dot blot and the reverse dot blot analysis (Tuzmen & Schechter, 2001).

Both RFLP and ARMS analysis are based on primer-specific polymerase amplification using a set of primers that are complementary to most common polymorphism within a population under study (Old *et al.,* 2005).

RFLP method can distinguish DNA sequences based on fragment lengths yielded by specific endonucleases and can also indicate point mutation on the β-globin gene (Lee *et al.,* 2002). On the other hand, ARMS analysis which is also referred to as the allele specific PCR method utilizes two PCR reactions with primers specific for the normal allele contained in one reaction and primers specific for the mutant allele contained in the other reaction. When gel electrophoresis is done, a band from the normal reaction correspond to the normal allele, a band from the mutant reaction corresponds to the mutant allele while bands from both reactions corresponds to the heterozygous allele. ARMS analysis was introduced for diagnosing thalassemia and is more accurate than the RFLP (Simsek *et al.,* 1999; Old *et al.,* 2000).

A recent method is the next generation sequencing (NGS) technique which is 99% accurate and sensitive and also has the advantage of either whole genome sequence analysis or a specific exon analysis (Scherer, 2015).

# Chapter 3

## 3.0 Materials and method

### 3.1 Chemicals

Invitrogen PureLink Genomic DNA Extraction Kit (Catalog No: K1820-02; Lot No: 174352) containing genomic wash buffer 1 and 2, Genomic Digestion Buffer, Genomic Lysis/Binding Buffer, Genomic Elution Buffer, Proteinase K, RNase A, genomic spin columns and collection tubes was obtained from life technologies, USA. Thermo Scientific PCR Kit (Catalog No: K0171) containing PCR Master Mix (2×) and nuclease-free water was obtained from Thermo Scientific Molecular Biology Lithuania (EU). The primers: rs11886868_Fwd and rs11886868_Rev; rs766433_A_Fwd, rs7664323_A_Rev, rs766432_C_Fwd and rs766432_C_Rev; rs9399137_C_Fwd, rs9399137_C_Rev, rs9399137_T_Fwd and rs9399137_T_Rev were obtained from Thermo Fisher Scientific, USA. *Mbo*II digestion enzyme was obtained from Thermo Scientific, USA. 50 bp DNA ladder (1 μg $\mu l^{-1}$), 100bp DNA ladder (1 μg $\mu l^{-1}$) and 10× blue juice gel loading buffer were obtained from Thermo Fisher Scientific, USA. Agarose gel (Lot No: 124543PR) was obtained from BIOMAX, USA. Ethidium bromide and EDTA were obtained from Sigma, USA. Tris base was purchased from Santa Cruz Biotechnology International, USA and Boric acid was obtained from Wisent Inc., Canada.

### 3.2 Methods

#### 3.2.1 Ethical approval

The study was approved by the Research and Publication Ethics Board of Eastern Mediterranean University. The procedure used in the study is in accordance with guidelines of the Declaration of Helsinki.

#### 3.2.2 Sample collection

Whole blood sample from each patient was collected in a collection tube with EDTA, labeled and stored appropriately prior to DNA isolation after obtaining their consent or that of their parents/guardians.

#### 3.2.3 DNA isolation

DNA was isolated using the PureLink Genomic DNA Mini Kit (Life Technologies) following the protocol recommended by the manufacturer as follows;

200 µl of defrosted whole blood was lysed in a fresh micro tube by adding 20 µl of Proteinase K (20 mg $l^{-1}$ in storage buffer) to breakdown the protein molecules in the blood, then 20 µL of RNase A (20 mg $l^{-1}$ in 50 mM Tris–HCl, 10 mM EDTA, pH 8.0) was added to breakdown the RNA molecules. 200 µl of Genomic Lysis/Binding Buffer was added and the mixture in the micro tube was properly mixed using a vortex then placed in the heat block to incubate at 55 °C for 20 min. The lysate was centrifuged at 16,873×*g* in a spin column placed in a collection tube for one minute at room temperature to bind the DNA molecules to the matrix of the spin column while collection tube was discarded.

The bound DNA molecules were washed using 500 µl of Wash Buffer 1 by centrifuging at 16,873×g for one min at room temperature, the collection tube was removed and substituted with a new one then the bound DNA molecules were washed again using 500 µl of Wash Buffer 2 by centrifuging at 16,873×g for three min at room temperature and the collection tube was discarded. The bound DNA molecules were eluted in a 1.5 ml sterile micro centrifuge tube by adding 100 µl of Elution Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) into the spin column placed in the 1.5 ml sterile micro centrifuge tube, incubated for one min at room temperature then centrifuged at 16,873×g for one min at room temperature. The eluted DNA was collected in the 1.5 ml sterile micro centrifuge tube and the spin column was discarded.

All the isolated DNA molecules were labelled accordingly and stored at 4 °C for short-term and at -20 °C for long-term storage.

### 3.2.4 Design of primers

The primers used for this research were adopted from Rujito *et al.,* (2016). The FASTA files for the single nucleotide polymorphisms were obtained from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) and the DNA sequences downloaded were used to determine the amplification patterns and sizes of the amplicons.

The details of the primers and restriction endonuclease used for this research are presented on **Table 3.1** below.

**Table 3.1** Details of primers and restriction endonuclease used for PCR-RFLP and tetra-primer ARMS-PCR amplification

| SNP Locus | Primers | Primers Sequence | Amplicon Size (bp) | Restriction Endonuclease | Interpretation of alleles |
|---|---|---|---|---|---|
| rs11886868 | rs11886868_Fwd<br>rs11886868_Rev | 5'-TTTGGTGCTACCCTGAAAGAC-3'<br>5'-ACTCAACAGTAGCAGAATGAAAGAG-3' | 540 | *Mbo*II | C: 478 bp, 62 bp<br>T: 540 bp |
| rs766432 | rs766432_A_Fwd<br>rs766432_A_Rev<br>rs766432_C_Fwd<br>rs766432_C_Rev | 5'-TTGTTTCGCTTTAGCTTTATTAAGGTACAA-3'<br>5'-GACGTGTTCTGTATCTTGATTTTGGT-3'<br>5'-CCAAACAGTTTAAAGGTTACAGACAGACT-3'<br>5'-AAAATGAATGACTTTTGTTGTATGTAGAG-3' | 193<br>135<br>116 | --- | A: 135 bp<br>C: 116 bp |
| rs9399137 | rs9399137_C_Fwd<br>rs9399137_C_Rev<br>rs9399137_T_Fwd<br>rs9399137_T_Rev | 5'-AATGTAATTAACTGAACATATGGTTAGTC-3'<br>5'-TTTATTGTTACAAGGTTAATTCACTGCC-3'<br>5'-GAAATACCATCACTGAGAAAAGCATAAG-3'<br>5'-CAGCAGGGTTGCTTGTGAAAAAACTTTA-3' | 365<br>178<br>243 | --- | C: 178 bp<br>T: 243 bp |

#### 3.2.4.1 Reconstitution of primers

The tubes containing the freeze-dried primers were briefly centrifuged to ensure that the primers settle at the bottom and appropriate volumes of Tris EDTA (TE) buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) was added to each tube.

#### 3.2.4.2 Reconstitution of rs11886868 primers

The rs11886868_Fwd primer was reconstituted to a concentration of 100 μM by adding 1572 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs11886868_Rev primer was reconstituted to a concentration of 100 μM by adding 1320 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

#### 3.2.4.3 Reconstitution of rs766432 primers

The rs766432_A_Fwd primer was reconstituted to a concentration of 100 μM by adding 1544 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs766432_A_Rev primer was reconstituted to a concentration of 100 μM by adding 1494 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs766432_C_Fwd primer was reconstituted to a concentration of 100 μM by adding 1478 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs766432_C_Rev primer was reconstituted to a concentration of 100 μM by adding 1728 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

#### 3.2.4.4 Reconstitution of rs9399137 primers

The rs9399137_C_Fwd primer was reconstituted to a concentration of 100 μM by adding 1565 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs9399137_C_Rev primer was reconstituted to a concentration of 100 μM by adding 1680 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs9399137_T_Fwd primer was reconstituted to a concentration of 100 μM by adding 1584 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

The rs9399137_T_Rev primer was reconstituted to a concentration of 100 μM by adding 1278 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0) and the solution was vortexed to mix properly and centrifuged briefly.

### 3.2.5 Genotyping of the rs11886868 (*Mbo*II) polymorphism

rs11886868 (*Mbo*II) polymorphism was determined using PCR-RFLP.

#### 3.2.5.1 Sample preparation for rs11886868 PCR

PCR amplification of rs11886868 was carried out using Thermo Scientific PCR master mix and pair of rs11886868 primers (rs11886868_Fwd and rs11886868_Rev primer) in a 25 μl PCR reaction mix containing 1× PCR master mix (0.625 U Taq polymerase, 2 mM $MgCl_2$, reaction buffer, 0.2 mM each of dTTP, dATP, dGTP and dCTP), 0.6 μM rs11886868_Fwd Primer, 0.6 μM rs11886868_Rev Primer, nuclease free water and genomic DNA template. The PCR master mix, primers, nuclease free water and DNA samples were thawed at room temperature. The PCR master mix and primers were vortexed and centrifuged briefly then placed on an arctic cold block. The thawed DNA samples were mixed gently and the PCR amplification was set up in a laminar flow cabin. A stock reaction mix containing 11.2 μl of sterile distilled water, 12 .5 μl of 2× PCR Master Mix, 0.15 μl rs11886868_Fwd primer and 0.15 μl rs11886868_Rev primer was prepared in an eppendorf tube and was mixed properly. 24 μl of the stock reaction mix was transferred into a PCR tube and 1 μl of genomic DNA template was added. The volumes of reagents used for the PCR amplification are presented on **Table 3.2a** and **3.2b** below.

**Table 3.2a.** Volumes of reagents used for rs11886868 stock reaction mix preparation

| **Reagent** | **Volume (μl)** |
|---|---|
| Sterile distilled water | 11.2 |
| PCR Master Mix 2× | 12.5 |
| rs11886868_Fwd primer | 0.15 |
| rs11886868_Rev primer | 0.15 |
| **Total** | 24 |

**Table 3.2b.** Volumes of reagents used for rs11886868 PCR amplification

| **Reagent** | **Volume (μl)** |
|---|---|
| Stock reaction mix | 24 |
| Genomic DNA template | 1 |
| **Total** | 25 |

#### 3.2.5.2 Thermal cycling conditions for rs11886868 PCR

Thermal cycling was carried out using Eppendorf Mastercycler gradient with thermal cycling conditions programmed to run the initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at a temperature of 95 °C for 30 s, annealing at a temperature of 57 °C for 30 s, extension at a temperature of 72 °C for 45 s followed by a final extension at a temperature of 72 °C for 10 min. The program was set to hold the reaction at 4 °C at the end of the thermal cycling. The rs11886868 thermal cycling conditions are presented on **Table 3.3.**

**Table 3.3** rs11886868 thermal cycling conditions

| | Step | Temperature (°C) | Time | Number of Cycles |
|---|---|---|---|---|
| **1** | Initial denaturation | 95 | 2 min | 1 |
| **2** | Denaturation | 95 | 30 s | |
| **3** | Annealing | 57 | 30 s | 30 |
| **4** | Extension | 72 | 45 s | |
| **5** | Final extension | 72 | 10 min | 1 |
| **6** | Hold | 4 | ∞ | |

#### 3.2.5.3 Visualization of the rs11886868 amplicons

The rs11886868 amplicons were visualized by agarose gel electrophoresis using 1% agarose gel pre-stained with 0.75 μl of 1 μg $ml^{-1}$ ethidium bromide. 100-bp DNA

ladder was used as a molecular marker to determine the molecular weights of the amplicons.

#### 3.2.5.3.1 Preparation of a 1% agarose gel

1% agarose gel was prepared in 50 ml of 1× TBE electrophoresis buffer by dissolving 0.5 g of agarose. The solution was melted in a microwave oven with swirling at intervals to melt into a clear solution. The heated gel was allowed to cool a bit then 0.75 µl of 1 µg $ml^{-1}$ ethidium bromide was added to stain the gel by swirling it gently to mix. The stained gel was then poured into the gel-casting cassette to cast the gel slap as it cooled to room temperature.

#### 3.2.5.3.2 Sample preparation for electrophoresis of rs11886868 amplicons

100-bp DNA ladder was prepared by mixing 1 µl of Blue juice DNA loading buffer, 0.5 µl of 100-bp DNA ladder and 8.5 µl of sterile distilled water on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 µl to ensure proper mixing then loaded into the first well of the gel slap. The samples were prepared by mixing 1 µl of Blue juice DNA loading buffer, 4 µl of sterile distilled water and 5 µl DNA amplicon on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 µl to ensure proper mixing then loaded into the wells of the gel slap starting from the third well, consecutively. The volumes of reagents used for preparation of samples for electrophoresis of rs11886868 amplicons are presented on **Table 3.4**.

**Table 3.4** Volumes of reagents used for preparation of samples for electrophoresis of rs11886868 amplicons

| | **Volumes (µl)** | |
|---|---|---|
| | **100 bp DNA ladder** | **Test samples** |
| **Blue juice DNA loading Buffer** | 1 | 1 |
| **100 bp DNA ladder** | 0.5 | -- |
| **Sterile distilled water** | 8.5 | 4 |
| **DNA amplicon** | -- | 5 |
| **Total volume** | 10 | 10 |

#### 3.2.5.3.3 Electrophoresis of rs11886868 amplicons

Electrophoresis was carried out using Bio-Rad mini-sub cell electrophoresis tank with the 1% agarose gel placed on the gel tray and submerged in the electrophoresis tank filled with 1× TBE electrophoresis running buffer. Electrophoresis was started at 70 V for 5 min to allow the loaded samples and DNA marker to align and start migrating in the gel at the time. The voltage was increased to 90 V after 5 min when the samples and marker have aligned and was allowed to migrate until they migrated to 2/3 of the gel.

#### 3.2.5.3.4 Visualization of rs11886868 DNA amplicons

The DNA amplicons were visualized under UV-light using MINIBIS PRO Gel Doc (Software: Gel capture) and the gel picture was named and saved with a bitmap ('bmp') format.

### 3.2.5.4 *Mbo*II digestion

The rs11886868 DNA amplicons were digested using *Mbo*II restriction endonuclease (5 U $\mu l^{-1}$) in a 32 μl total volume at 37 °C for 4 hours as follows;

2 μl of 10× buffer B (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg $ml^{-1}$ BSA, pH 7.5) was diluted 10× using 18 μl of sterile distilled water in a sterile eppendorf tube. 10 μl of DNA amplicon was added and 2 μl of *Mbo*II restriction endonuclease (5 U $\mu l^{-1}$) was added and mixed gently. The reaction mixture was placed in a heat block to incubate at 37 °C for 4 hours. The volumes of reagents used for the *Mbo*II digestion are presented on **Table 3.5** below.

**Table 3.5** Volumes of reagents used for *Mbo*II digestion

| **Reagents** | **Volume (μl)** |
|---|---|
| **Nuclease free water** | 18 |
| **10× buffer B** | 2 |
| **DNA amplicon** | 10 |
| ***Mbo*II endonuclease** | 2 |
| **Total volume** | 32 |

### 3.2.5.5 rs11886868 *Mbo*II genotyping

The *Mbo*II restriction endonuclease digests the 540-bp DNA amplicon of the homozygous C-allele into two DNA fragments of 478 bp and 62 bp long respectively.

The homozygous T-allele remains undigested. Hence, it remains as a single band of DNA that is 540 bp long. In the case of the heterozygous allele, one strand of the DNA amplicon containing the C-allele is digested into two DNA fragments of 478 bp and 62 bp long respectively while the other strand of the DNA amplicon containing the T-allele remains undigested. Hence, the heterozygous allele is partially digested yielding three DNA bands that are 540 bp, 478 bp and 62 bp long respectively.

The digested DNA bands were visualized on a 2% agarose gel pre-stained with 0.75 μl of 1 μg $ml^{-1}$ ethidium bromide.

#### 3.2.5.5.1 Preparation of a 2% agarose gel

2% agarose gel was prepared in 50 ml of 1× TBE electrophoresis buffer by dissolving 1 g of agarose. The solution was melted in a microwave oven with swirling at intervals to melt into a clear solution. The heated gel was allowed to cool a bit then 0.75 μl of 1 μg $ml^{-1}$ ethidium bromide was added to stain the gel by swirling it gently to mix. The stained gel was then poured into the gel-casting cassette to cast the gel slap as it cooled to room temperature.

#### 3.2.5.5.2 Sample preparation for electrophoresis of digestion products

50-bp DNA ladder was prepared by mixing 1 μl of Blue juice DNA loading buffer, 0.5 μl of 50-bp DNA ladder and 8.5 μl of sterile distilled water on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 μl to ensure proper mixing then loaded into the first well of the gel slap. The samples were prepared by mixing 1 μl of Blue juice DNA loading buffer, 4 μl of sterile distilled water and 5 μl DNA amplicon on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 μl to ensure proper mixing then loaded into the wells of the gel slap starting from the third well, consecutively. The volumes of reagents used for preparation of samples for electrophoresis of digested DNA amplicons are presented on **Table 3.6**.

**Table 3.6** Volumes of reagents used for preparation of samples for electrophoresis of digestion products

| | Volumes (μl) | |
|---|---|---|
| | **50 bp DNA ladder** | **Test samples** |
| **Blue juice DNA loading buffer** | 1 | 1 |
| **50 bp DNA ladder** | 0.5 | -- |
| **Sterile distilled water** | 8.5 | 4 |
| **Digestion product** | -- | 5 |
| **Total volume** | 10 | 10 |

#### 3.2.5.5.3 Electrophoresis of digestion products

Electrophoresis was carried out using Bio-Rad mini-sub cell electrophoresis tank with the 2% agarose gel placed on the gel tray and submerged in the electrophoresis tank filled with 1× TBE electrophoresis running buffer. Electrophoresis was started at 70 V for 5 min to allow the loaded samples and DNA marker to align and start migrating in the gel at the time. The voltage was increased to 90 V after 5 min when the samples and marker have aligned and was allowed to migrate until they migrated to 2/3 of the gel.

#### 3.2.5.5.4 Visualization of digested DNA amplicons

The digested DNA amplicons were visualized under UV-light using MINIBIS PRO Gel Doc (Software: Gel capture) and the gel picture was named and saved with a bitmap (‘bmp’) format.

### 3.2.6 Genotyping of rs766432 polymorphism

The rs766432 polymorphism was genotyped using tetra-primer ARMS.

#### 3.2.6.1 Sample preparation for rs766432 tetra-primer ARMS

The rs766432 tetra-primer ARMS was carried out using Thermo Scientific PCR master mix and two pairs of rs766432 primers (rs766432_A_Fwd primer, rs766432_A_Rev primer and rs766432_C_Fwd primer, rs766432_C_Rev primer) in a 25 μl PCR reaction mix containing 1× PCR master mix (0.625 U Taq DNA polymerase, 2 mM $MgCl_2$, reaction buffer, 0.2 mM each of dTTP, dATP, dGTP and dCTP), 0.6 μM of each primer, nuclease free water and genomic DNA template. The PCR Master Mix, primers, nuclease free water and DNA samples were thawed at room temperature. The PCR Master Mix and primers were vortexed and centrifuged

briefly then placed on an arctic cold block. The thawed DNA samples were mixed gently and the PCR amplification was set up in a laminar flow cabin. A stock reaction mix containing 10.9 µl of sterile distilled water, 12 .5 µl of 2× PCR Master Mix, 0.15 µl of rs76632_A_Fwd primer, 0.15 µl of rs766432_A_Rev primer, 0.15 µl of rs766432_C_Fwd primer and 0.15 µl of rs766432_C_Rev primer was prepared in an eppendorf tube and was mixed properly. 24 µl of the stock reaction mix was transferred into a PCR tube and 1 µl of genomic DNA template was added. The volumes of reagents used for the tetra-primer ARMS amplification are presented on **Table 3.7a** and **3.7b** below.

**Table 3.7a.** Volumes of reagents used for rs766432 stock reaction mix preparation

| **Reagent** | **Volume (µl)** |
|---|---|
| Sterile distilled water | 10.9 |
| PCR Master Mix 2× | 12.5 |
| rs766432_A_Fwd primer | 0.15 |
| rs766432_A_Rev primer | 0.15 |
| rs766432_C_Fwd primer | 0.15 |
| rs766432_C_Rev primer | 0.15 |
| **Total** | 24 |

**Table 3.7b.** Volumes of reagents used for rs766432 PCR amplification

| **Reagent** | **Volume (µl)** |
|---|---|
| Stock reaction mix | 24 |
| Genomic DNA template | 1 |
| **Total** | 25 |

### 3.2.6.2 Thermal cycling conditions for rs766432 tetra-primer ARMS

Thermal cycling was carried out using Eppendorf MasterCycler Gradient with thermal cycling conditions programmed to run the initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at a temperature of 95 °C for 30 s, annealing at a temperature of 55 °C for 30 s, extension at a temperature of 72 °C for 45 s followed by a final extension at a temperature of 72 °C for 10 min. The program was set to hold the reaction at 4 °C at the end of the thermal cycling. The rs766432 tetra–primer ARMS-PCR thermal cycling conditions are presented on **Table 3.8**.

**Table 3.8** rs766432 thermal cycling conditions

| | Step | Temperature (°C) | Time | Number of Cycles |
|---|---|---|---|---|
| **1** | Initial denaturation | 95 | 2 min | 1 |
| **2** | Denaturation | 95 | 30 s | |
| **3** | Annealing | 55 | 30 s | 30 |
| **4** | Extension | 72 | 45 s | |
| **5** | Final extension | 72 | 10 min | 1 |
| **6** | Hold | 4 | ∞ | |

#### 3.2.6.3 rs766432 tetra-primer ARMS genotyping

In this tetra-primer ARMS, the outer primers are used to synthesize the outer control band while the inner primers, which are allele specific, are used for the synthesis of DNA fragment containing the specific allele for the rs766432 polymorphism. For the homozygous A-allele, two DNA bands are synthesized; the outer control band that is 193 bp long and the inner A-allele band that is 135 bp long. For the homozygous C-allele, two DNA bands are synthesized; the outer control band that is 193 bp long and the inner C-allele band that is 116 bp long. For the heterozygous alleles, three DNA bands are synthesized; the outer control band, the inner A-allele band and inner C-allele band, which are 193 bp, 135 bp and 116 bp long respectively.

The tetra-primer ARMS amplicons were visualized on a 2.5% agarose gel pre-stained with 0.75 μl of 1 μg $ml^{-1}$ ethidium bromide.

##### 3.2.6.3.1 Preparation of a 2.5% agarose gel

2.5% agarose gel was prepared in 50 ml of 1× TBE electrophoresis buffer by dissolving 1.25 g of agarose. The solution was melted in a microwave oven with swirling at intervals to melt into a clear solution. The heated gel was allowed to cool a bit then 0.75 μl of 1 μg $ml^{-1}$ ethidium bromide was added to stain the gel by swirling it gently to mix. The stained gel was then poured into the gel-casting cassette to cast the gel slap as it cooled to room temperature.

##### 3.2.6.3.2 Sample preparation for electrophoresis of rs766432 tetra-primer ARMS amplicons

50-bp DNA ladder was prepared by mixing 1 μl of Blue juice DNA loading buffer, 0.5 μl of 50-bp DNA ladder and 8.5 μl of sterile distilled water on the inside of a

clean paraffin paper. The mixture was mixed several times using the pipette set at 10 µl to ensure proper mixing then loaded into the first well of the gel slap. The samples were prepared by mixing 1 µl of Blue juice DNA loading buffer, 4 µl of sterile distilled water and 5 µl DNA amplicon on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 µl to ensure proper mixing then loaded into the wells of the gel slap starting from the third well, consecutively. The volumes of reagents used for preparation of samples for electrophoresis of rs766432 tetra-primer ARMS amplicons are presented on **Table 3.9**.

**Table 3.9** Volumes of reagents used for preparation of samples for electrophoresis of rs766432 tetra-primer ARMS amplicons

| | **Volumes (µl)** | |
|---|---|---|
| | **50 bp DNA ladder** | **Test samples** |
| **Blue juice DNA loading Buffer** | 1 | 1 |
| **50 bp DNA ladder** | 0.5 | -- |
| **Sterile distilled water** | 8.5 | 4 |
| **DNA amplicon** | -- | 5 |
| **Total volume** | 10 | 10 |

#### 3.2.6.3.3 Electrophoresis of rs766432 tetra-primer ARMS amplicons

Electrophoresis was carried out using Bio-Rad mini-sub cell electrophoresis tank with the 2.5% agarose gel placed on the gel tray and submerged in the electrophoresis tank filled with 1× TBE electrophoresis running buffer. Electrophoresis was started at 70 V for 5 min to allow the loaded samples and DNA marker to align and start migrating in the gel at the time. The voltage was increased to 90 V after 5 min when the samples and marker have aligned and was allowed to migrate for 25 min. The voltage was again increased to 120 V and allowed to migrate until they migrated to 2/3 of the gel.

#### 3.2.6.3.4 Visualization of rs766432 tetra-primer ARMS amplicons

The rs766432 DNA amplicons were visualized under UV-light using MINIBIS PRO Gel Doc (Software: Gel capture) and the gel picture was named and saved with a bitmap (‘bmp’) format.

### 3.2.7 Genotyping of rs9399137 polymorphism

rs9399137 polymorphism was genotyped using tetra-primers ARMS.

#### 3.2.7.1 Sample preparation for rs9399137 tetra-primer ARMS

rs9399137 tetra-primer ARMS was carried out using Thermo Scientific PCR master mix and two pairs of rs9399137 primers (rs9399137_A_Fwd primer, rs9399137_A_Rev primer and rs9399137_C_Fwd primer, rs9399137_C_Rev primer) in a 25 µl PCR reaction mix containing 1× PCR Master Mix (0.625 U Taq DNA polymerase, 2 mM$MgCl_2$, reaction buffer, 0.2 mM each of dTTP, dATP, dGTP and dCTP), 2.0 µM of rs9399137_C_Fwd primer, 2.0 µM of rs9399137_C_Rev primer, 0.15 µM of rs9399137_T_Fwd primer, 0.3 µM of rs9399137_T_Rev primer, nuclease-free water and genomic DNA template. The PCR Master Mix, primers, nuclease free water and DNA samples were thawed at room temperature. The PCR master mix and primers were vortexed and centrifuged briefly then placed on an arctic cold block. The thawed DNA samples were mixed gently and the PCR amplification was set up in a laminar flow cabin. A stock reaction mix containing 10.3875 µl of sterile distilled water, 12 .5 µl of 2× PCR Master Mix, 0.5 µl of rs9399137_C_Fwd primer, 0.5 µl of rs9399137_C_Rev primer, 0.0375 µl of rs9399137_T_Fwd primer and 0.075 µl of rs9399137_T_Rev primer was prepared in an eppendorf tube and was mixed properly. 24 µl of the stock reaction mix was transferred into a PCR tube and 1 µl of genomic DNA template was added. The volumes of reagents used for the tetra-primer ARMS-PCR amplification are presented on **Table 3.10a** and **3.10b** below.

**Table 3.10a.** Volumes of reagents used for rs9399137 stock reaction mix preparation

| **Reagent** | **Volume (µl)** |
|---|---|
| Sterile distilled water | 10.9 |
| PCR Master Mix 2× | 12.5 |
| rs9399137_C_Fwd primer | 0.5 |
| rs9399137_C_Rev primer | 0.5 |
| rs9399137_T_Fwd primer | 0.0375 |
| rs9399137_T_Rev primer | 0.075 |
| **Total** | 24 |

**Table 3.10b.** Volumes of reagents used for rs9399137 PCR amplification

| **Reagent** | **Volume (μl)** |
|---|---|
| Stock reaction mix | 24 |
| Genomic DNA template | 1 |
| **Total** | 25 |

### 3.2.7.2 Thermal cycling conditions for rs9399137 tetra-primer ARMS

Thermal cycling was carried out using Eppendorf Mastercycler gradient with thermal cycling conditions programmed to run the initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at a temperature of 95 °C for 30 s, annealing at a temperature of 45 °C for 30 s, extension at a temperature of 72 °C for 45 s followed by a final extension at a temperature of 72 °C for 10 min. The program was set to hold the reaction at 4 °C at the end of the thermal cycling. The rs9399137 tetra-primer ARMS thermal cycling conditions are presented on **Table 3.10.1**

**Table 3.10.1** rs9399137 thermal cycling conditions

| | Step | Temperature (°C) | Time | Number of Cycles |
|---|---|---|---|---|
| **1** | Initial denaturation | 95 | 2 min | 1 |
| **2** | Denaturation | 95 | 30 s | |
| **3** | Annealing | 45 | 30 s | 40 |
| **4** | Extension | 72 | 45 s | |
| **5** | Final extension | 72 | 10 min | 1 |
| **6** | Hold | 4 | ∞ | |

### 3.2.7.3 rs9399137 tetra-primer ARMS genotyping

In this tetra-primer ARMS, the outer primers are used to synthesize the outer control band while the inner primers, which are allele specific are used for the synthesis of DNA fragment containing the specific allele for the rs9399137 polymorphism. For the homozygous C-allele, two DNA bands are synthesized; the outer control band that is 365 bp long and the inner C-allele band that is 178 bp long. For the homozygous T-allele, two DNA bands are synthesized; the outer control band that is 365 bp long and the inner T-allele band that is 243 bp long. For the heterozygous alleles, three DNA bands are synthesized; the outer control band, the inner T-allele band and inner C-allele band, which are 365 bp, 243 bp and 178 bp long respectively.

The tetra-primer ARMS amplicons were visualized on a 2% agarose gel pre-stained with 0.75 µl of 1 µg $ml^{-1}$ ethidium bromide.

#### 3.2.7.3.1 Preparation of a 2% agarose gel

2.5% agarose gel was prepared in 50 ml of 1× TBE electrophoresis buffer by dissolving 1 g of agarose. The solution was melted in a microwave oven with swirling at intervals to melt into a clear solution. The heated gel was allowed to cool a bit then 0.75 µl of 1 µg $ml^{-1}$ ethidium bromide was added to stain the gel by swirling it gently to mix. The stained gel was then poured into the gel-casting cassette to cast the gel slap as it cooled to room temperature.

#### 3.2.7.3.2 Sample preparation for electrophoresis of rs9399137 tetra-primer ARMS amplicons

50-bp DNA ladder was prepared by mixing 1 µl of Blue juice DNA loading buffer, 0.5 µl of 50-bp DNA ladder and 8.5 µl of sterile distilled water on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 µl to ensure proper mixing then loaded into the first well of the gel slap. The samples were prepared by mixing 1 µl of Blue juice DNA loading buffer, 4 µl of sterile distilled water and 5 µl DNA amplicon on the inside of a clean paraffin paper. The mixture was mixed several times using the pipette set at 10 µl to ensure proper mixing then loaded into the wells of the gel slap starting from the third well, consecutively. The volumes of reagents used for preparation of samples for electrophoresis of rs9399137 tetra-primer ARMS amplicons are presented on **Table 3.10.2**

**Table 3.10.2** Volumes of reagents used for preparation of samples for electrophoresis of rs9399137 tetra-primer ARMS amplicons

| | **Volumes (µl)** | |
|---|---|---|
| | **50 bp DNA ladder** | **Test samples** |
| **Blue juice DNA loading Buffer** | 1 | 1 |
| **50 bp DNA ladder** | 0.5 | -- |
| **Sterile distilled water** | 8.5 | 4 |
| **DNA amplicon** | -- | 5 |
| **Total volume** | 10 | 10 |

#### 3.2.7.3.3 Electrophoresis of rs766432 tetra-primer ARMS amplicons

Electrophoresis was carried out using Bio-Rad mini-sub cell electrophoresis tank with the 2% agarose gel placed on the gel tray and submerged in the electrophoresis tank filled with 1× TBE electrophoresis running buffer. Electrophoresis was started at 70 V for 5 min to allow the loaded samples and DNA marker to align and start migrating in the gel at the time. The voltage was increased to 90 V after 5 min when the samples and marker have aligned and was allowed to migrate for 25 min. The voltage was again increased to 110 V and allowed to migrate until they migrated to 2/3 of the gel.

#### 3.2.7.5.4 Visualization of rs9399137 tetra-primer ARMS amplicons

The rs9399137 DNA amplicons were visualized under UV-light using MINIBIS PRO Gel Doc (Software: Gel capture) and the gel picture was named and saved with a bitmap (‘bmp’) format.

# Chapter four

## 4. Results

### 4.1 Genotyping

#### 4.1.1 rs11886868 (*Mbo*II) genotyping

rs11886868 (*Mbo*II) polymorphism was determined by PCR-RFLP using *Mbo*II restriction enzyme to digest the PCR amplicons. The digested fragments were visualized using agarose gel electrophoresis on a 2% agarose gel which was pre-stained with ethidium bromide.

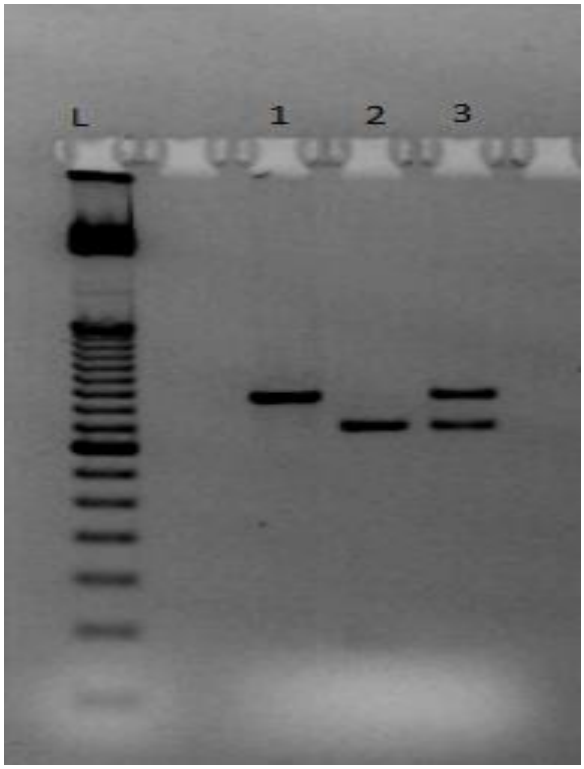



**Figure 4.1:** Gel picture showing the PCR-RFLP results for the genotyping of the rs11886868 polymorphism using the *Mbo*II restriction enzyme. Lane L contains the 50-bp DNA ladder, lane 1 contains the homozygous TT, lane 2 contains the homozygous CC and lane 3 contains the heterozygous TC.

#### 4.1.2 rs766432 genotyping

rs766432 polymorphism was genotyped by tetra-primer ARMS. The PCR amplicons were visualized using agarose gel electrophoresis on a 2.5% agarose gel which was pre-stained with ethidium bromide.

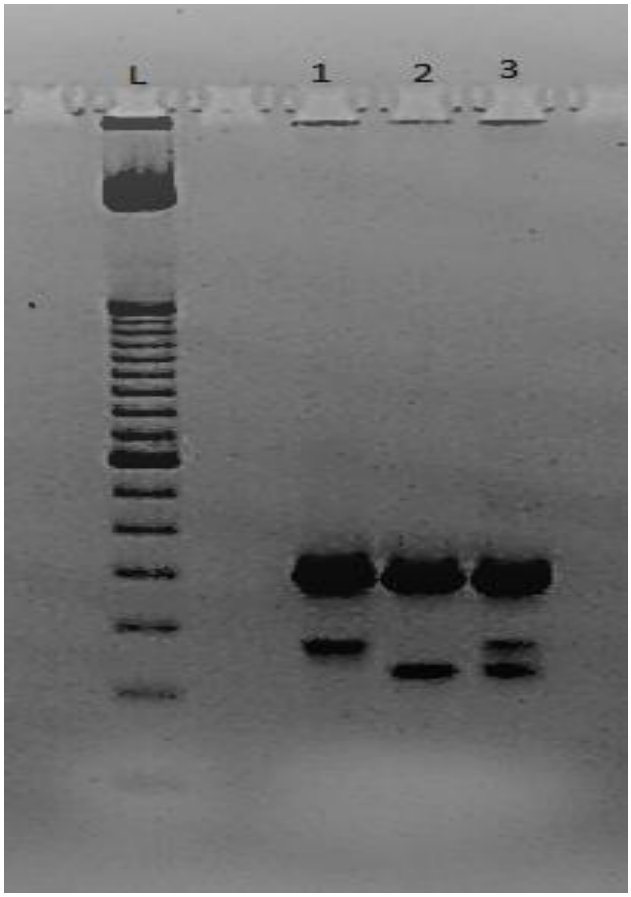



**Figure 4.2:** Gel picture showing tetra-primer ARMS result of rs766432 genotyping. Lane L contains the 50-bp DNA ladder, lane 1 contains the homozygous AA, lane 2 contains the homozygous CC and lane 3 contains the heterozygous AC.

### 4.1.3 rs9399137 genotyping

rs9399137 polymorphism was determined by tetra-primer ARMS. The PCR amplicons were visualized using agarose gel electrophoresis on a 2% agarose gel which was pre-stained with ethidium bromide.

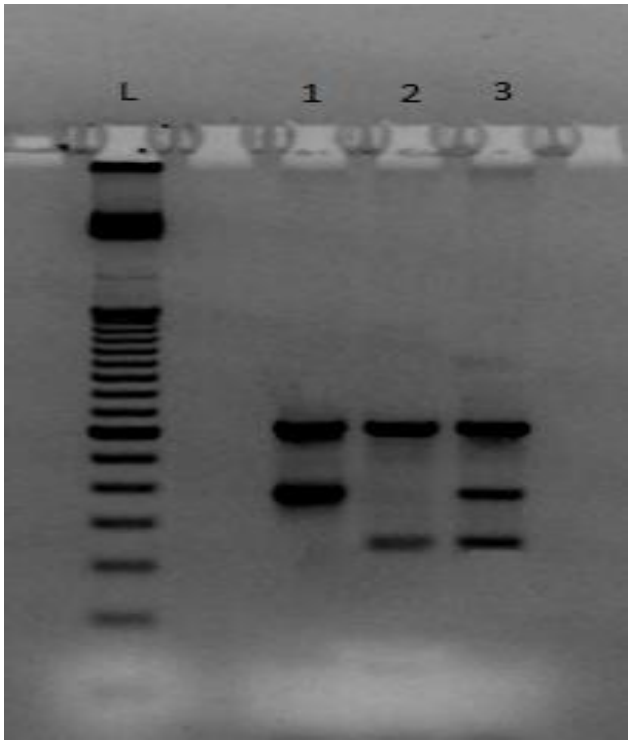



**Figure 4.3:** Gel picture showing tetra-primer ARMS result of rs9399137 genotyping. Lane L contains the 50-bp DNA ladder, lane 1 contains the homozygous TT, lane 2 contains homozygous CC and lane 3 contains the heterozygous TC.

### 4.2 Statistics

Statistical analysis was done using SNPStats and SPSS version 18 for Windows. SNPAstats was used to compute the genotype frequencies, allele frequencies and Hardy–Weinberg equilibrium. SPSS Chi square test (Fisher's exact test) was used to compute the statistical significance of association between the genotypes carrying the protective allele and the two groups of β-thalassemia patients. A significance value, α of 0.05 was used. *P* value > 0.05 was taken to be insignificant.

#### 4.2.1 Estimation of genotype frequencies

**Table 4.1** rs11886868 (*Mbo*II) polymorphism genotype frequency

| Patient group | rs11886868 (*Mbo*II) polymorphism genotype | | |
|---|---|---|---|
| | TT | CC | TC |
| **Thalassemia Major (*n*=42)** | 11 (0.262) | 12 (0.286) | 19 (0.452) |
| **Thalassemia Intermedia (*n*=25)** | 5 (0.20) | 4 (0.16) | 16 (0.64) |
| **Total** | 16 (0.239) | 16 (0.239) | 35 (0.522) |

Numbers in parenthesis represent the genotype frequencies

**Table 4.2** rs766432 polymorphism genotype frequency

| Patient group | rs766432 polymorphism genotype | | |
|---|---|---|---|
| | AA | CC | AC |
| **Thalassemia Major (*n*=42)** | 20 (0.476) | 3 (0.071) | 19 (0.452) |
| **Thalassemia Intermedia (*n*=25)** | 9 (0.36) | 3 (0.12) | 13 (0.52) |
| **Total** | 29 (0.433) | 6 (0.09) | 32 (0.478) |

Numbers in parenthesis represent the genotype frequencies

**Table 4.3** rs9399137 polymorphism genotype frequency

| Patient group | rs9399137 polymorphism genotype | | |
|---|---|---|---|
| | TT | CC | TC |
| **Thalassemia Major (*n*=42)** | 28 (0.667) | 3 (0.071) | 11 (0.262) |
| **Thalassemia Intermedia (*n*=25)** | 11 (0.44) | 3 (0.12) | 11 (0.44) |
| **Total** | 39 (0.582) | 6 (0.09) | 22 (0.328) |

Numbers in parenthesis represent the genotype frequencies

### 4.2.2 Estimation of allele frequencies

**Table 4.4** rs11886868 (*Mbo*II) polymorphism allele frequency

| Patient group | rs11886868 (*Mbo*II) polymorphism allele | | Hardy-Weinberg *P* Value |
|---|---|---|---|
| | T | C | |
| **Thalassemia Major** | 41 (0.488) | 43 (0.512) | 0.81 |
| **Thalassemia Intermedia** | 26 (0.52) | 24 (0.48) | |
| **Total** | 67 (0.50) | 67 (0.50) | |

Numbers in parenthesis represent the allele frequencies

**Table 4.5** rs766432 polymorphism allele frequency

| Patient group | rs766432 polymorphism allele | | Hardy-Weinberg *P* Value |
|---|---|---|---|
| | A | C | |
| **Thalassemia Major** | 59 (0.702) | 25 (0.298) | 0.59 |
| **Thalassemia Intermedia** | 31 (0.620) | 19 (0.380) | |
| **Total** | 90 (0.672) | 44 (0.328) | |

Numbers in parenthesis represent the allele frequencies

**Table 4.6** rs9399137 polymorphism allele frequency

| Patient group | rs9399137 polymorphism allele | | Hardy-Weinberg *P* Value |
|---|---|---|---|
| | T | C | |
| **Thalassemia Major** | 67 (0.798) | 17 (0.202) | 0.33 |
| **Thalassemia Intermedia** | 33 (0.66) | 17 (0.34) | |
| **Total** | 100 (0.746) | 34 (0.254) | |

Numbers in parenthesis represent the allele frequencies

**Table 4.7** Relationship between genotypes carrying the protective allele and patient group

| SNP | Patient group | Genotype | Frequency | *P* Value |
|---|---|---|---|---|
| **rs11886868** | Thalassemia Major | CT + TT | 30 (0.714) | 0.375 |
| | | CC | 12 (0.286) | |
| | Thalassemia Intermedia | CT + TT | 21 (0.84) | |
| | | CC | 4 (0.16) | |
| **rs766432** | Thalassemia Major | AC + CC | 22 (0.524) | 0.447 |
| | | AA | 20 (0.476) | |
| | Thalassemia Intermedia | AC + CC | 16 (0.64) | |
| | | AA | 9 (0.36) | |
| **rs9399137** | Thalassemia Major | TC + CC | 14 (0.333) | 0.080 |
| | | TT | 28 (0.667) | |
| | Thalassemia Intermedia | TC + CC | 14 (0.56) | |
| | | TT | 11 (0.44) | |

# Chapter five

## 5. Discussion

The *BCL11A* locus and *HBS1L–MYB* intergenic locus are known to be among the quantitative trait loci (QTL) associated with persistent high HbF levels (Thein, 2008; Wonkam *et al*., 2014).

Mean HbF levels were shown by Terali *et al*., (2016) to be significantly higher in β-thalassemia intermedia (TI) group than in β-thalassemia major (TM) group suggesting that the high levels of HbF have ameliorating effect on the clinical phenotype of the β-thalassemia patients in the same population.

In this research, two groups of β-thalassemia patients; β-thalassemia intermedia (TI) and β-thalassemia major (TM) were genotyped for SNPs on the *BCL11A* locus and *HBS1L-MYB* intergenic locus.

The rs11886868 T>C polymorphism on the *BCL11A* gene was determined by PCR-RFLP using *Mbo*II restriction enzyme to digest the PCR amplicons. The digested rs11886868 fragments (478 bp and 62 bp) were interpreted as the C-allele while the undigested rs11886868 amplicon (540 bp) was interpreted as the T-allele (**Figure 4.1**).

Genotype frequencies, allele frequencies and Hardy-Weinberg equilibrium of the rs11886868 T>C polymorphism was calculated using SNPStats to show the genotype distribution and allele distribution between the two groups of patients.

The TM group was found to have genotype frequencies of 0.262, 0.286 and 0.452 for TT, CC and TC genotypes respectively. Genotype frequencies of 0.20, 0.16 and 0.64 for TT, CC and TC genotypes respectively were found for the TI group. From the total population, 0.239 were genotyped TT, 0.239 were genotyped CC and 0.522 were genotyped TC (**Table 4.1**).

rs11886868 T>C (*Mbo*II) polymorphism was detected in 51 (76.1%) of the patients. 16/51 were homozygous and 35/51 were heterozygous for the rs11886868 (*Mbo*II) polymorphism (**Table 4.1**).

Both the T and C alleles were found to have equal allele frequencies of 0.50 each in the total population (**Table 4.4**). This result differs from the result obtained for

rs11886868 polymorphism among β-thalassemia patients of Indonesian origin where the T allele was reported to have an allele frequency of 0.22 and the C allele was reported to have an allele frequency of 0.78 (Rujito *et al*., 2016). This is not surprising because data from the updated HapMap project shows that the rs11886868 genotype distribution varies among populations of the world (dbSNP, 2018a). The rs11886868 alleles were found to comply with the Hardy-Weinberg equilibrium in the entire groups of patients showing that the alleles were independently inherited and that no allele was dropped out during genotyping (*P* value = 0.81) (**Table 4.4**). No significant association was established between the rs11886868 genotypes carrying the protective alleles and the two groups of patients (*P* value = 0.375) (**Table 4.7**).

The rs766432 A>C polymorphism in the *BCL11A* gene was genotyped by tetra-primer ARMS. The outer forward (rs766432_C_Fwd) and outer reverse (rs766432_A_Rev) primers were used to synthesize the outer control fragment, which is 193bp long. The A-allele specific primer (rs766432_A_Fwd) and the outer reverse primer (rs766432_A_Rev) were used to synthesize the A-allele fragment, which is 135bp long. The C-allele specific primer (rs766432_A_Rev) and the outer forward primer (rs766432_C_Fwd) were used to synthesize the C-allele fragment. The rs766432 amplicons were interpreted based on the sizes of the amplicons (**Figure 4.2**).

Genotype frequencies, allele frequencies and Hardy–Weinberg equilibrium of the rs766432 A>C polymorphism was calculated using SNPStats to show the genotype distribution and allele distribution between the two groups of patients.

The TM group had genotype frequencies of 0.476, 0.071 and 0.452 for AA, CC and AC genotypes respectively. In the TI group, genotype frequencies of 0.36, 0.12 and 0.52 were found for AA, CC and AC genotypes respectively. From the total population, 0.433 were genotyped AA, 0.09 were genotyped CC and 0.478 were genotyped AC (**Table 4.2**).

The rs766432 A>C polymorphism was detected in 38 (56.8%) of the patients. 6/38 were homozygous and 32/38 were heterozygous for the rs766432 polymorphism (table 4.2).

In the total population, the alleles frequencies were found to be 0.672 for the A allele and 0.328 for the C allele (**Table 4.5**). This result is in agreement with the report of

Rujito *et al*., (2016) for the rs766432 polymorphism among β-thalassemia patients of Indonesian origin where they reported the rs766432 A-allele as the major allele, having allele frequency of 0.81 and the C-allele as the minor allele, having allele frequency of 0.19. Similarly, both the genotype and frequencies and allele frequencies for the rs766432 polymorphism are in agreement with the frequencies reported for most populations of the world (dbSNP, 2018b). The rs766432 alleles were found to comply with the Hardy-Weinberg equilibrium in the entire groups of patients showing that the alleles were independently inherited and that no allele was dropped out during genotyping (*P* value = 0.59) (**Table 4.5**). No significant association was established between the rs766432 genotypes carrying the protective alleles and the two groups of patients (*P* value = 0.447) (**Table 4.7**).

The rs9399137 T>C polymorphism in the *HBS1L-MYB* intergenic region was genotyped by tetra-primer ARMS. The outer forward primer (rs9399137_T_Fwd) and the outer reverse primer (rs9399137_C_Rev) were used to synthesize the outer control fragment, which is 365bp long. The outer forward primer (rs9399137_T_Fwd) and the T-allele specific primer (rs9399137_T_Rev) were used to synthesize the T-allele fragment, which is 243 bp long. The C-allele specific primer (rs9399137_C_Fwd) and the outer reverse primer (rs9399137_C_Rev) were used to synthesize the C-allele fragment, which is 178 bp long. The rs939917 amplicons were interpreted based on the sizes of the amplicons (**Figure 4.3**).

Genotype frequencies, allele frequencies and Hardy-Weinberg equilibrium of the rs9399137 T>C polymorphism was calculated using SNPStats to show the genotype distribution and allele distribution between the two groups of patients.

The TM group was found to have genotype frequencies of 0.071, 0.667 and 0.262 for the CC, TT and TC genotypes respectively. Genotype frequencies of 0.12, 0.44 and 0.44 were found for the CC, TT and TC genotypes respectively in the TI group. From the total population, 0.582 were genotyped TT, 0.09 were genotyped CC and 0.328 were genotyped TC (**Table 4.3**).

The rs9399137 polymorphism was detected in 28 (41.8%) of the patients. 6/28 were homozygous and 22/28 were heterozygous for the rs9399137 polymorphism (**Table 4.3**).

In the total population, the T allele was found to be the major allele with an allele frequency of 0.746 while the C allele was found to be the minor allele with an allele frequency of 0.254 (**Table 4.6**). This result is similar to the report of Rujito *et al*., (2016) for the rs9399137 allele in β-thalassemia patients of Indonesian origin where they reported the rs9399137 T-allele as the major allele, having allele frequency of 0.82 and the C allele as the minor allele, having allele frequency of 0.18. In a comparison with other populations, both the genotype frequencies and the allele frequencies follow the same pattern of genotype and allele distribution with other populations of the world (dbSNP, 2018c).

The rs9399137 alleles were found to comply with the Hardy-Weinberg equilibrium in the entire groups of patients showing that the alleles were independently inherited and that no allele was dropped out during genotyping (*P* value = 0.33) (**Table 4.6**). No significant association was established between the rs9399137 genotypes carrying the protective alleles and the two groups of patients (*P* value = 0.080) (**Table 4.7**).

# Chapter six

## 6. Conclusion

In this study, the frequency of rs11886868 T>C polymorphism in the *BCL11A* locus, rs766432 A>C polymorphism on the *BCL11A* locus and rs9399137 T>C polymorphism on the *HBS1L-MYB* intergenic region was determined in β-thalassemia patients of Turkish-Cypriot origin.

For the rs11886868 T>C (*Mbo*II) polymorphism, 76.1% of the patients was found to carry the polymorphic C allele with genotype distribution of 0.239, 0.239 and 0.522 for the TT, CC and TC genotypes respectively. For the rs766432 A>C polymorphism, 56.8% of the patients was found to carry the polymorphic C allele with genotype distribution of 0.433, 0.09 and 0.478 for the AA, CC and AC genotypes respectively. For the rs9399137 T>C polymorphism, 41.8% of the patients was found to carry the polymorphic C allele with genotype distribution of 0.582, 0.09 and 0.328 for the TT, CC and TC genotypes respectively.

All the alleles for the three polymorphisms were found to comply with the Hardy-Weinberg equilibrium and no significant association was established between the genotypes carrying the protective alleles and the two groups of β-thalassemia patients for any of the three polymorphisms.

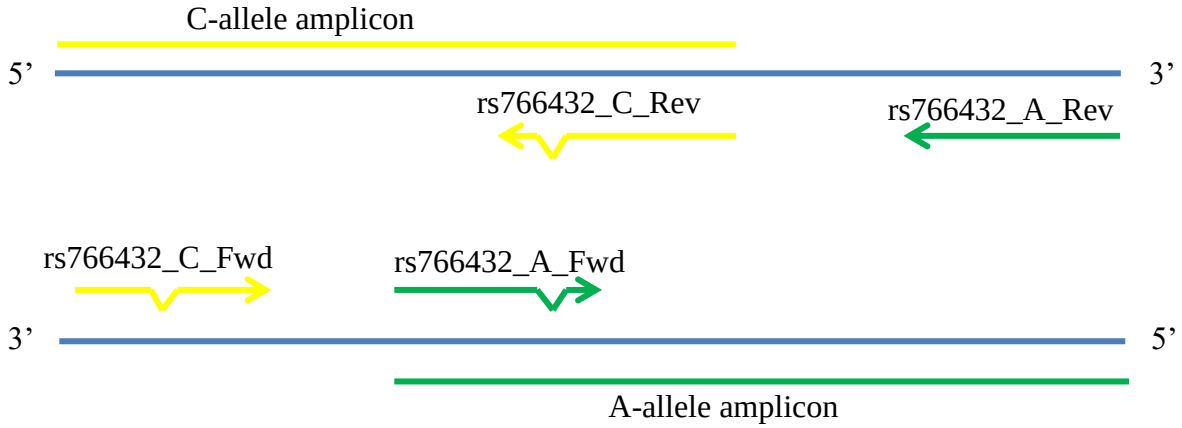
# APPENDIX

## Appendix I


Amplification pattern of rs11886868 PCR

Amplicon size: 540 bp

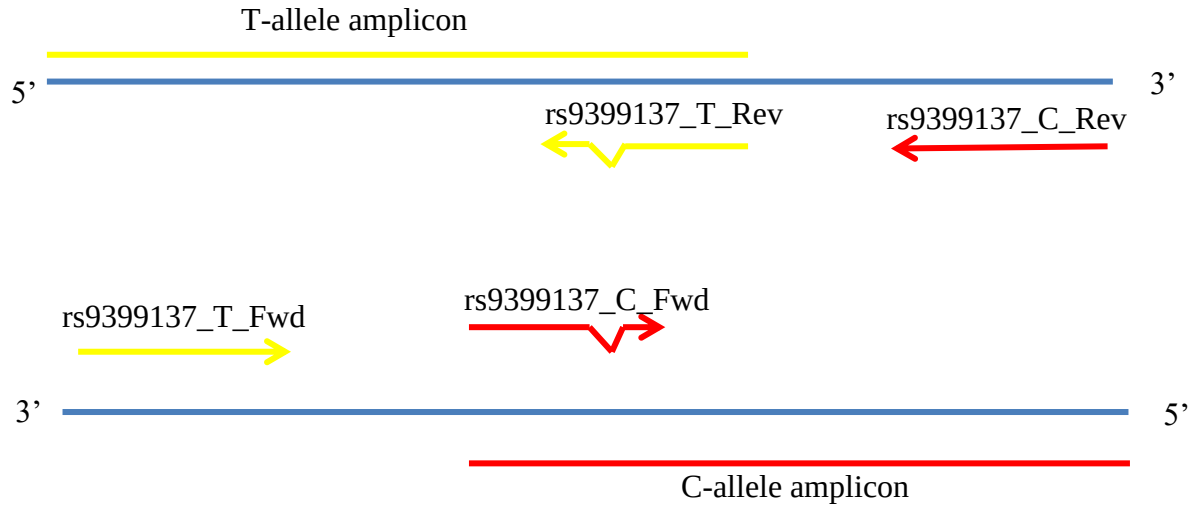
# Appendix II




Amplification pattern of rs766432 tetra-primer ARMS

| Amplicons | Size |
|---|---|
| **Common Strand** | 193 bp |
| **A-allele Strand** | 135 bp |
| **C-allele Strand** | 116 bp |

## Appendix III




Amplification pattern of rs9399137 tetra-primer ARMS

| Amplicons | Size |
|---|---|
| **Common Strand** | 365 bp |
| **C-allele Strand** | 178 bp |
| **T-allele Strand** | 243 bp |

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