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MOLECULAR CHARACTERIZATION OF MUTATIONS GIVING RISE TO β-THALASSEMIA IN VIETNAM

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Ph.D Thesis

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ATTESTATION OF AUTHORSHIP

I hereby declare that this study is my own work to the best of my knowledge. It doesn't contain any publication of other previous authors except contents appearing in the citations.

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LIST OF ABBREVIATION

α-thal	α-thalassemia
β-thal	β-thalassemia
cd	codon
CE	Capillary electrophoresis
dNTP	Deoxynucleotide
ddNTP	Dideoxynucleotide
Hb	Hemoglobin
Hb-pathies	Hemoglobinopathies
HPLC	High performance liquid chromatography
IVS	Intervening sequence
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
NTDT	Non-transfusion dependent thalassemia
RBC	Red blood cell
TDT	Transfusion dependent thalassemia
TI	Thalassemia intermedia
TM	Thalassemia major
UTR	Untranslated region

ABSTRACT

Thalassemias, sickle cell anemia and Hb E, are indisputably the most common monogenic diseases worldwide. In Vietnam, both β-thalassemia and Hb E are prevalent, being distributing all over the country. The frequency of β-thalassemia carrier ranges from 1.5 – 25% and prevalence of Hb E is from 1 – 9% depending on ethnical population groups. The spectrum of mutations among regional populations shows a marked heterogeneity. Nevertheless, most of previous researches focused on detection of mutations mainly in transfusion dependent thalassemia patients, therefore possibly misidentifying mutations that only partially affect the synthesis of β-globin chain. Therefore the overall objectives of this study were to define the molecular basis of mutations affecting β-globin gene not only in patients affected by the severe, and intermediate severe, forms of the anemia but also in a number of heterozygotes living in Central Vietnam, and to correlate the relationship between phenotype and genotype in β-thalassemia patients, with particularly emphasis to β-thalassemia intermedia.

The sample consisted of 226 subjects including 138 β-thalassemia carriers, 57 patients affected by thalassemia intermedia, and 31 affected by transfusion dependent thalassemia major patients. A total of 314 chromosomes were examined by the sequence of β-globin genes. Moreover, the DNA of 88 thalassemia patients was evaluated for the detection of the α-globin gene mutations common in SEA: $-\alpha^{3.7}$, $-\alpha^{4.2}$, --SEA, and Hb CS. Finally, with the aim to investigate if the co-inheritance of determinants such as the -158 (C>T) $^{G}\gamma$ polymorphism might be associated with an increased γ-globin chains production, the promoter region of the fetal $^{G}\gamma$-globin gene was sequenced.

Twelve known mutations along the β-globin gene were observed, among them four most common 26 (G>A) or Hb E, codon 17 (A>T), codons 41/42 (-TTCT) and IVS-I-1 (G>T) accounted for 86.94%. Other mutations with lower frequencies: codons 71/72 (+A), -28 (A>G), codon 95 (+A), codon 26 (G>T) were observed in 11.46% of chromosomes. The other four mutations have to be considered rare or very rare including -198 (A>G), -72 (T>A), -50 (G>A) and codons 14/15 (+G) with allelic frequencies 0.64%, 0.32%, 0.32% and 0.32%, respectively.

Several different combinations of β-globin genotypes were present in thalassemia patients: the genotypes β^E/β^E, β^E/β^+ and β^+/β^+ were observed only in patients with the intermediate, non-transfusion dependent, form of the disease whereas the β^o/β^o genotype was exclusive of patients affected by thalassemia major transfusion dependent. The coinheritance of α-thalassemia genes was confirmed to ameliorate the severity of the disease by reducing the amount of unpaired α-globins. On the other hand, no manifest correlation between -158 (C>T) $^G\gamma$ polymorphism and β-thalassemia genotypes as a factor ameliorating the phenotype was observed.

1. INTRODUCTION

1.1. GLOBIN GENE CLUSTER AND HEMOGLOBIN SYNTHESIS

1.1.1. Hemoglobin

Human hemoglobin (Hb) is a globular protein having the fundamental role to carry oxygen (O_2) and carbon dioxide (CO_2) molecules throughout the body. As shown in Figure 1.1, it is a tetrameric molecule consisting of four subunits, each covalently bound with a heme prosthetic group: two identical α-globin chains and two identical β-globin chains. Each subunits are held together by non-covalent bonds [68].

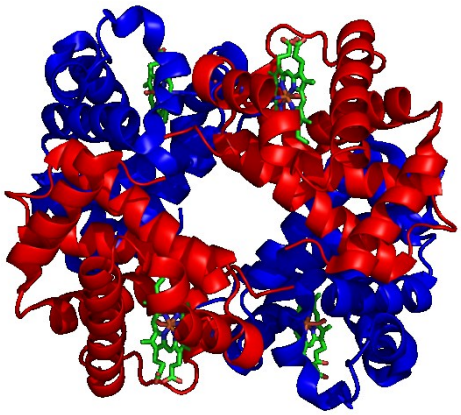


Figure 1.1. *Quaternary structure of Hb. The four subunits are shown in different colors. The heme groups are shown in green. The α and β subunits are in red and blue, and the iron-containing heme groups in green.*

The heme group is covalently bound to a N atom of the "proximal histidine, His", located at position 93 of the primary structure. This His stabilizes the heme group within each subunit. The O_2 molecule binds to the Fe ion that is opposite of the proximal His by the sixth coordination bond, in front of another His residue which is located at position 3364. This His is not directly bound to the heme group nor to the O_2 molecule (it is thus termed as the "distal His") even though it forces O_2 to bind heme with the axis at an angle thus weakening the binding of O_2 to Hb [68].

1.1.2. Switching of globin gene expression

As showed in Figure 1.2, human Hb consists of three different "α like" globin chains (ζ, α, and θ), and five different "β like" globin chains (ε, $^{G}\gamma$, $^{A}\gamma$, β, and δ) which

give rise, during the different stages of development, because of two switches, to seven different Hbs: the production of embryonic Hbs Gower1 ($\zeta_2\varepsilon_2$), Gower2 ($\alpha_2\varepsilon_2$) and Portland ($\zeta_2\gamma_2$) switches during the first two months of gestation to the production of fetal Hb (Hb F). Due to the presence of two different γ-globin genes ($^{G}\gamma$ and $^{A}\gamma$) there are two different fetal Hbs: $\alpha_2{}^{G}\gamma_2$ and $\alpha_2{}^{A}\gamma_2$. Just before birth, because of a new switch, the major adult Hb A ($\alpha_2\beta_2$) and minor adult Hb A_2 ($\alpha_2\delta_2$) tetramers are synthesized. As the result of the second switch, the circulating Hb at birth contains 70 to 80% of Hb F whereas 6 months later Hb F will be less than 4-5% of the total. The adult Hb pattern is reached at one year of life, when Hb A comprises ~97%, Hb A_2 ~2% and Hb F ~1%. It is at this stage that mutations affecting the β gene become clinically apparent [68].

At birth, Hb F contains $^{G}\gamma$ and $^{A}\gamma$ chains with the 70:30 ratio. Since the switch from Hb F to Hb A production is not complete, in the small amounts of Hb F produced in adult life the proportion of the two chains reverses to 40:60. The residual amounts of Hb F in adult life is due to a subset of erythrocytes called F-cells which are genetically controlled [48,68].

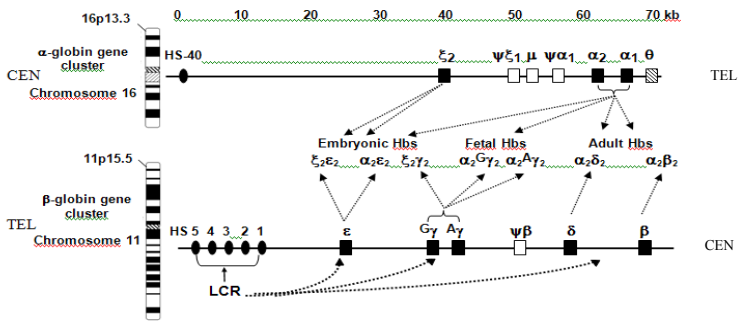


Figure 1.2. *Organization of human α- and β-globin genes and their expression during ontogenesis. The complex of α-globin gene is located close to the telomere of the short arm of chromosome 16. The β-globin gene complex is located on the short arm of chromosome 11. The functional genes are shown as black boxes, named according to the globin encoded. The non-functional pseudo-globin genes are shown as white boxes. The θ-globin gene is shown as a streaked box. The 5' → 3' transcriptional orientation is from left to right. The composition of Hb produced during developmental stages is given between the clusters. It is also shown the stage specific interaction between the Locus Control Region (β-LCR) and the gene cluster.*

1.1.3. Globin gene cluster

Hb subunits are encoded at two separate loci: the α-like globin gene cluster which is located on chromosome 11p15.5, and the β-like globin gene cluster on the terminus of chromosome 16p13.3. In each cluster the active genes are arrayed on the chromosome in the same order they are expressed developmentally.

1.1.3.1. The α-globin gene cluster

Position of the α-globin gene cluster is band 16p13.3, on the short arm of chromosome 16. From the direction 5' to 3', these genes are ζ, ψζ, ψα2, ψα1, α2, α1 and θ, respectively (Figure 1.3). Among them, genes ζ, α2 and α1 are three functional genes; three pseudogenes include ψζ, ψα2, ψα1 which are nonfunctional genes and θ gene that has still undetermined function. HS-40, the major regulatory element of the α globin gene cluster, is located 40 kb upstream of the CAP site of ζ gene [18].

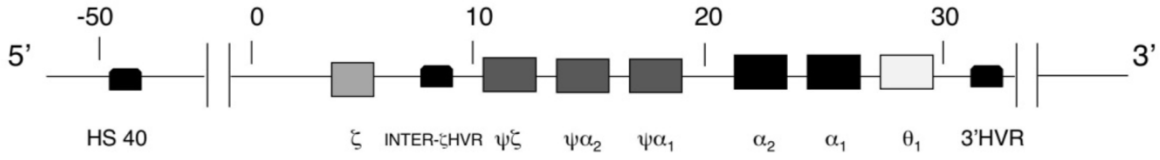


Figure 1.3. Structure of α-globin gene cluster [30]

1.1.3.2. The β-globin gene cluster

This cluster is on the short arm of chromosome 11, band 11p15.5, and spans approximately 60 kb DNA segment. Following the 5' to 3' direction, it is arranged as ε - ^{G}γ - ^{A}γ - ψβ - δ - β (Figure 1.4). The Locus Control Region (LCR) contains five elements such as HS-1, HS-2, HS-3, HS-4 and HS-5 [18].

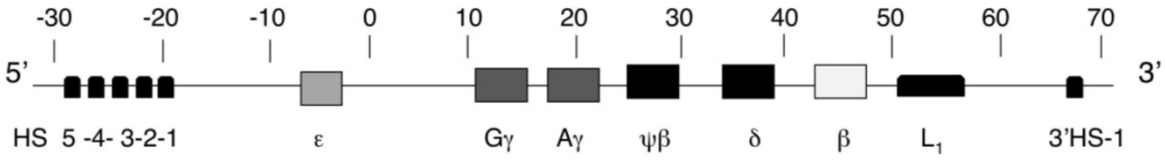


Figure 1.4. Structure of β-globin gene cluster [30]

1.1.3.3. α- and β-globin gene structure

All globin genes have the same general structure. The transcribed region includes three exons and two intervening sequences (IVS) or introns. The length of each region is variable between these genes: α-globin gene or *HBA1,* and *HBA2* [1] have two IVSs interrupting the coding sequence between codon 31 and codon 32 and between codon 99 and codon 100. Otherwise, the β-globin gene or *HBB* [1] have two IVSs located between codon 30 and codon 31, codon 104 and codon 105, respectively (Figure 1.5) [18]

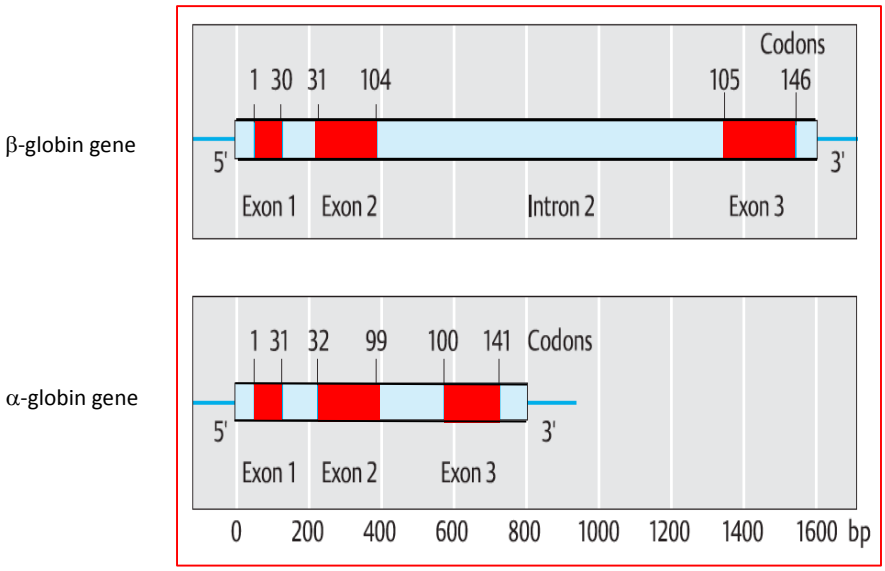


Figure 1.5. Structure of α- and β-globin gene [59]

1.2. HEMOGLOBINOPATHIES

Hemoglobinopathies (Hb-pathies), are a large group of inherited disorders of the synthesis of Hb that are considered the most common monogenic diseases worldwide, the highest frequency being observed in poor countries where appropriate services for control are hampered by the absence of knowledge about their molecular bases as well as their prevalence [81]. Hb-pathies are inherited as single-gene disorders resulting in the abnormal structure of one of the two pairs of globin chains (examples are the Hb variants) or in the underproduction of any normal globin chains, such as α-thalassemia, β-thalassemia, γ-thalassemia, δ-thalassemia, etc. In most cases, Hb-pathies are inherited as autosomal co-dominant traits. Some of them are very common, such are the cases of sickle-cell (Hb S) and Hb E diseases, which may reach the 30-40% frequencies. As is the

case of Hb E, Hb Knossos and others, abnormal structure and underproduction may overlap since mutations responsible for abnormalities in sequence may also affect production. Many Hb variants, however, do not cause pathology or anemia, and thus are not considered pathologies [48,68].

1.2.1. Thalassemia

Thalassemia is the disease which is characterized by decreased or absent synthesis of normal globin subunits. Thus, depending on which globin whose synthesis is ineffective, the disease is called: α-, β-, γ-, δ-, δβ- or εγδβ-thal [11]. Undoubtedly, α-thalassemia (α-thal) and β-thalassemia (β-thal) are the most relevant clinical forms [82].

1.2.1.1. The β-thalassemia

The β-thalassemia is due to mutations affecting the β-globin gene having the effect to completely prevent the synthesis of β-globin chain (β^o-thal). If the effect of the mutation is a lower than normal synthesis of β-globin chains, the disease is called β^+-thal [77].

*** *<u>Molecular basis of β thalassemia</u>***

The molecular basis of β-thal are extremely heterogenous. As many as 933 different mutations affecting the structure of the β-globin gene has been described thus far, nearly 300 of which affecting the rate of synthesis of β-globin [60]. Mutations are single nucleotide substitution, small insertion or deletions leading to frameshift. Rarely β thalassemia is cause by major gene deletions [27,83].

Mutations may affect gene expression at any level of the structural gene: from the 5'-untranslated region (UTR) to the 3'-UTR (Figure 1.6.) Depending on the effect on different stage of gene expression, mutations can be divided as follows [16,83]:

+ Transcriptional mutations: These include promoter mutations such as in the CACCC, CCAAT or TATA boxes. Generally, such mutations are mild forms of β-thal (of the β^+ or β^{++} type).

+ RNA processing mutations: The primary RNA transcript is modified by the "capping" reaction, which consists of the addition of 7-methylguanylate cap (m7G) at the 5' end of mRNA by means of the unusual 5' to 5' triphosphate bridge. At the opposite 3'

end of the pre-mRNA another post-transcriptional RNA processing a string of adenine bases are linked by poly (A) polymerase enzyme, which recognizes the sequence AAUAAA as a signal for the addition. This string of adenine is called the "poly A tail". Mutations result in phenotype of mild β^{+}-thal.

+ Maturation of pre-mRNA: A quite large number of β-thal mutations at the level of RNA splicing are observed. Mutations affect either the invariant GT dinucleotide motif at the 5' donor site or the AG motif at the 3' acceptor site at the level of intron/ exon junctions having the effect to abolish normal splicing. As the consequence there will be no β-globin synthesis (β^{o}-thal). Mutations may also occur within the splicing consensus sequences. In this case, the efficiency of normal splicing (and then the synthesis of β-globin) is reduced giving rise to a range from mild to severe of β^{+}-thal. Mutations in exon and intron might activate cryptic splice sites resulting in β^{+}-thal with phenotypes from mild to severe.

+ RNA stability: Mutations affecting the untranslated 5' or 3' regions also give rise to mild β-thal phenotype.

+ mRNA translation: Mutations such as nonsense, frameshift, initiation codon mutations result in β^{o}-thal.

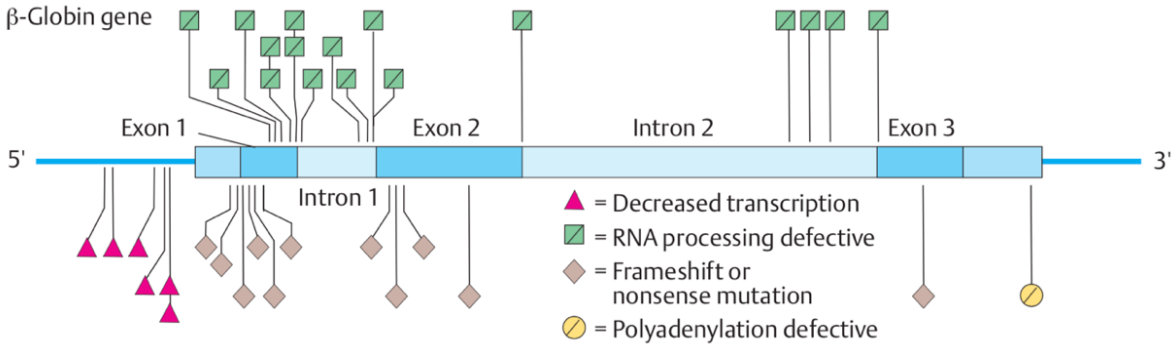


Figure 1.6. Different mutations causing β-thalassemia [59]

*** *Clinical classification of β-thalassemia***

β-thalassemia includes three clinical form: thalassemia minor, thalassemia intermedia and thalassemia major [36].

- Thalassemia minor

A subject affected by "thalassemia minor" is a heterozygous for a β-globin gene mutation, either of the β^o or the β^+ type. The carrier condition is usually clinically asymptomatic and normally detected by means of determination of red blood cells parameters and by the determination of the minor Hb A_2 fraction.

The characteristic of heterozygous β thalassemia, either β^o or β^+, is microcytosis (low mean corpuscular volume - MCV), hypochromia (low mean corpuscular hemoglobin - MCH) and increased Hb A_2 level. The increased Hb A_2 percentage is the most important feature for identifying heterozygous β-thal [8].

Table 1.1. Hematological features of β-thalassemia minor [7]

	Indices
Red blood cell indices	MCV < 80 fL, MCH < 27 pg/cell
Hemoglobin pattern	Hb A 92 – 95%, Hb A_2 > 3.8%, Hb F 0.5 – 4%

- Thalassemia major

Thalassemia major (TM), first described by Thomas Cooley in 1925, also known as "Cooley's anemia" or "Mediterranean anemia". This is the most severe β-thal. Clinical presentation occurs between 6 to 24 months and patients require regular transfusion to survive. The β genotype may be β^o/β^o, β^o/β^+ or β^+/β^+.

- Thalassemia intermedia

The term thalassemia intermedia (TI) describes patients whose clinical phenotype is too severe to be called minor and too mild to be called major. TI is extremely heterogenous and has a wide clinical spectrum: mildly affected patients might be asymptomatic until adult life, only have mild anemia and maintain Hb level between 7 to 10 g/dl. The more severe TI patients present symptoms between 2 to 6 years old [12].

At the genotype level TI is quite heterogeneous: depending on the number of mutations in a given population, patients may be homozygous or compound heterozygous for β^+, β^o, or both type of mutations. Heterozygotes for only one β mutation having symptoms of TI are less common and might have inherited other kind of mutations such as α-thal genes.

The basis and severity of thalassemia syndromes are mainly due to globin chain imbalance. To the absence or the reduced number of β-globin chains corresponds a proportional excess of mismatched α-globin chains. The free α chains early precipitate in the bone marrow erythroid precursors leading to membrane damage and cell death. This "ineffective erythropoiesis" is thus the major factor of anemia. Moreover, in the circulating peripheral erythrocytes the precipitates of oxidized α-chain, (also called inclusions bodies or Heinz bodies) have the effect to induce hemolysis. In homozygous or compound heterozygous β-thal, the factors reducing α/non-α globin synthesis imbalance often result in milder clinical forms.

The milder clinical manifestation of TI compared with TM is considered to be associated with inherited form of mild to silent β-thal mutations, or the coinheritance of α-thal, leading to a more balanced α-/non-α globin chains ratio, to the coinheritance of determinants associated with the increase of γ-chain production [25]. In fact, the increased production of γ-globin chain of Hb F not only helps to neutralize large portion of unbound α-chains but also produces a net increase in total hemoglobin synthesis. Several genetic determinants enhancing γ-globin synthesis have been identified: the intrinsic to β-gene mutations (such as 5' β-promoter deletion, deletion δβ- thal, and Hb Lepore), $^{G}\gamma$ or $^{A}\gamma$ promoter mutations (-196 C → T $^{A}\gamma$, and -158 C → T $^{G}\gamma$), or related to coinherited heterocellular hereditary persistence of fetal hemoglobin (HPFH) (deletion and non-deletion).

The most common is a sequence variant (C → T) at position -158 upstream of the $^{G}\gamma$-globin gene, termed the "Xmn-1 $^{G}\gamma$ polymorphism". It was originally recognized that under condition of erythropoietic stress such as homozygous β-thal and sickle cell anemia, the presence of Xmn-1 $^{G}\gamma$ polymorphism lead to higher Hb F response [41,66]. Although, there are no conclusive evidence for its effect in heterozygous β-thal and in normal individual, the influence of Xmn-1 polymorphic site through its interactions with transcription factors, and polymorphism in the transcription factors could be influencing Hb F expression, conditional on the Xmn-1 $^{G}\gamma$ site. Table 1.2 shows some criteria to differentiate between TM and TI.

Table 1.2. Criteria to differentiate between TM and TI at presentation [72]

Parameter	TM more likely	TI more likely
Clinical		
Presentation (years)	< 2	> 2
Liver/spleen enlargement	Severe	Moderate to severe
Hematology		
Hb (g/dl)	6 – 7	7 – 10
Hb F (%)	> 50	10 – 50 (may up to 100 %)
Hb A_2 (%)	< 3.5	> 3.5
Molecular		
Type of mutation	Severe	Mild/Silent
Co-inheritance of α-thalassemia	No	Yes
Hereditary persistence of Hb F	No	Yes
δβ-thalassemia	No	Yes
$^{G}\gamma$ Xmn-1 polymorphism	No	Yes

**** Hematological features***

- β-thalassemia carrier state

Heterozygotes (carriers) of β-thal are clinically asymptomatic. The main, and characteristic, hematological features are reduced red blood cell (RBC) volume (microcytosis), reduced RBC Hb content (hypochromia), increase level of the minor Hb A_2 ($\alpha_2\delta_2$), and slightly imbalanced $\alpha/(\beta + \gamma)$ globin chain synthesis. Thus, the Hb pattern of β-thal heterozygotes is characterized by 92-95% Hb A, 3.8% Hb A_2, and variable, although minute, amount of Hb F (0.5-4%). Additionally, the blood smear confirms microcytosis, hypochromia, and marked variations in size and shape of RBC.

- Thalassemia major

Patients have a severe microcytic and hypochromic anemia, with increased number of RBCs and low MCV and MCH. Blood smear shows, in addition to microcytosis and hypochromia, anisocytosis, spiculated tear drop and elongated cells (poikilocytosis), and erythroblasts (nucleated RBC), the number of which being related to the degree of anemia.

Hb pattern, as determined by different methods, such as cellulose acetate electrophoresis, high performance liquid chromatography (HPLC), capillary electrophoresis, varies according to the type of β-thal: in β^o-thal homozygotes, characterized by the lack of synthesis of the β globin, Hb A is absent, Hb F is 95-98%, and Hb A_2 is from 2 to 5%. In β^+-thal homozygotes and in β^o/β^+-thal double heterozygotes, having a residual ability to synthesize β-globin, the Hb pattern shows Hb A between 10 and 30%, Hb F between 70 and 90%, and Hb A_2 from of 2 to 5%.

Because of the complete, or quite complete, absence of β-chain synthesis and the marked excess of α-globins and inadequate levels of γ-globins of Hb F, the bone marrow appears extremely cellular as a result of marked erythroid hyperplasia, with a myeloid/erythroid ratio reversed from the normal 3 or 4 to 0.1 or less.

- Thalassemia intermedia

Patients have a moderate anemia and show a markedly heterogeneous hematological picture, ranging in severity from that of the β-thal heterozygous to that of thalassemia major [7].

1.2.1.2. The α-thalassemia

Normal individuals have four α-globin genes, two on each chromosome 16 and the genotype is written as αα/αα. The α2 and α1 genes are highly homologous, whereas 5'UTR, coding regions, introns are highly similar, the 3'UTR is markedly diverged [50]. Both genes are all expressed and encode identical protein. However, the α2 gene (5' position) synthesis protein from 2 to 3 folds more than α1 (3' position). The dominant expression of α2 gene predicts a more severe impact of mutation on α2 gene compared to α1 gene [47].

The most frequent mutations observed in α-thal are deletions involving one (-α) or both (--) α genes on chromosome. Non-deletional mutations occurring on α2 gene ($\alpha^T\alpha$) or α1 gene ($\alpha\alpha^T$) are less common. According to α-globin chain synthesis, there are either α^o-thal if no product is expressed or α^+-thal if the product is the result of a reduced expression. Figure 1.7 classifies α-globin gene defects and clinical phenotypes.

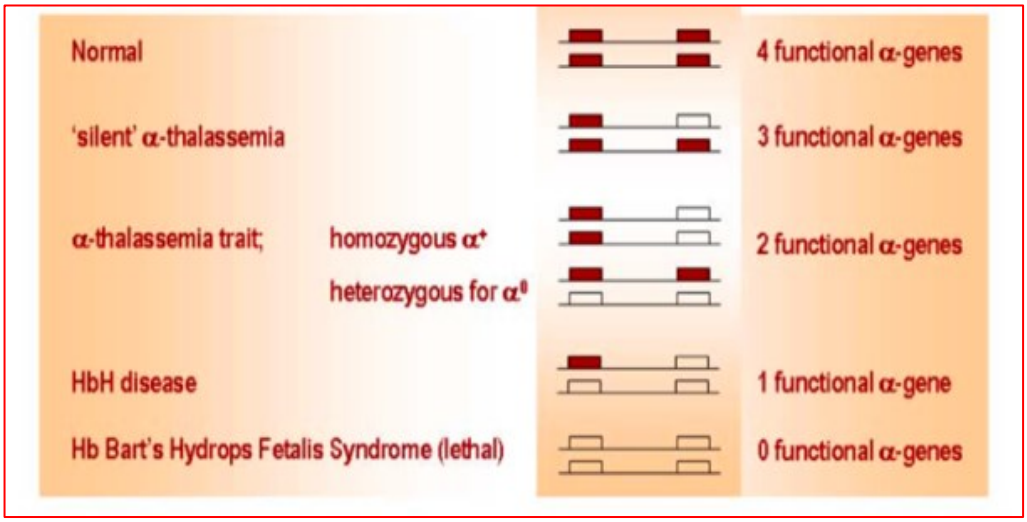


Figure 1.7. The classification of α-globin gene defects and clinical phenotype [32]

*** *α^{+}-thalassemia due to deletions***

The α-globin genes are located within two homologous DNA segments, each approximately 4 kb in length. Each region has three homologous subsegments including X, Y and Z, among them Zα1 and Zα2 segments contains α1 and α2 genes, respectively [33]. The common -α$^{3.7}$ deletion is caused by reciprocal recombination between mispaired Z segments. In the same way, a reciprocal recombination occurs between mispaired X segment results in -α$^{4.2}$ deletion (Figure 1.8).

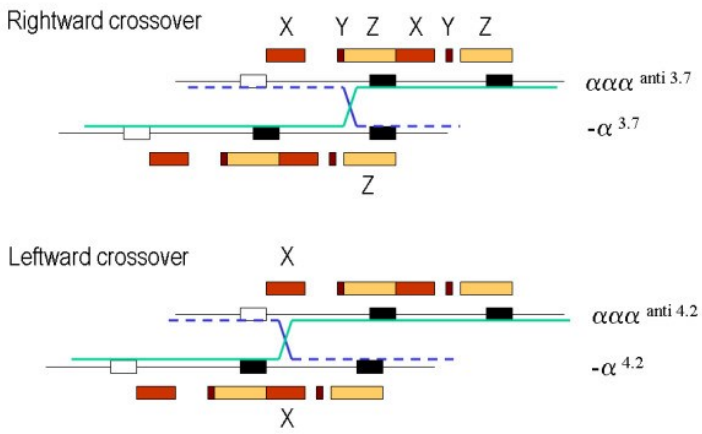


Figure 1.8. Deletions that cause α^{+}-thalassemia [32]

*** α^{+}-thalassemia due to non-deletion**

With respect to deletional type of chromosomes, non-deletional α^{+}-thal may severely reduce α-globin chain synthesis than deletional type. Many mutations have been reported that affect mRNA processing, mRNA translation or α-globin stability [83].

*** α^{o}-thalassemia due to deletions**

Completely or partially delete both α genes in *cis* leads to no α-chain product directed by these chromosomes (Figure 1.9). Homozygous for these mutations gives rise to the Hb Bart's hydrops fetalis syndrome.

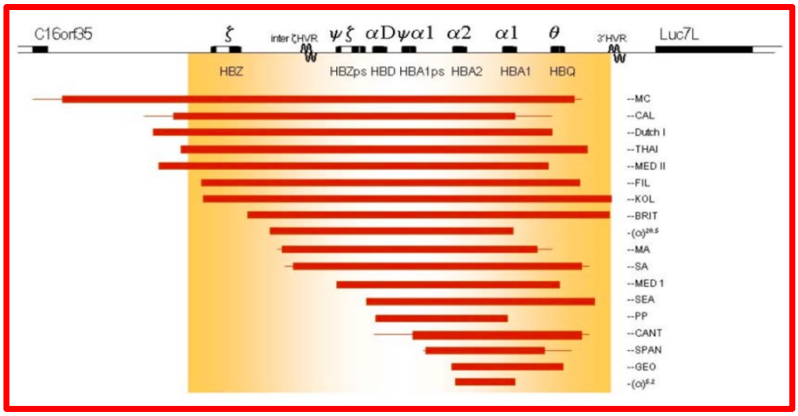


Figure 1.9. α^{o}-thalassemia due to deletion both α gene [32]

*** Clinical phenotype of α-thalassemia**

The clinical phenotypes of most individuals with α-thalassemia are mild. There are three groups including α-thal trait, Hb H disease and Hb Bart's hydrops fetalis syndrome.

The α-thal trait or heterozygous of α-thal is clinically asymptomatic and has from mild to moderate microcytic hypochromic anemia.

The Hb H disease is due to the deletion of three α-globin genes out of the four, and patients affected may show different phenotypes. Patients with non-deletional Hb H disease have more severe phenotype than patients with deletional form and may need occasional or frequent transfusion requirement.

Hb Bart's hydrops fetalis is caused by deletion of all four α-globin genes (Figure 1.7 and 1.9). Due to the absence of α-globin chains, most of the Hb in these infants are made by the non-functional homotetramers γ_4 and β_4, together with the fetal Hb Portland ($\zeta_2\gamma_2$), which is the only functional Hb. These infants die in utero or shortly after birth [32].

1.2.2. Structural hemoglobin variant

Hemoglobin variants result from mutations which alter amino acid sequence. This group is mostly composed of missense mutations which cause substitution of single amino acid in globin chain. Hemoglobin variants are less commonly associated with other mutation types such as deletions, insertions and fusions one or more adult globin genes [63,78].

Hemoglobin variants show a range of biochemical abnormalities. Despite many variants are clinically silent, some of them produce clinically significant symptoms of varying severity. The most common and important variants are Hb S, Hb C and Hb E. Hemoglobin S is mainly distributed in Sub-Saharan Africa, parts of Mediterranean, Indian subcontinent and Middle East, in where the frequencies of carrier are between 5 – 40% or more. Hb E is most prevalent throughout Southeast Asia with carrier rates may exceed 60% [82]. Hb C is the third most common variants worldwide. It is observed in 17 – 28% of West African people and 2 – 3% of African Americans [63].

Hb E is cause by a G → A substitution at codon 26 of *HBB* gene, which results in replacement glutamic acid to lysine. This mutation not only alters structure of globin gene but also activates new cryptic splice site, which affects mRNA processing. The degree of normally spliced β^E mRNA is decreased results from the competition between normal donor site and new splice site [56]. Therefore, Hb E acts like a mild form of β-thal [21].

In Southeast Asia, Hb E and β-thal are both high prevalent, therefore coinheritance of Hb E and β-thal frequently occurs. This condition gives a major public health problem in this region [23]. Hb E/β-thal is classified as: *severe* form with Hb 4 – 5 g/dl, transfusion dependent, clinical symptoms same as β TM; *moderate* form with Hb 6 – 7 g/dl, transfusion independent, clinical symptoms same as β TI; *mild* form with Hb 9 – 12 g/dl, transfusion independent, usually not have clinically significant problems [71].

1.2.3. Transfusion dependent thalassemia and non-transfusion dependent thalassemia

The classification of thalassemia including thalassemia minor, intermedia and major has left out several clinical thalassemia syndromes, especially α-thal and hemoglobin structural variants combined to thalassemia such as Hb E/β-thal.

In 2012, the new terminology for clinical classification of thalassemia was proposed including transfusion dependent thalassemia (TDT) and non-transfusion dependent thalassemia (NTDT). These new terminologies are adopted by the Thalassemia International Federation in their guidelines and publications [11,71].

Patients with TDT require regular blood transfusion to survive. TDT consist of patients with β-thal major, severe Hb E/ β-thal and non-deletional Hb H disease [11].

The term NTDT is used for patients who do not require lifelong regular transfusion for survival, however they may occasionally or even frequently require transfusion in certain clinical settings. According to molecular defect, NTDT encompasses three main group: β-thal intermedia, mild and moderate forms of Hb E/β-thal and Hb H disease [71].

1.3. WORLD DISTRIBUTION AND POPULATION SCREENING FOR β-THALASSEMIA

1.3.1. Distribution of β-thalassemia mutations

Thalassemia is distributed in a broad region extending from Mediterranean, Africa, Middle East, Indian subcontinent, Southeast Asia, Melanesia and the Pacific (Figure 1.10). The highest β-thalassemia carrier frequency is observed in the Mediterranean islands of Cyprus (14%), Sardinia (10.3%) and Southeast Asia [27].

Figure 1.10. World distribution of α- and β-thalassemia [82]

More than 300 mutations causing β-thal have been so far described. Many of them are rare, often reported only one, although the most are rather common. The spreading of mutations is heterogeneous, geographically and ethnically specific. The list of the most common mutations in Figure 1.11 indicates a breakdown of the distribution: in Europe and Middle East, nearly three-quarters of the β-thal alleles are represented by codon (cd) 39 C → T, IVS-I-110 G → A, IVS-I-1 G →A and IVS-I-6 T → C. In Southeast Asia, Hb E is largely prevalent, reaching 50 – 60% at the Thailand, Laos and Cambodia triangle, IVS-I-5 G → C, and cd 41/42 -TTCT mutations being also common. Without reference to the Hb S mutation, in Africa, just two β-thal alleles, the -29 A → G and -88 C →T account for 81% [22,82].

Figure 1.11. Distribution of common β-thalassemia mutations [82]

1.3.2. Population screening for thalassemia

Since the years 1970s, large screening programs for β-thal have been introduced in Sardinians, Continental Italians, Greeks and Cypriots representing at risk populations in Mediterranean area. A number of procedures for the identification of β-thal heterozygotes were adopted [8,10] mainly based on the MCV, and MCH determination, followed by Hb A_2 quantification for individuals with low MCV or low MCH. Analysis of Hb by means of column HPLC, rather than the electrophoresis on cellulose acetate, allows to quantify Hb A_2 and to detect most common Hb variants. Individual with low MCV, MCH and elevated Hb A_2 is diagnosed as being β-thal carrier. Thanks to this simple and low cost procedure, it was possible to successfully prevent the birth of thalassemia major [9].

Procedures for carrier identification have to consider some factors that may modify hematology. One of these factors is iron deficiency which may decrease high Hb A_2 level. This level, however, unless an associated severe anemia, remains within the

range for β-thal [28] or may not affect to level of Hb A_2 [67]. There are some mild β^+-thal mutations that may be associated to normal or borderline levels of Hb A_2, as well as double heterozygous for β- and δ-thal. It also important to consider that double heterozygous for β- and α-thal might show normal MCV and MCH and variable levels of Hb A_2. Thus in populations having high prevalence of both α- and β-thal, the first test of screening have to include determination of both MCV, MCH and Hb A_2 detection [8].

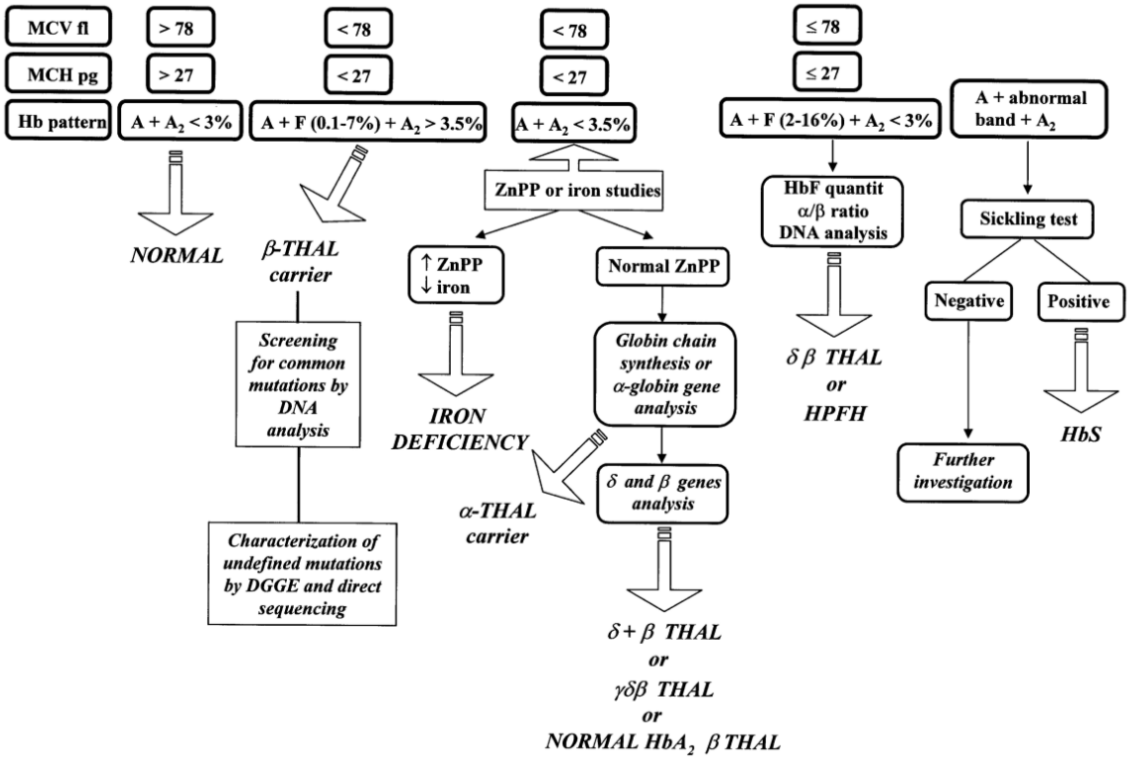


Figure 1.12. Flow chart of screening thalassemia in Sardinia [9]

The screening approaches for thalassemia in developed countries have to consider the presence of a mixed, multiethnic population. Thus, due to the amplitude of heterogeneity of the β-thal mutations and their occurrence in different populations, knowledge of ethnic background is essential and the flowchart for screening adapted [44].

1.4. SCREENING AND DIAGNOSTIC TECHNIQUES FOR HEMOGLOBINOPATHIES

1.4.1. Complete red blood cells count

Full red blood cell count on an automated, or semi-automated, hematology analyzer is the primary screening approach which also gives the indices that indicate thalassemia trait: hemoglobin concentration per liter (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). If the MCV < 80 fL or MCH < 27 pg, the next step is hemoglobin analysis by electrophoresis or HPLC quantifying Hb A_2 and Hb F [11,44].

1.4.2. Hemoglobin analysis

1.4.2.1. High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was first described by Wilson et al. in 1983 [84] and widely applied for detecting β-thalassemia trait and some structural hemoglobin variants.

Hemoglobins are separated by an analytical cartridge in HPLC system using programmed buffer gradient with increasing ionic strength and pH to the cartridge. In this method, the different concentrations phosphate buffers (mobile phase) pass through an ionic exchange column (stationary phase) under pressure.

The stationary phase consists of analytical cartridge which contains resins of anionic or cationic particles. Hemoglobins are separated based on their ionic interaction with stationary phase. The separated fractions then pass through a flow cell, where absorbance at 415 nm is measured and additional absorbance at 690 nm is also valued for reducing background noise [42,55]. Each hemoglobin has its own retention time which is measured from the sample injection to the apex of each hemoglobin peak time. By comparing to known hemoglobin retention times, the hemoglobin types can be identified [35]. In addition, the percentage of each hemoglobin component are detected include Hb F, Hb A_{1c}, Hb A, Hb A_2 and, eventually, abnormal Hb.

Many researches show HPLC as a single, highly reproducible system for screening thalassemias and hemoglobin variants. This technique is widely applied for routine clinical laboratory based on high resolution, rapid assay time, precise quantification of

hemoglobin components as well as minimal sample preparation, automatic analysis [11,24,35,39,64].

However, HPLC technique have some limitations. Hb E and the δβ fusion globin Hb Lepore, are co-eluted with Hb A_2, therefore their presence gives falsely high percentage of Hb A_2 (> 10%). So, samples with Hb A_2 level more than 10% should be further investigated. Due to co-elution with Hb S, falsely increased Hb A_2 may be seen in Hb S heterozygotes. Hb H and Hb Bart's can be observed, but not quantified because their earlier elution time [24].

1.4.2.2. Capillary electrophoresis

Capillary electrophoresis (CE) is an alternative tool for separating and quantifying hemoglobin fractions performed in narrow capillaries filled with buffer [40,55]. It is based on the same properties of any Hb separation by means of electrophoretic mobility of charged molecules, and electric osmotic flow. The internal surface of capillary is charged negatively thus attracting the positive charged ions of the buffer leading to an electrical double layer. When a voltage across the capillary is applied, cations in the layer move towards the cathode, carrying the bulk solution with them. Hemoglobin fractions are directly detected and quantitated at 415 nm at the cathodic terminal of capillary. From the anode to cathode the order of normal and abnormal variants elution is: Hb H, Hb J, Hb Bart's, Hb A, Hb F, Hb S, Hb E, Hb A_2, Hb CS, Hb C, and Hb D [55].

Capillary electrophoresis is of great importance in countries where Hb E variant is present since it provides accurate identification and quantification of Hb A_2 when Hb E is present. This technique is rapid, fully automated, and provides good resolution and high accuracy [29].

Both HPLC and CE might be complementary technique for routinely clinical practice in laboratories that can afford it economically [19,37].

1.4.3. Molecular diagnosis

There are many different PCR-based techniques using for detecting globin gene mutations. Depend on type and variety of mutations, particular condition of each laboratory, as well as the advantages and disadvantages of methods, the appropriate methods may be chosen for detecting mutations.

1.4.3.1. Gap-PCR

In gap-PCR, specific primers are designed to flank of known deletion. Gap-PCR is based on the inability of two flanking primers to generate PCR product in the normal allele because the distance between two primers is too large for successful amplification. The deletion joins the flanking sequences together, so amplicon occurs in the mutant allele [43].

Gap-PCR is a rapid, simple and efficient for identifying α-thalassemia deletion mutations. This method has been applied for diagnosis of the seven most common α-thalassemia deletions including $-\alpha^{3.7}$, $-\alpha^{4.2}$, --SEA, --MED, $-(\alpha)^{20.5}$, --FIL and --THAI [14,73].

1.4.3.2. DNA sequencing

There are two method for DNA sequencing: chemical method developed by Maxam and Gilbert in 1980 [49] and enzymatic method or dideoxy sequencing method first developed by Sanger. The vast majority of current DNA sequencing uses dideoxy sequencing method.

Dideoxynucleotides (ddNTPs) have closely similar structure to the normal deoxynucleotides (dNTPs). ddNTPs differ only in that they lack a hydroxyl group at position 3'. A ddNTP can be incorporated into the growing DNA chain by forming a phosphodiester bond with previously incorporated nucleotide. However, since ddNTPs lack a hydroxyl group at position 3', any ddNTP incorporated into a growing DNA chain cannot perform phosphodiester bond at its position 3' leading to terminate chain synthesis.

There are four parallel reactions, each reaction contains four dNTPs plus a small proportion of one of the four ddNTPs (ddATP, ddGTP, ddCTP, ddTTP). The primer or one of the four dNTPs is labeled with a distinctive radioisotope group or fluorophore, therefore the growing DNA strand becomes labeled.

In each reaction, there will be competition between normal dNTP and specific ddNTP for inclusion in the growing DNA chain, if a dNTP is included, chains continue extend, but occasionally a ddNTP is incorporated causing chain termination. The chain termination randomly occurs at one of the possible choices for a specific type of base in any one DNA strand. The synthesized DNA fragments in each reaction include a range of

different sizes. Fragments which are different in size, even by a single nucleotide can be size-fractionated on a denaturing polyacrylamide gel. After exposing the sequencing gel to an X-ray film, the sequence is manually read by following successive bands on autoradiograph. This cumbersome approach has been resolved by automated DNA sequencing methods.

Automated DNA sequencing uses fluorescence labeling and capillary electrophoresis instead of radioisotope and electrophoresis on polyacrylamide gel. The use of different fluorophores in four base specific reactions allow all four reactions can be loaded into a single land. A monitor detects and records different fluorescence signals and then information is automated analyzed [69].

Most β thalassemia are due to point mutations, therefore Sanger sequencing is the most efficiently current method for identifying all possible mutations in *HBB* gene [65].

2. RESEARCH OBJECTIVE

As discussed in paragraph 1, Southeast Asia is characterized by the presence of α- and β-thal, Hb E and Hb CS with gene frequencies in some cases really conspicuous. In the case of α-thal, the 30 – 40% prevalence was described in Northern Thailand and Laos, 4.5% in Malaysia and 5% in the remote island of the Philippines, whereas β-thal vary between 1 and 9%. Similarly, the Hb E structural variant reaches the 50 – 60% frequency at the junction of Thailand, Laos, and Cambodia. From 1 to 8% frequencies of the thalassemic Hb CS gene were also assessed. As to be expected, the combination of such a number of abnormal genes gives dozens of different thalassemia syndromes [23].

In Vietnam, both β-thal and Hb E are prevalent. They were described as being distributed all over the country. The frequency of β-thal carrier ranges from 1.5% to 25% and prevalence of Hb E is from 1 – 9% depending on ethnical population groups [70]. The first study for β-thal early performed in North Vietnam, then the spectrum of β-thal mutations in South Vietnam were soon reported and the Central Vietnam mutations have just recently published. The spectrum of mutations among three main regions shows marked heterogeneity [15,17,31,61,70]. Nevertheless, most of researches focused on detection mutations in transfusion dependent thalassemia patients, therefore possibly misidentifying mutations that only partially affect synthesis of β globin chain (β^+) mutations.

The aim of this research project is to contribute to the knowledge of the molecular lesions at the level of β-globin gene characterizing β-thal defects in Central Vietnam, with the following objectives:

1. To define the molecular basis of mutations affecting β-globin gene in Central Vietnam.

2. To define relationship between phenotype and genotype in β-thal patients, with particularly emphasis to β-thal intermedia.

3. To compare result with the previous studies on β-thal conducted in North and South Vietnam, and in neighboring countries.

4. To contribute to guide appropriate future preventive and control actions of the disease at the level of the whole country.

3. MATERIALS AND METHODS

3.1. STUDY DESIGN

This consisted with a cross-sectional study that was conducted between October 2016 and September 2019, at following settings:

- Medical Genetics Department of Hue University of Medicine and Pharmacy (HUMP), Vietnam
- Hematology Department of HUMP, Vietnam.
- Hematology Department of Hue Central Hospital, Vietnam.
- Clinical Hematology Department of Hue Central Hospital, Vietnam.
- Department of Biomedical Sciences of the University of Sassari, Italy.
- Institute of Research and Biomedical Genetics, National Research Council, Sassari, Italy.

3.2. STUDY POPULATION

This study includes patients who admitted to Medical Genetics Department of HUMP, Hematology Department of Hue Central Hospital and Clinical Hematology Department of Hue Central Hospital, Vietnam. The explanation about the study and informed consent were made from all the patients.

The patients were chosen based on the following criteria:

3.2.1. Inclusion criteria

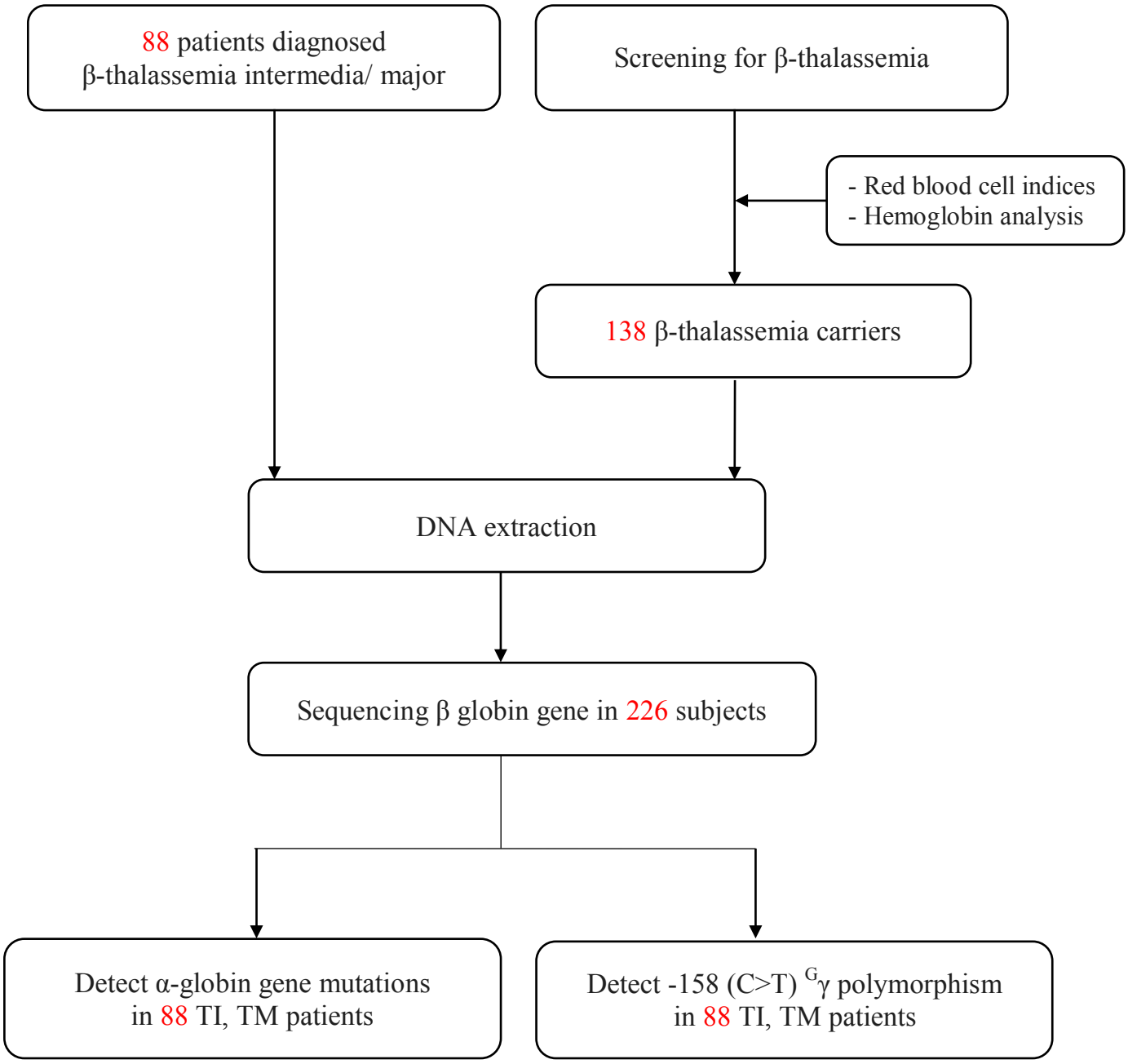
- Patients determined as β-thal carrier based on MCV < 80 fL, MCH < 28 pg and Hb $A_2 \geq 3.5\%$.
- Patients diagnosed as severely affected β-thal required blood transfusion.

3.2.2. Exclusion criteria

- Patients refuse to join in this study.

Figure 3.1. WORKFLOW OF STUDY

138 β-thalassemia carriers were identified and 88 patients diagnosed β-thalassemia intermedia or major were included. The flowing procedure is shown below.



3.3. METHODS

3.3.1. Complete blood count (CBC)

Whole blood was collected using EDTA as anticoagulant.

Red blood cell indices and complete cell analysis were determined using an automatic cell counter (SX 1000i of Sysmex, Japan). The hematological parameters for the study included: Hb (g/dl), MCV (fL), MCH (pg).

Samples with MCV < 80 fL and/or MCV < 27 pg were chosen for analyzing in the next step.

3.3.2. Hemoglobin analysis

Analysis hemoglobin were performed by high performance column liquid chromatography (HPLC) at Medical Genetics Department of HUMP or by capillary electrophoresis coupled to agarose gel electrophoresis, at Hematology Department of Hue Central Hospital.

3.3.2.1. HPLC

A. Equipment and reagent

- Thermo Scientific^{TM} UltiMateTM 3000 RSLCnano system.
- Chromsystems kit for β-thal testing includes buffer A, buffer B, hemolysis reagent, wash buffer and HPLC column.

B. Procedure

B.1. Collection of samples

- 3 ml whole blood sample was collected using EDTA as anticoagulant.
- Stored the sample at 2 – 8^{o}C.

B.2. Preparation of HPLC system

- Switched on HPLC system and computer, opened Chromeleon analyzer.
- Checked buffer A and buffer B.
- Turned on UV light, installed at 415 nm.
- Removed old buffer inside the system.
- Chose analyzed column.
- Washed automatic sample injection system.

- Run about 20 minutes by Buffer B with speed 1.5 ml/minute for washing column, stabilize pressure of mobile phase and UV light signal before running samples.
- Prepared list of samples.

B.3. Procedure

- Diluted 5 to 8 µl whole blood in bottle already containing 1 ml hemolysis reagent, then mixed well.
- Put the bottle on the correct position in plate, appropriate to position installed.
- Entered "Start batch" to start running samples.
- After finish running, washed the column by buffer B with speed 1.5 ml/minutes in 15 minutes to remove residual.
- Next, installed buffer A to move with speed 1.5 ml/minute in 5 minutes to preserve column.
- Switched off system.

Based on retention time of each hemoglobin, the Chromeleon software identify hemoglobin types and quantify percentage of each hemoglobin.

3.3.2.2. Capillary electrophoresis

A. Equipment and reagent

- MINICAP FLEX-PIERING instrument, (SEBIA, Evry Cedex, France) Sebia.
- MINICAP HEMOGLOBIN(E) kit: it is designed for the separation of normal hemoglobins including Hb A, Hb A_2 and Hb F, and also for identification of major hemoglobin variants such as Hb S, Hb C, Hb E and Hb D. The MINICAP HEMOGLOBIN(E) kit consist of five components:
  + MINICAP HEMOGLOBIN(E) buffer contains alkaline buffer, pH 9.4
  + Hemolyzing Solution
  + Washing Solution
  + Reagent cups
  + Filters

B. Procedure

B.1. Collect samples

- 3 ml whole blood sample was collected using EDTA as anticoagulant.
- Stored the sample at 2 – 8^{o}C.

B.2. Sample preparation

- Used directly whole blood samples.
- Checked that all tubes containing at least 1 ml of blood and were perfectly closed. The tubes were closed with their corresponding caps designed for the MINICAP HEMOGLOBIN(E) procedure with the MINICAP FLEX-PIERCING instrument.
- Vortexed the blood samples for 5 seconds.

B.3. Procedure

- Switched on MINICAP FLEX-PIERCING instrument and computer.
- In order to start the instrument, positioned at least one new reagent cup in the automate loading system for cups on MINICAP FLEX-PIERCING.
- Set up the software, the instrument automatically started.
- Selected 'HEMOGLOBIN(E)" analysis program, placed the MINICAP HEMOGLOBIN(E) buffer via in position "B2" in instrument.
- Positioned new reagents cups on the automated loading system for cups of MINICAP FLEX-PIERCING.
- Positioned a bin for used cups in MINICAP FLEX-PIERCING at the location intended for this purpose.
- Checked reagent vials, added more if necessary and emptied the waste container. In the window "Check reagent levels", updated the software by moving the cursors buttons.
- Placed up to 26 capped sample tubes on the rotating sampler with specific centering rings (positions number 1 to 26), the barcode of each tube had to be visible in the opening of the rotating sampler.
- Put the hemolyzing buffer in an uncapped hemolyzing tube, identified by hemolyzing solution barcode label, without air bubbles: 2 ml for analyzing one or two samples, 5 ml for twelve samples. Placed this tube in position number 27.
- Placed the normal Hb A_2 control in position number 28 on the rotating sampler and selected the number of analyses of the normal Hb A_2 control.
- Slid the rotating sampler into the MINICAP FLEX-PIERCING instrument.
- Closed the doors of the MINICAP FLEX-PIERCING instrument, the analysis started automatically.

- After analyses, removed the rotating sampler with analyzed sample tubes.
- At the end of each analyses, shut down procedure of MINICAP FLEX-PIERCING in order to store capillaries in optimal condition.

At the end of the analysis, the hemoglobin fractions are automatically identified, the relative quantification of each hemoglobin was automatically performed. The potential positions of different hemoglobin variants that were identified in zones form Z1 to Z15 were shown on the screen of system. When the software identified a hemoglobin fraction in a defined zone, the zone's name was framed.

3.3.3. DNA extraction

After analyzing hemoglobin components, DNA was extracted from whole blood samples.

A. Reagent

- Wizard Genomic DNA Purification kit (Promega).
- 70% ethanol, room temperature.
- Isopropanol, room temperature.

B. Procedure

- Added 900 µl of Cell Lysis Solution to a sterile 1.5 ml microcentrifuge tube.
- Gently rocked the blood tube until thoroughly mixed, then transferred 300 µl blood to the tube containing the Cell Lysis Solution. Inverted the tube 5 – 6 times to mix.
- Incubated the mixture for 10 minutes at room temperature (inverted 2 – 3 times once during the incubation) to lyse the red blood cells. Centrifuge at 13,000 x *g* for 20 seconds at room temperature.
- Removed and discarded as much as possible the supernatant without disturbing the visible white pellet.
- Vortexed the tube vigorously until the white blood cells were resuspended (about 10 – 15 seconds).
- Added 300 µl Nuclei Lysis Solution to the tube containing the resuspended cells. Pipetted the solution 5 – 6 times to lyse the white blood cells. The solutions should become very viscous.

- Added 100 µl Protein Precipitation Solution to the nuclear lysate, and vortexed vigorously for 10 – 20 seconds.
- Centrifuged at 13,000 x *g* for 3 minutes at room temperature.
- Transferred 300 µl supernatant to a clean 1.5 ml microcentrifuge tube containing 300 µl of room temperature isopropanol.
- Gently mixed the solution by inversion until the white thread-like strands of DNA form a visible mass.
- Centrifuged at 13,000 x *g* for 1 minute at room temperature. The DNA was visible as a small white pellet.
- Decanted the supernatant, added 300 µl of room temperature 70% ethanol to the DNA. Gently inverted the tube several times to wash DNA pellet and the sides of microcentrifuge tube.
- Centrifuged at 13,000 x *g* for 1 minute at room temperature.
- Carefully aspirated the ethanol. Inverted the tube on clean absorbent paper and air-dry the pellet for 10 – 15 minutes.
- Added 100 µl DNA Rehydration Solution to the tube and rehydrated the DNA by incubating at 65^{o}C for 1 hour.
- Quantified DNA concentration and assessed purity of DNA (based on ratio A260/A280) by NanoDrop 2000.
- Store the DNA at -20^{o}C.

3.3.4. Detection of β-thalassemia mutations by DNA sequencing

The complete β-globin gene (*HBB*) was sequenced, which covered the region from -293 to +1674 with respect to the Cap site.

3.3.4.1. PCR amplification

Four PCR reactions were performed that amplified complete *HBB* gene, encompassed the proximal promoter region to the IVS I; exon 1, IVS I, exon 2 and part of IVS II; part of IVSII; the final part of IVS II and the exon 3.

A. Reagent

- 10X PCR Buffer with 15 mM $MgCl_2$.
- dNTP Set 100 mM of each (EuroClone, Italy).

- Taq DNA Polymerase 5 U/µl (VWR Life Science).
- The list of primers, the annealing temperatures and the size of amplicons of four PCR reactions were shown in Table 3.1.

Table 3.1. List of primers for amplified *HBB* gene

Primer	Sequence	Annealing temperature (Ta)	Amplicon size
β1-F	5'-CTTACCAAGCTGTGATTCCA-3'	58°C	530 bp
β1-R	5'-GTCAGTGCCTATCAGAAACC-3'		
β2-F	5'-AACCTCAAACAGACACCATG-3'	58°C	595 bp
β2-R	5'-ACTTCCACACTGATGCAATC-3'		
β3-F	5'-TGGAAGTCTCAGGATCGTTT-3'	57°C	534 bp
β3-R	5'-GCTATTGCCTTAACCCAGAA-3'		
β4-F	5'-GCCTCTTTGCACCATTCTAA-3'	55°C	576 bp
β4-R	5'-TTTAAATGCACTGACCTCCC-3'		

B. Procedure

PCR components included 1X PCR buffer, 250 µM each dNTPs, 0.2 µM each primer, 0.5 U of Taq polymerase, 30 ng template DNA and deionized H_2O up to 15 µl final reaction volume.

The amplification conditions were as followed: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at Ta that were shown in table 3.1 for each reaction for 30 seconds, extension at 72°C for 1 minute; and a final extension cycle at 72°C for 10 minutes. PCR was performed on GeneAmp PCR system 9700 (Applied Biosystem).

PCR products were loaded on a 1.2% agarose gel stained with SYBR Green (Invitrogen, USA). Electrophoresis was performed under 110V in 20 minutes. The bands were observed under the ultraviolet light.

3.3.4.2. PCR purification

- Added 5 µl PCR product with 2 µl of ExoSAP-IT (Affymetrix), mixed well. Kept on ice while pipetting.
- Run on thermal cycler following this condition: 37°C for 30 minutes, then 80°C for 15 minutes.

The DNA was ready for direct sequencing.

3.3.4.3. Direct sequencing

A. Reagent

- BigDye^{TM} Terminator v1.1 Cycle Sequencing Kit (Applied Biosystem, USA).
- BigDye^{TM} Terminator v1.1 & v.3.1 5X Sequencing Buffer (Applied Biosystem, USA).
- Sephadex^{TM} G-50 Superfine (GE Healthcare Life Sciences, Sweden).

B. Procedure

**** Sequencing reaction***

Reaction components included 1 µl Sequencing buffer, 1 µl BigDye Terminator kit, 0.17 µM of primer (forward or reverse) and 7 µl purified PCR product.

The conditions of sequencing reactions were 25 cycles of denaturation at 96^{o}C for 10 seconds; annealing for 5 seconds at 57^{o}C for β1 or β2 PCR product, 56^{o}C for β3 PCR product and 54^{o}C for β4 PCR product; extension at 60^{o}C for 2 minutes, run on GeneAmp PCR system 9700 (Applied Biosystem). Kept on 4^{o}C until purification.

**** Purification sequencing product***

The sequenced products were purified by Sephadex, followed by these steps:

- Prepared Sephadex plate: filled all wells of plate with Sephadex powder, then added 300 µl deionized H_2O in each well on plate for the first time. Stored at 4^{o}C until using.
- Stuck another plastic plate under the Sephadex plate to obtain H_2O.
- Centrifuged these plates at 1700 rpm for 2 minutes to remove H_2O.
- Centrifuged again at 1700 rpm for 1 minutes to remove all H_2O.
- Removed the plastic plate under the Sephadex plate, put another clean plastic line (96 Well Plate Model (P), Costar) for collecting sequence products.
- Put 10 µl sequencing product of each sample on each well of Sephadex plate.
- Centrifuged at 1700 rpm for 3 minutes to collect sequencing product into the clean plastic line.
- Transferred all sequencing product that already purified to the plate compatible with Applied Biosystem Genetic Analyzer system that already containing 10 µl Formamide in each well. Mixed well.

- Put the cover.
- Centrifuged this plate at 1700 rpm for 1 minute.

**** Electrophoresis on ABI PRISM 3130xl Genetic Analyzer system***

- Run on thermal cycler for 95^{o}C for 2 minutes for denaturation.
- Run on ABI PRISM 3130*xl* Genetic Analyzer system.

**** Sequenced analysis***

The resulting DNA sequences were analyzed by Lasergene SeqMan (DNASTAR) or by 4Peaks software. The obtained sequences were compared with the reference sequence present in GenBank accession U01317.1.

3.3.5. Gap-PCR for detecting common α thalassemia deletions

A multiplex PCR were performed for detecting common α thalassemia deletions in Vietnam such as -$\alpha^{3.7}$, -$\alpha^{4.2}$, --SEA mutations [5,52].

A. Reagent

- 10X Key Buffer (15 mM $MgCl_2$) (VWR Life Science).
- dNTP Set 100 mM of each (EuroClone, Italy).
- Taq DNA Polymerase 5 U/µl (VWR Life Science).
- Betaine solution 5 M (Sigma-Adrich).
- DMSO 10% (Sigma-Adrich).
- All the primers used for identifying three deletions were shown in Table 3.2 [4,14].

Table 3.2. Primer sequences for detecting common α-thalassemia deletions

Primer	Sequence	Amplicon size
LIS1-F	5'-GTCGTCACTGGCAGCGTAGATC-3'	2503 bp
LIS1-R	5'-GATTCCAGGTTGTAGACGGACTG-3'	
α2/3.7-F	5'-CCCCTCGCCAAGTCCACCC-3'	2022/2029 bp
3.7-R	5'-AAAGCACTCTAGGGTCCAGCG-3'	
α2/3.7-F	See above	1800 bp
α2-R	5'-AGACCAGGAAGGGCCGGTG-3'	
4.2-F	5'-GGTTTACCCATGTGGTGCCTC-3'	1628 bp
4.2-R	5'-CCCGTTGGATCTTCTCATTTCCC-3'	
SEA-F	5'-CGATCTGGGCTCTGTGTTCTC-3'	1349 bp
SEA-R	5'-AGCCCACGTTGTGTTCATGGC-3'	

B. Procedure

PCR components included 1X Key buffer, 0.35 mM each dNTPs, 0.75 M betaine, 0.5% DMSO, 0.3 μM each primer, 5 U Taq polymerase, 75 ng template DNA and distilled H_2O up to 50 μl final reaction volume.

The amplification conditions were as followed: initial denaturation at 95°C for 10 minutes; 35 cycles of denaturation at 95°C for 45 seconds, annealing at 65°C for 45 seconds, extension at 68°C for 2 minutes and 30 seconds; and a final extension cycle at 72°C for 10 minutes. PCR was performed on GeneAmp PCR system 9700 (Applied Biosystem).

PCR products were loaded on a 1.2% agarose gel stained with SYBR Green (Invitrogen, USA). Electrophoresis was performed under 80V in 2 hours. The bands were observed under the ultraviolet light.

3.3.6. DNA sequencing to identify Hb CS

Procedure for identifying Hb CS mutation which occurs at codon 142 TAA → CAA (Ter → Gln) of α2 globin *(HBA2)* gene.

3.3.6.1. PCR amplification

A. Reagent

- 10X Key Buffer (15 mM $MgCl_2$) (VWR Life Science).
- dNTP Set 100 mM of each (EuroClone, Italy).
- Taq DNA Polymerase 5 U/μl (VWR Life Science).
- The sequence of primers for amplification a part of *HBA2* gene consisting position of mutation lead to HbCS formation were:
  HbCS-F: 5'-ACCCGGTCAACTTCAAGGT-3'
  HbCS-R: 5'-ACATTCCGGGACAGAGAGAA-3'

B. Procedure

PCR components included 1X PCR buffer, 250 μM each dNTPs, 0.2 μM each primer, 0.5 U of Taq polymerase, 30 ng template DNA and deionized H_2O up to 15 μl final reaction volume.

The amplification conditions were as followed: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30

seconds, extension at 72°C for 1 minute; and a final extension cycle at 72°C for 10 minutes. PCR was performed on GeneAmp PCR system 9700 (Applied Biosystem).

PCR products were loaded on a 1.2% agarose gel stained with SYBR Green, under 110V in 20 minutes and observed under the ultraviolet light.

3.3.6.2. PCR purification, direct sequencing

Other steps such as PCR purification and direct sequencing had the same procedures that already described before on the DNA sequencing for detecting β thalassemia mutations description (3.3.4).

The annealing temperature for sequencing reaction was 56°C.

The obtained sequences were compared with the reference sequence present in GenBank accession NC_000016.10.

3.3.7. DNA sequencing to identify -158 C → T $^{G}\gamma$ polymorphism

3.3.7.1. PCR amplification

A. Reagent

- 10X Key Buffer (15 mM $MgCl_2$) (VWR Life Science).
- dNTP Set 100 mM of each (EuroClone, Italy).
- Taq DNA Polymerase 5 U/µl (VWR Life Science).
- The sequence of primers for amplification a promoter region of $^{G}\gamma$ gene were:

  HBG2-F: 5'-GGCCTAAAACCACAGAGAGTAT-3'

  HBG2-R: 5'-TGATAACCTCAGACGTTCCAGA-3'

B. Procedure

PCR components included 1X PCR buffer, 250 µM each dNTPs, 0.2 µM each primer, 0.5 U of Taq polymerase, 30 ng template DNA and deionized H_2O up to 15 µl final reaction volume.

The amplification conditions were as followed: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute; and a final extension cycle at 72°C for 10 minutes. PCR was performed on GeneAmp PCR system 9700 (Applied Biosystem).

PCR products were loaded on a 1.2% agarose gel stained with SYBR Green, under 110V in 20 minutes and observed under the ultraviolet light.

3.3.7.2. PCR purification, direct sequencing

Other steps such as PCR purification and direct sequencing had the same procedures that already described before on the DNA sequencing for detecting β-thal mutations description (3.3.4).

The annealing temperature for sequencing reaction was 56^{o}C.

The obtained sequences were compared with the reference sequence present in GenBank accession U01317.1.

3.4. STATISTICAL METHODS

- Statistical analyses were performed using Microsoft Excel and SPSS version 20 (SPSS Inc., Chicago, IL).
- To compare the mean difference of hematological parameters, the ANOVA test and Kruskal-Wallis test according to parametric or nonparametric distribution.
- To compare proportions, Fisher's exact test was applied.
- P – value < 0.05 was considered statistically significant.

4. RESULTS

4.1. STUDY POPULATION

A total of 226 subjects were enrolled in this study. Among them, 53.5% (121/226) were female, and 46.5% (105/226) were male. The mean age was 22.9 ± 14.4, the lowest age 1, and the highest 72 years old.

By means of the application of the standard methodology described in section 3 (i.e. red blood cells indices, qualitative and quantitative determinations of Hb components), a total of 138 subjects resulted β-thal heterozygotes, whereas 57 were diagnosed as being affected by the mild form, non-transfusion-dependent, of the thalassemia disease. The remaining 31 subjects examined resulted patients affected by the β-thal major (Cooley's anemia) subjected to, more or less regularly, blood transfusions in the Hue Central Hospital. Figure 3.1 schematizes the approach utilized.

4.2. MOLECULAR BASIS OF MUTATIONS AFFECTING β-GLOBIN GENE

4.2.1. β-thalassemia mutations detecting by DNA sequencing

Table 4.1 lists the twelve known mutations along the β-globin gene observed in a total of 314 chromosomes studied. Their parental transmission was verified when possible.

Table 4.1. The twelve β-globin gene alleles found in 314 chromosomes.

Name and type of Mutation	HGVS nomenclature [60]
-198 (A>G) β^+	HBB:c.-248A>G
-72 (T>A) β^+	HBB:c.-122T>A
-50 (G>A) β^+	HBB:c.-100G>A
-28 (A>G) β^+	HBB:c.-78A>G
Codons 14/15 (+G) β^o	HBB:c.45_46insG
Codon 17 (A>T) AAG(Lys) >TAG(stop cd) β^o	HBB:c.52A>T
Codon 26 (GAG->AAG) β^+, or Hb E	HBB:c.79G>A
Codon 26 (G>T); GAG(Glu) >TAG(stop cd) β^o	HBB:c.79G>T
IVS-I-1 (G>T); AG^GTTGGT>AGTTTGGT β^o	HBB:c.92+1G>T
Codons 41/42 (-TTCT) β^o	HBB:c.126_129delCTTT
Codons 71/72 (+A) β^o	HBB:c.216_217insA
Codon 95 (+A); AAG(Lys)->AAAG β^o	HBB:c.287_288insA

As it is shown in Table 4.2, four mutations accounted for 86.94% of the total: the most common cd 26 (G>A) or Hb E, cd 17 (A>T), cds 41/42 (-TTCT) and IVS-I-1 (G>T). The remaining mutations showed lower frequencies. Four mutations cds 71/72 (+A), -28 (A>G), cd 95 (+A), cd 26 (G>T) accounted for a total 11.46%, whereas others have to be considered rare or very rare: the promoter mutations -198 (A>G), -72 (T>A), -50 (G>A), and cds 14/15 (+G), having the allelic frequencies 0.64%, 0.32%, 0.32% and 0.32%, respectively.

Table 4.2. β-thalassemia mutations and allelic frequency observed in 314 chromosomes

Mutation	Type	β^{Th}/β^{Th}	β^{Th}/β^{A}	Number of alleles	Allelic frequency (%)
cd 26 (G>A) or Hb E	β^{+}	84	46	130	41.4
cd 17 (A>T)	β^{o}	38	48	86	27.39
cds 41/42 (-TTCT)	β^{o}	21	13	34	10.83
IVS-I-1 (G>T)	β^{o}	12	11	23	7.32
cds 71/72 (+A)	β^{o}	4	8	12	3.82
-28 (A>G)	β^{+}	8	2	10	3.18
cd 95 (+A)	β^{o}	4	4	8	2.55
cd 26 (G>T)	β^{o}	4	2	6	1.91
-72 (T>A)	β^{+}	1	1	2	0.64
-50 (G>A)	β^{+}	0	1	1	0.32
-198 (A>G)	β^{+}	0	1	1	0.32
cds 14/15 (+G)	β^{o}	0	1	1	0.32
Totals		**176**	**138**	**314**	**100**

4.2.2. The molecular mechanisms of the β-thalassemia mutations found

The common basis is consistent with the absent or decreased synthesis of β-globin chains of Hb A, which results in the accumulation of the excess α-globin chains that, in turn, is responsible for the pathophysiology of the disorder. The severity of the phenotype is thus related to the degree of imbalance between α- and non-α-globin chain synthesis, and the size of the free α-chain pool. Hence severity is related to the type of β allele (β^{o}, β^{+}, β^{++}) with respect to the residual amount, ameliorated by an interacting α-thal (by reducing the α-chain excess) and any increased production of γ-chains (that decrease the excess of free α-chains to form Hb F [77].

The following mechanisms concern mutations found in this study (of all mutations found, it is shown the portion of the gene sequence containing the mutation):

- The -198 (A>G) β^+: the mutation occurs in the upstream region of the HBB gene, slightly reducing the synthesis of mRNA.

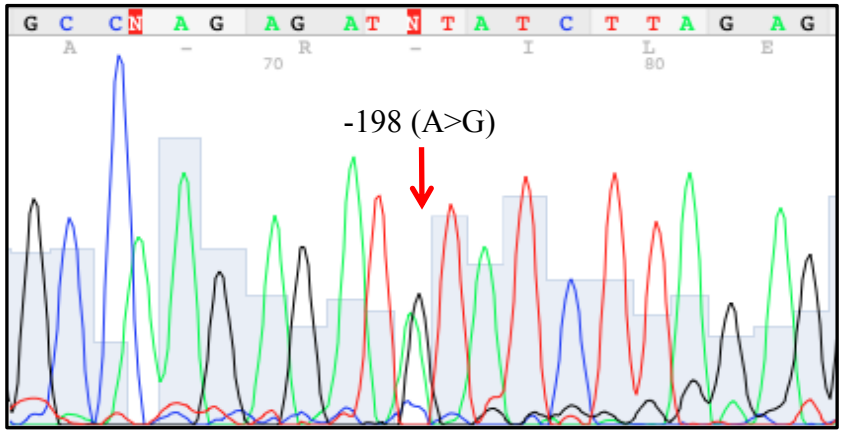


Figure 4.1. The sequence in the -208 to -188 promoter region of the β-globin gene in a heterozygote subject. The red arrow shows that at position -198, with respect to the Cap site, there are two bases, the normal A, and G, due to the A > G substitution.

- The -72(T>A) β^+: the mutation occurs within the conserved CCAAT box of the β-globin gene promoter, having the effect to slightly reducing the synthesis of β-globin mRNA.

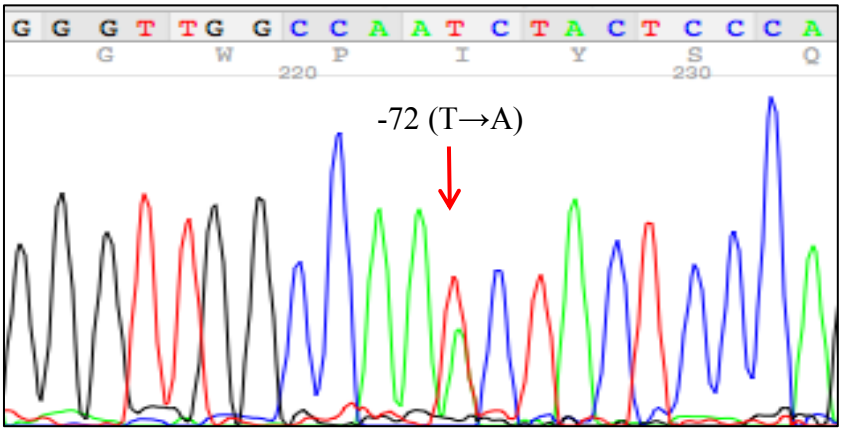


Figure 4.2. The sequence in the -83 to -63 promoter of the gene in a heterozygote. At position -72 there are two bases, T and A, due to the T > A substitution.

- The -50 (G>A) β^+: mutation located between the CCAAT and TATA boxes containing a highly conserved element of a 10 bp sequence.

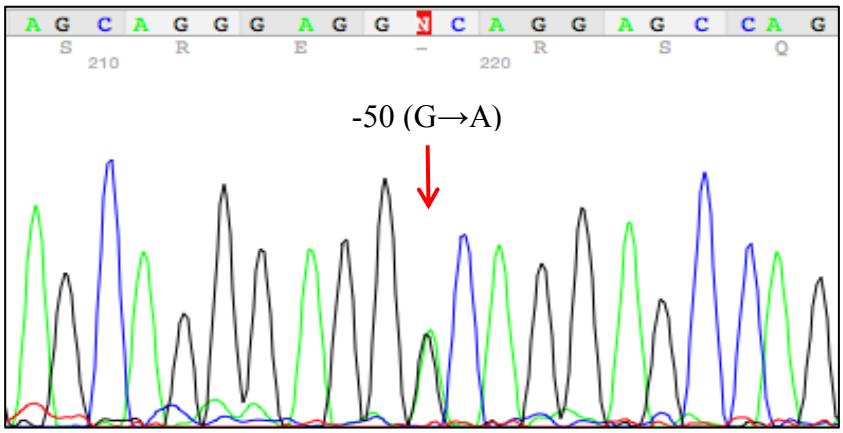


Figure 4.3. The sequence in the -60 to -40 promoter in a heterozygote. At position -50, with respect to the Cap site, there are two bases, G and A, due to the G > A substitution.

- The -28 (A>G) β^+: it is responsible for a decreased transcription because of reduced binding of erythroid factors.

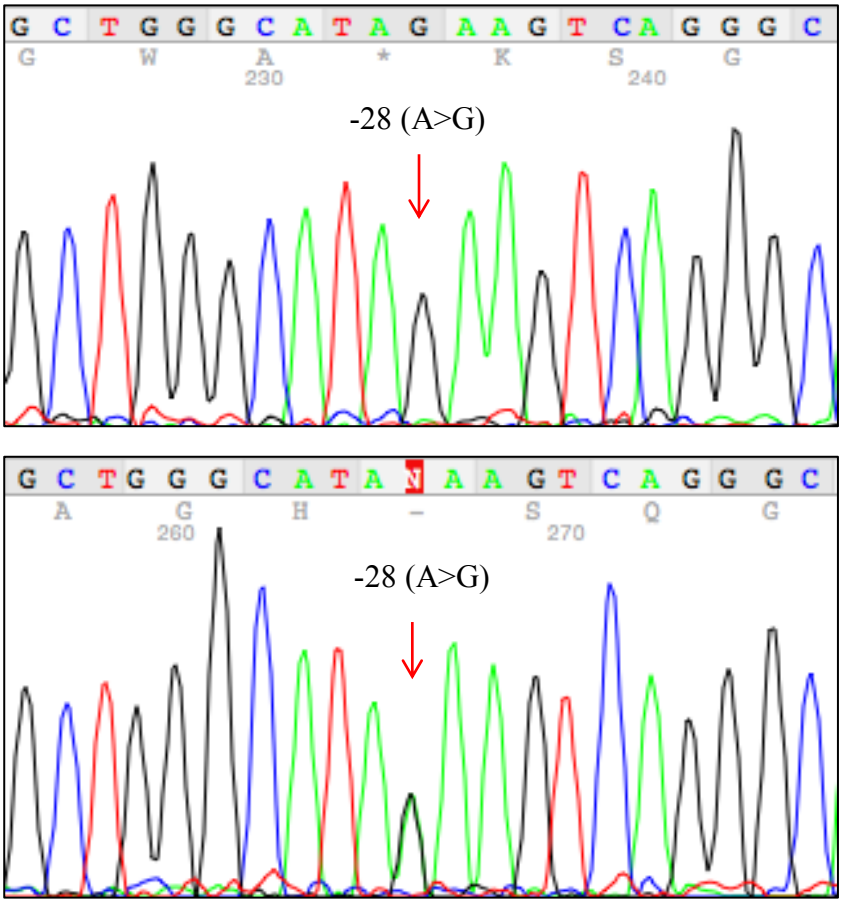


Figure 4.4. The sequence in the promoter region of a homozygote subject (upper sequence), and in a heterozygote (lower sequence) with the A>G substitution at position -28.

- The codons 14/15 (+G) β^o: due to the insertion of G between cds 14 and 15 of the gene. This frameshift mutation results in the termination of translation at codon 22 (TGA). The C-terminal sequence is modified as follows: (15)Val-Gly-Gln-Gly-Glu- Arg-(21)-Gly-COOH.

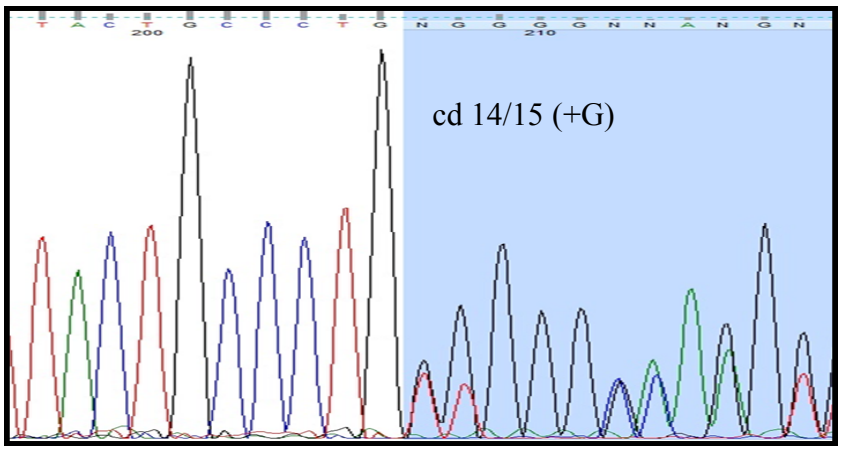


Figure 4.5. Heterozygote for the Cds 14/15 (+G). The blue box indicates the overlap of two bases.

- The codon 17 (A>T) AAG >TAG(stop cd) β^o: change of one nucleotide resulting in termination of translation at codon 17.

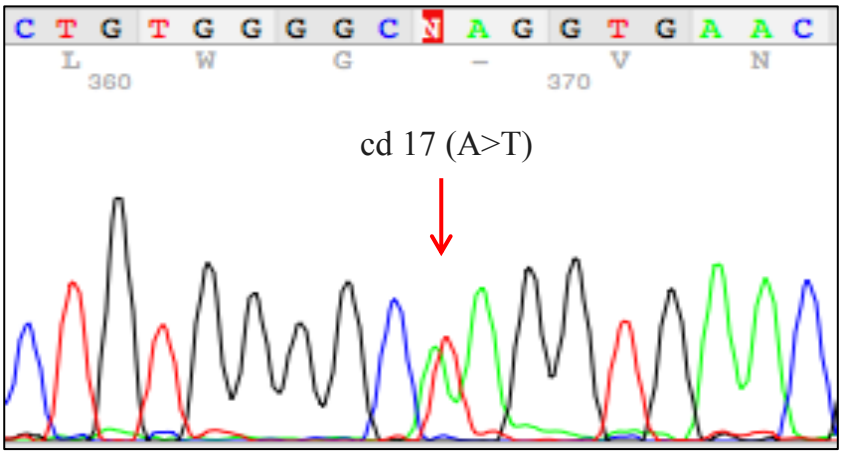


Figure 4.6. The sequence from cd 14 to 19. The arrow shows the AAG>TAG mutation at codon 17 to which the Lys>stop substitution corresponds.

- The codon 26 (GAG->AAG) β^{+}, or Hb E: the base substitution creates a splice donor site within the exon leading to loss of the last 16 bases of the exon. The frameshift results in a nonsense-mediated mRNA decay.

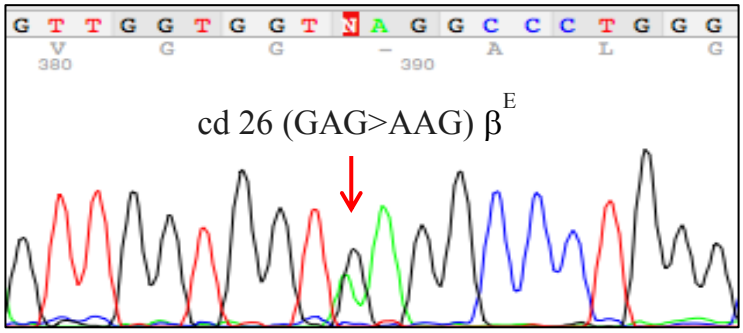


Figure. 4.7. The sequence from cd 23 to 29. The red arrow indicates the GAG>AAG mutation at cd 26 to which the substitution Glu>Lys corresponds. The variant Hb is called Hb E.

- The mutation at first base of codon 26 (G>T); GAG(Glu) >TAG(stop) β^{o}: this G>T mutation changes codon 26 into a stop codon and terminates translation.

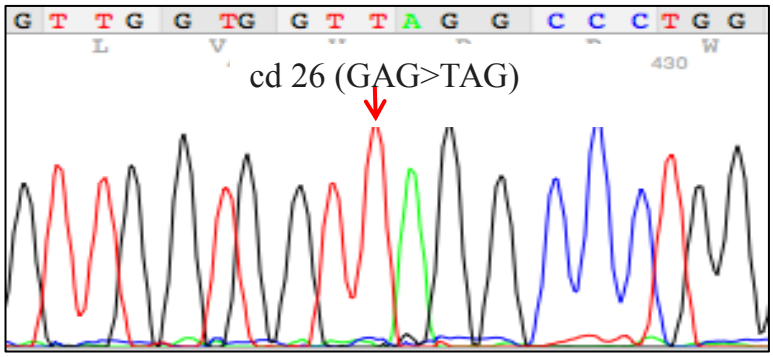


Figure 4.8. Sequencing showing the GAG>TAG (Glu>stop) mutation at codon 26, corresponding to nucleotide 79 of the coding sequence.

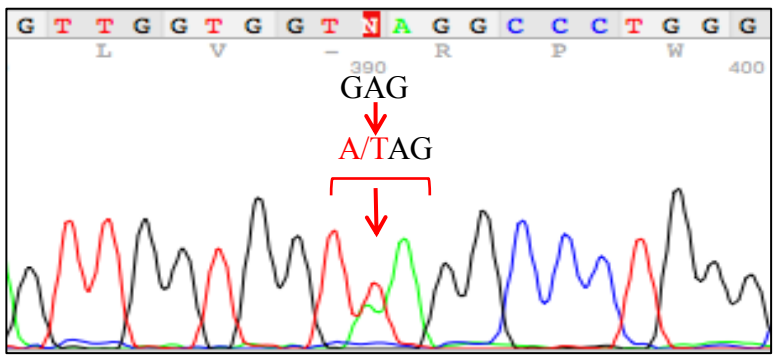


Figure 4.9. Sequencing of the β-globin gene of a compound heterozygote having one cd 26 with the AAG sequence (Hb E), and the other with the TAG sequence (β^{E}/β^{o}).

- The IVS-I-1 (G>T); AG^GTTGGT>AGTTTGGT β^{o}: this G>T changes the GT dinucleotide required for normal splicing; as the result, it abolishes the process and no normal mRNA is formed.

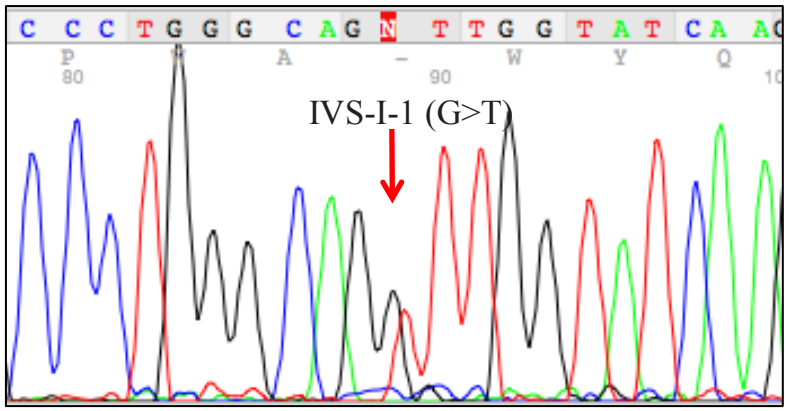


Figure 4.10. In the IVS-I-1, the G>T mutation abolishes the splicing of the first intron of the β-globin gene.

- The codons 41/42 (-TTCT) β^{o}: the insertion of T between cds 40 and 41 modifies the C-terminal sequence: (41)Phe-(42)Leu-COOH, and the frameshift leads to a terminating codon at position 43, thus ending translation.

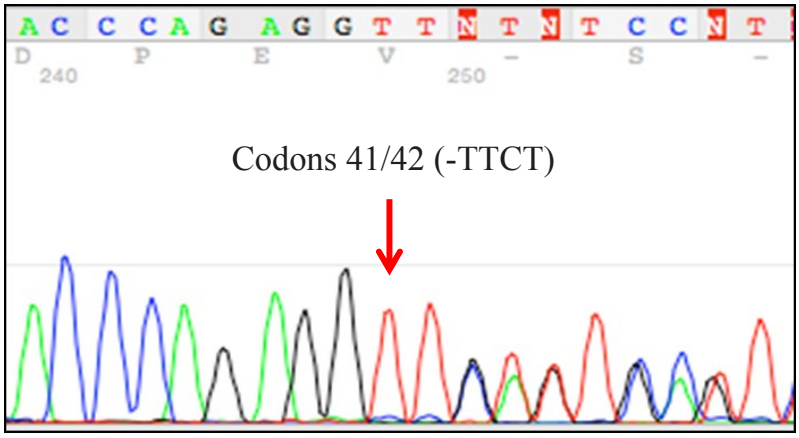


Figure 4.11. The sequence from cds 38 to 42. Due to the deletion of TTCT, cd 41 (TTC) is deleted and cd 42 (TTT) lacks a nucleotide T.

- The codons 71/72 (+A) β^o: the insertion of A between cds 71 and 72 of β-globin gene changes the reading frame and terminates translation at the new cd 73 (TGA).

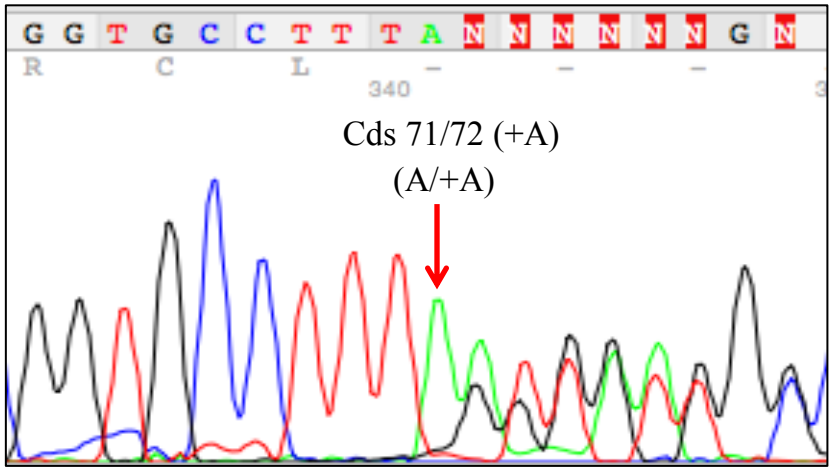


Figure 4.12. The sequence from cd 69 to 73. The insertion of A after the first base of cd 72 changes the reading of the frame to AAG.TGA, the latter being a stop codon.

- The codon 95 (+A); AAG(Lys)->AAAG β^o: insertion of A within cd 95 (AAAG) (or between cds 94 and 95) changes the reading frame with termination of translation at cd 101 which is a stop cd (TGA).

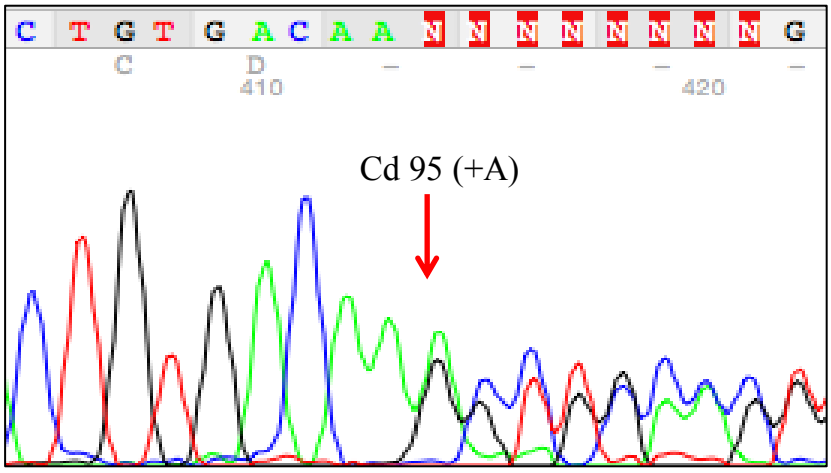


Figure 4.13. Heterozygote for the Cd 95(+A). The red arrow indicates that two bases overlap from the third base of codon 95 due to the insertion of A (AAG >AAAG).

4.2.3. β-globin genotype of thalassemia patients

The twelve different mutations gave rise, as expected, to several different combinations, either homozygous or compound heterozygous, such as β^E/β^E, β^E/β^+, β^E/β^o, β^+/β^+, β^+/β^o, β^o/β^o. Some interaction might give rise to a genotype characterized by a slight or a severe reduction of β-globin synthesis whereas other in a severe or absent reduction.

As showed in Table 4.3, five different combinations of heterozygote genotypes resulted in a mild, non-transfusion-dependent, thalassemic disease (TI or NTDT), whilst three (β^E/β^o, β^+/β^o, β^o/β^o) appeared affected by thalassemia major (TM or TDT) which is characterized by the need of regular blood transfusions. Thalassemia intermedia (TI) is characterized by Hb levels steadily around 7 – 10 g/dl without the need for regular transfusions, more severe RBC abnormalities than thalassemia minor, spleen enlargement, susceptibility to infections, and skeletal changes. Most TI patients are homozygotes or compound heterozygotes for β-thal of the β^+ type. It might be thus rather surprising to notice, in Table 4.3, 43 β^E/β^o compound heterozygotes were non-transfusion dependent. As referred in the Introduction chapter 1, the mild clinical features of TI may result from three different mechanisms: the inheritance of a mild β-gene mutation, the co-inheritance of α-thal, the co-inheritance of determinants associated with an increased γ-globin production.

Accordingly, the TI patients identified in this thesis resulted to have different combination of β-genotypes. The most common was β^E/β^o found in 43/57 (75.4%). The β^E/β^E genotype was observed in 9/57 (15.8%). Other genotypes were β^E/β^+ and β^+/β^+ both having the frequency 2/57 (3.5%), and finally β^+/β^o, 1/57 (1.8%).

The most common genotype in the TM patients was β^E/β^o, 21/31 (67.7%), next is genotype β^o/β^o, 8/31 (25.8%), and other was β^+/β^o in 2/31 (6.5%).

Less frequently, subjects are observed with only a single β-globin locus affected. Of particular interest is thus one subject of the 46 with the β^E/β^A heterozygous genotype. Subject's RBC indices were similar, or poorer, than those clearly classified as being TI patients: Hb 7.7 g/dl, MCV 62.6 fL, MCH 15.2 pg/cell, Hb F 6.1%. The patient, however, resulted also the carrier of the --SEA/$\alpha^{CS}\alpha$ genotype, thus supporting that the

consequent strong suppression of α-chains synthesis may be the interacting cause of the remarkable anemia.

Table 4.3: The β-globin genotype and the severity of β-thalassemia

β-genotype	β-thal carriers	Thalassemia intermedia	Thalassemia major
β^E/β^A	46*	0	0
β^+/β^A	5	0	0
β^o/β^A	87	0	0
β^E/β^E	0	9	0
β^E/β^+	0	2	0
β^+/β^+	0	2	0
β^E/β^o	0	43	21
β^+/β^o	0	1	2
β^o/β^o	0	0	8
Total subjects	**138**	**57**	**31**

**A heterozygous β^E/β^A which showed the typical signs of NTDT because of interacting HbH*

4.2.4. β-globin genotype, hematology and hemoglobin components associated to β-thalassemia carriers

Table 4.4. β-globin genotype, RBC indexes and hemoglobin components associated to β-thal heterozygotes

β-genotype		**Hematology**			**% Hemoglobin components**			
		Hb (g/dl)	MCV (fL)	MCH (pg/cell)	Hb A	Hb F	Hb A_2	Hb E
β^E/β^A	**46**	11.3 ± 2.1*	73.0 ± 6.9*	23.5 ± 3.1*	72.5 ± 6.6	0.3 ± 1.0	4.1 ± 1.9*	22.8 ± 6.6
cd 26 (G>A)/wild	46							
β^+/β^A	**5**	12.3 ± 2.1	70.1 ± 6.3	21.6 ± 2.3	93.7 ± 4.0	2.4 ± 4.6	3.9 ± 1.7	-
-198 (A>G)/wild	1							
-72 (T>A)/wild	1							
-50 (G>A)/wild	1							
-28 (A>G)/wild	2							
β^o/β^A	**87**	10.3 ± 1.9*	61.4 ± 7.3*	19.4 ± 2.8*	92.7 ± 2.9	1.4 ± 3.1	5.8 ± 0.9*	-
cd 17 (A>T)/wild	48							
cds 41/42 (-TTCT)/wild	13							
IVS-I-1 (G>T)/wild	11							
cds 71/72 (+A)/wild	8							
cd 95 (+A)/wild	4							
cd 26 (G>T)/wild	2							
cds 14/15 (+G)/wild	1							

*: *Significantly different from β^o-thal trait, $p < 0.05$.*

The two most common mutation found in thalassemia carrier were cd 17 (A>T) and cd 26 (G>A) with frequencies 34.8% (48/138) and 32.6% (45/138), respectively. The lowest frequencies were observed in carriers of β^+ mutations such as -198 (A>G), -72 (T>A), -50 (G>A) and in β^o mutation cds 14/15 (+G), all of them having the same 0.7% (1/138) prevalence.

As expected, the hematology of thalassemia carriers showed the typical characteristic of β-thalassemia heterozygous including mild anemia, microcytosis, hypochromia, and increased Hb A_2. Similarly, the hematological pattern of Hb E heterozygous shows the association with relatively milder phenotype compared to β^o-thal. As shown in Table 4.4, significant difference was observed for Hb, MCV, MCH and Hb A_2 levels for Hb E heterozygote in comparison to β^o-thal trait.

4.2.5. β-globin genotype, and associated RBC indices of thalassemia intermedia patients

Table 4.5: β-genotype and associated hematological parameters of thalassemia intermedia patients

β-genotype		Hb (g/dl)	MCV (fL)	MCH (pg/cell)	Hb A (%)	Hb F (%)	Hb E (%)
β^E/β^E							
cd 26 (G>A)/cd 26 (G>A)	**9**	10.3 ± 1.7	61.9 ± 7.7	21.3 ± 2.3	0	1.9 ± 2.4	83.2 ± 7.4
β^E/β^+							
-28 (A>G)/cd 26 (G>A)	**2**	11.1 ± 0.2	70.4 ± 1.7	24.2 ± 1.8	20.3 ± 0.9	36.4 ± 6.8	38.3 ± 5.7
β^+/β^+							
-28 (A>G)/-28 (A>G)	**2**	9.1 ± 0.2	64.9 ± 1.0	21.2 ± 1.1	54.4 ± 7.7	39.7 ± 8.5	0
β^E/β^o	**43**	7.7 ± 1.1	67.6 ± 8.4	21.0 ± 2.5	19.9 ± 21.6	33.4 ± 13.3	41.6 ± 10.4
cd 26 (G>A)/cd 17 (A>T)	21						
cd 26(G>A)/cds 41/42 (-TTCT)	11						
cd 26 (G>A)/IVS-I-1 (G>T)	7						
cd 26 (G>A)/cd 95 (+A)	2						
cd 26 (G>A)/cds 71/72 (+A)	1						
cd 26 (G>A)/cd 26 (G>T)	1						
β^+/β^o							
-72 (T>A)/cd 17 (A>T)	**1**	7.4	74.1	21.8	75.5	16.6	0
		$p < 0.001$	$p > 0.05$	$p > 0.05$			

Table 4.5 refers to RBC indices associated to β-globin genotypes of TI patients. Among patients with homozygous or compound heterozygous mutations, the lowest Hb was found in patients with genotype β^E/β^o, there was significant difference of Hb level between different genotypes. The Hb F level was high in patients with genotype β^E/β^+, β^+/β^+ and β^+/β^o and β^E/β^o.

4.3. THE β- GLOBIN GENOTYPE OF THALASSEMIA INTERMEDIA AND THALASSEMIA MAJOR, AND THE ROLE OF INTERACTING α-THALASSEMIA, AND OF THE Xmn-1 $^G\gamma$ POLYMORPHISM FOR THE INCREASED PRODUCTION OF Hb F

Table 4.6 shows the specific genotypes found in 57 patients with β-thal of intermediate severity and in 31 β-thal transfusion dependent patients: the homozygous or compound heterozygous β^E/β^E, β^E/β^+, β^+/β^+ only belong to TI patients. The homozygous or compound heterozygous β^o/β^o belong to TM patients.

Depending on the β^+ mutations, the patients with genotype β^+/β^o could fit either to TI or TM groups: the -72 (T>A)/cd 17 (A>T) compound heterozygous was TI, whereas the -28 (A>G)/cd 17 (A>T), and -28 (A>G)/cd 95 (+A) patients were found in TM patients. This difference is dependent to the relative severity of the β^+-thal allele co-inherited, the -28 (A>G) being a severe mutation due his nearness to the Cap site.

There were patients with the same genotype being TI, whereas others were TM patients. For example, 21 compound heterozygous cd 26 (G>A)/cd 17 (A>T), were TI patients, while at the same time, 12 patients resulted TM.

To verify the hypothesis on the possible effect due to the co-inheritance of α-thal genes lowering the synthesis of α-chains, thus reducing the α/β-globin imbalance, a search for α-globin gene mutations was carried out. This was the case of 2/9 patients with the same genotype β^E/β^E, and 4/43 patients with genotype β^E/β^o found in TI patients.

There were no TM patients having co-inherited deletion mutations or Hb CS mutation in α-globin genes.

Table 4.6. β-globin genotype of total 88 patients

β-genotype		TI	TM
β^E/β^E	cd 26 (G>A)/cd 26 (G>A)	9	
β^E/β^+	-28 (A>G)/cd 26 (G>A)	2	
β^+/β^+	-28 (A>G)/-28 (A>G)	2	
β^E/β^o	cd 26 (G>A)/cd 17 (A>T)	21	12
	cd 26 (G>A)/cds 41/42 (-TTCT)	11	5
	cd 26 (G>A)/IVS-I-1 (G>T)	7	2
	cd 26 (G>A)/cd 95 (+A)	2	1
	cd 26 (G>A)/cds 71/72 (+A)	1	1
	cd 26 (G>A)/cd 26 (G>T)	1	
β^+/β^o	-72 (T>A)/cd 17 (A>T)	1	
	-28 (A>G)/cd 17 (A>T)		1
	-28 (A>G)/cd 95 (+A)		1
β^o/β^o	cd 17 (A>T)/cd 26 (G>T)		1
	cd 17 (A>T)/cds 41/42 (-TTCT)		1
	cd 17 (A>T)/cds 71/72 (+A)		1
	cd 26 (G>T)/cd 26 (G>T)		1
	cds 41/42 (-TTCT)/cds 41/42 (-TTCT)		1
	cds 41/42 (-TTCT)/cds 71/72 (+A)		1
	IVS-I-1 (G>T)/IVS-I-1 (G>T)		1
	IVS-I-1 (G>T)/cds 41/42 (-TTCT)		1
TOTALS		**57**	**31**

A number of studies suggested that the C → T change at position -158 5' to the $^G\gamma$-globin gene, also known as the Xmn-1 $^G\gamma$ polymorphism, is associated to an increased propensity for Hb F production in β thalassemia [26,41,75]. In both African-Asian and Italian populations this polymorphism was the only sequence variation observed in several patients affected by β^o-thal homozygotes whose clinical course was from moderate to severe TI. Comparisons of the β-globin gene restriction fragment length polymorphism (RFLP) haplotypes of patients with TI and TM was a support on the above suggestions: homozygotes β^o- or β^+-thal resulted also homozygotes for the change at -158 to the $^G\gamma$ gene and have a milder disease [6,34]. This seems particularly evident in the case described by Khelil et al. of the β^o-thal IVS1 +2 T → G mutation which was identified in two Tunisian sisters having no adult Hb A [38]. In the two patients, resulting Xmn-1 homozygotes (TT), Hb was 10.1 g/dl, and Hb F accounted for ~98% of total. The

same was for the mother, homozygous for the Xmn-1 polymorphism, whose Hb was also unusually high (10%). Similar high Hb F levels (from 7.1 to 10.4%) were found in six β-thal carriers in association, heterozygous or homozygous, with the Xmn-1 polymorphism. Nonetheless, despite the association of the Xmn-1 polymorphism has been validated in several reports, Thein et al. (2008) suggested that other *cis*-acting factors may have an intrinsic propensity in increasing the synthesis of Hb F as well as deletional forms of β-thal removing the 5' end of the β-globin gene [74].

The possible effect of the Xmn-1 $^{G}\gamma$ polymorphism was investigated in the present work. The promoter region of the $^{G}\gamma$-globin gene of the 57 TI, and of 24 TM was sequenced in the promoter region. Result, showed in Table 4.7, indicates that 7/48 (14.6%) of patients affected by β-thal intermedia resulted to have T at position -158 in both chromosomes (TT). The % Hb F levels in the three genotypes where almost the same, from 31.8 to 36.8%. Among TM, 3/24 patients resulted homozygotes for T at position -158 of the $^{G}\gamma$-gene. On the whole, result indicates that in the Vietnamese population the Xmn-1 polymorphism is not associated with increasing of Hb F synthesis.

Table 4.7. $^{G}\gamma$ -158 genotype and Hb F concentration in TI and TM patients

$^{G}\gamma$ -158 genotype	**Phenotype**		**Hb F (%)**
	TI (n = 48) *	**TM (n = 24)**	**TI**
CC	6 (12.5%)	6 (25%)	33.2 ± 10.9
CT	35 (72.9%)	15 (62.5%)	31.8 ± 15.5
TT	7 (14.6%)	3 (12.5%)	36.8 ± 11.6

: 9 TI patients with genotype β^{E}/β^{E} were not included

Since homozygous Hb E result in hypochromic microcytosis, minimal anemia, and only slightly elevated Hb F level, nine patients were not included.

Finally, the presence of co-inherited α-thal mutations in intermediate and severely affected patients was determined in some instances.

Table 4.8: The β-, α-globin genotypes and $^{G}\gamma$ -158 genotype in thalassemia intermedia patients

β-genotype	No.	α-genotype	$^{G}\gamma$-158 genotype	Hb (g/dl)	MCV (fL)	MCH (pg)	Hb F (%)
β^E/β^E	3	αα/αα	CT	10.5 ± 2.3	60.8 ± 3.6	22.0 ± 1.0	3.1 ± 2.3
	4	αα/αα	TT	9.9 ± 0.5	59.6 ± 8.1	19.8 ± 2.2	0
	1	$-\alpha^{3.7}/\alpha\alpha$	CT	-	-	-	0.3
	1	$-\alpha^{3.7}/\alpha\alpha$	TT	10.8	71.8	23.6	5.5
β^E/β^+	1	αα/αα	CT	10.9	68.7	22.4	29.6
	1	αα/αα	CT	11.2	72	26	43.2
β^+/β^+	1	αα/αα	CC	8.9	63.9	20.1	31.2
	1	αα/αα	CC	9.3	65.8	22.2	48.2
β^E/β^o	3	αα/αα	CC	7.8 ± 0.1	63.8 ± 4.1	21.7 ± 1.3	34.4 ± 5.2
	29	αα/αα	CT	7.5 ± 1.1	68.7 ± 7.7	20.7 ± 2.5	29.4 ± 14.4
	7	αα/αα	TT	7.7 ± 1.0	67.2 ± 8.8	21.1 ± 1.9	33.0 ± 12.1
	4	$-\alpha^{3.7}/\alpha\alpha$ or $-\alpha^{4.2}/\alpha\alpha$	CT	8.2 ± 1.3	67.6 ± 11.8	23.7 ± 1.5	35.6 ± 18.6
β^+/β^o	1	αα/αα	CC	7.4	74.1	21.8	16.6

Table 4.8, which refers to β- and α-globin genotype, and $^{G}\gamma$ -158 genotype in non-transfusion dependent patients, in accordance with the literature, confirms that the concomitant inheritance of α-thal modulates β-thal phenotype. This is because the lower amount of free α-globin chains reduces the amount of oxidized globins in the red cell precursors in the bone marrow forming inclusion bodies and damaging membrane proteins. No transfusion dependent patients were found to have coinherited common deletion mutations or Hb CS mutation of α-globin gene.

5. DICUSSION

5.1. THE MOLECULAR BASIS OF MUTATIONS AFFECTING β-GLOBIN GENE

5.1.1. The spectrum of β-thalassemia mutations

In the actual absence of data from extensive epidemiological screening programs in Vietnam, this thesis focused both on heterozygotes peoples (138 subjects), patients affected by the severe form of thalassemia major disease (31 patients) and those affected by thalassemia of intermediate severity (57 patients) all referring to the Hematology departments of the Hue University of Medicine and Pharmacy and of the Central Hospital for examinations. The total chromosomes investigated were thus 314. Because of the samples collection was from selected peoples, the calculation of the percent of prevalence of the mutations among the population was not possible. Despite of this, with respect of previous observations [15], the actual result provides new, more accurate, insights into the number and frequency of the alleles characterizing the population of Central Vietnam.

Because of this new observation, it is now possible to better understand about types and distribution of β-thal mutations not only in the central province but in all provinces of the country.

As showed in Table 5.1, which compares % frequencies in the three regions of Vietnam with those of the neighboring Thailand and South West China, the cd 26 (G>A) or Hb E, cd 17 (A>T), and the cds 41/42 (-TTCT) mutations, which were reported as the most common mutations in North and South Vietnam, appear not to have the same frequency all over the country. Filon et al. (2000) in their study on 23 severely affected thalassemia patients showed five β-globin gene mutations, the highest frequencies being nearly the same obtained in this research [17]. Similarly, most recently, Vo et al. published a new investigation on three β-thal mutations in North Vietnam among 244 β-thal subjects and confirmed Hb E, cds 41/42 (-TTCT) and cd 17 (A>T) as being the three most common mutations despite the frequency appears to be lower with respect to this study (26.4%, 19.4% and 16.4%, respectively) [80]. Due to the higher number of samples analyzed, the new % frequencies are to be considered the most reliable.

In the first report on β-thal mutations in South Vietnam, Hao et al. obtained the same result among 35 transfusion dependent patients: Hb E accounted for the similar 38%, whereas the cds 41/42 (-TTCT) mutation resulted to have a double frequency, whereas cd 17 (A>T) showed a lower frequency [31].

Table 5.1. The frequencies (%) of β-thalassemia mutations in three Vietnam regions, in Thailand, and Southwestern China

Mutation	Central Vietnam [this study]	North Vietnam [17]-[80]*	South Vietnam [31]-[70]	Thailand [76]	China SW [86]
Cd 26 (G>A), Hb E	41.4	37.0 / 26.4	38.0 / 31.3	40.2	30.5
Cd 17 (A>T)	27.39	30.0 / 16.4	8.8 /17	6.2	20.8
Cds 41/42 (-TTCT)	10.83	22.0 / 19.4	29.0 / 24	30.4	17.5
IVS-I-1 (G>T)	7.32	-	3.0 / 3.9	1.0	0.6
Cds 71/72 (+A)	3.82	2.0 / -	6.0 / 4.7	0.5	2.42
-28 (A>G)	3.18	-	3.0 / 4.96	6.2	6.95
Cd 95 (+A)	2.55	9.0 / 0.6	- / 7.1	-	-
Cd 26 (G>T)	1.91	-	-	-	-
-72 (T>A)	0.64	-	-	-	-
-50 (G>A)	0.32	-	-	-	-
-198 (A>G)	0.32	-	-	-	-
Cds 14/15 (+G)	0.32	-	-	0.5	-
-86 (C>G)	-	-	-	0.5	-
Cd 5 (-CT)	-	-	-	-	0.3
Cd 19 (A>G)	-	-	-	1.0	-
Cds 27/28 (+C)	-	-	-	-	0.91
Cd 35 (C>A)	-	-	-	1.5	-
IVS-I-5 (G>C)	-	-	-	3.1	-
IVS-II-654 (C>T)	-	-	8.8 / 4.8	6.7	17.2
Others	-	-	9.0 / 1.0	2.1	2.1
No. of alleles	**314**	**46 / 640***	**34 / 99**	**116**	**331**

**Uncomplete data: 37.2% of mutations were not investigated*

The IVS-I-1 (G>T) was found to have a slightly high prevalence in Central Vietnam, with frequency as 7.32 %. In South Vietnam, this mutation appeared to have the less 3.0% frequency [31]. On the contrary, this mutation was not observed in the two studies concerning the North of the country.

The remaining mutations showed lower frequencies, whereas other were rare or even very rare. An exception is the promoter mutation -28 (A>G), which have the similar frequency (3.82 %) to that observed in South (from 3.0 to 4.9 %).

At present, there are five mutations that seems to be present only in Central Vietnam: the -72 (T>A) mutation, described in a family from Central Vietnam [61] , the -50 (G>A), observed in a transfusion dependent 3-years-old Chinese in combination with codon 41/42 (-TTCT) [46], and in a cohort of 528 Thai β^+-thal carriers with the 0.2% frequency [85], the -198 (A>G) never observed in Vietnam before, and very recently observed in Azerbaijan [3], cd 26 (G>T) and cds 14/15 (+G). The cds 14/15 (+G), cd 26 (G>T) were sporadically observed in Thai population [20,76].

With reference to the IVS-II-654 (C>T), which is relatively common in South Vietnam, it seems not to be present in Central Vietnam.

On the whole, with the exception of the three most common mutations cd 26 (G>A), Hb E, cd 17 (A>T), and cds 41/42 (-TTCT), the spectrum of mutations observed in this study indicates rather high differences in comparison either with North and South Vietnam as well as with Thailand and Southwestern China.

It is necessary to emphasize that the approach to the study on the spread of β-thal mutations adopted in this study, as well as in a number of other countries, which focuses on the detection of mutations in patients with β-thal major, doesn't allow identification of mild β^+-thal mutations which only partially affect the synthesis of the β-globin chain. Result obtained in this thesis compared with that of other areas of Vietnam, and of the adjoining and neighboring countries, underlines the need to conduct future large screening campaigns, and strengthens the importance for a better control and prevention of β-thal by means of micro-mapping studies in adequate size of samples collected from different areas of the same country.

5.1.2. β-globin genotype of thalassemia patients

As shown in Table 4.3, the most common heterozygous genotype found in this, as well as other, report is the β^o type, found in 87/138 (63.1%), followed by heterozygous Hb E in 46/138 (33.3%), whereas the lower frequency was by the heterozygous β^+, found in only 5/138 (3.6%). In the case of the mutation giving rise of the structural variant Hb E, which is consistent with the activation of a cryptic splice site which affects mRNA processing, the amount of abnormal Hb E in heterozygotes is about 30% less. Hb E thus behaves like a mild form of β^+-thal [21].

Homozygous or compound heterozygous for β-thal may develop either TM or TI. As described, the main pathophysiological determinant of the severity of β-thal is the imbalance of α-/non α- globin chain. Any factor that is capable of reducing the imbalance of α-/non-α- globin chain may ameliorate the clinical severity [7].

The most important mechanism resulting in TI is due to the coinheritance of mild β-globin gene mutations either in homozygous or compound heterozygous conditions. Accordingly, patients with the genotypes β^E/β^E, β^E/β^+ and β^+/β^+ were only observed in TI patients (3/57, 22.8%). Conversely, patients with genotype β^o/β^o, observed in 8/31 instances (25.8%) were only transfusion dependents.

According to β^+/β^o genotype, there was just one TI patient showing the -72 (T>A)/cd 17 (A>T) genotype, and two TM with compound heterozygosity of -28 (A>G) with cd 17 (A>T) or cd 95 (+A) mutations. The β^+ type mutations have variable effects on globin gene expression. Since rather close to the Cap site, the -28 (A>G) has a quantitative effect on β-globin gene expression. This mutation directs 3 to 5 folds less β-mRNA than normal [57]. On the other hand, the -72 (T>A) within the conserved CCAAT box resulted in a low 19.35% reduction of β-mRNA level [61].

The genotype β^E/β^o was the most common genotype of both TI and TM, 43/57 (75.4%) and 21/31 (67.7%), respectively. As shown in Table 4.6, there were six different β^o-thal mutations in *trans* to the β^E in these patients. Although it is expected that β^o-thal mutations were more common in TM, a number of them were also detected in TI patients. The two most common were cd 17 (A>T) and cds 41/42 (-TTCT). A study

conducted in Northeast Thailand evaluated 103 TM and 45 TI patients associated with Hb E/β-thal produced the same results of this study: β^E/β^o was the most common in TM as well as in TI patients, and the two most common β^o were cds 41/42 (-TTCT) and cd 17 (A>T) with frequencies 52.0% and 23.0%, respectively [53].

In summary, compound heterozygotes for a mild and a severe β mutations result in variability of clinical phenotype, ranging from mild to severe forms.

5.2. THE ROLE OF INTERACTING α-THALASSEMIA, AND OF THE Xmn-1 $^G\gamma$ POLYMORPHISM FOR THE INCREASED PRODUCTION OF Hb F

5.2.1. The coinheritance of α-thalassemia among thalassemia patients

As discussion before, the coinheritance with α-thal lead to reduce severity of clinical phenotype. This was the case of 2/9 patients with the same genotype β^E/β^E, and 4/43 patients with genotype β^E/β^o.

O-Riordan et al. (2010) performed large scale screening for hemoglobin disorders in Southern Vietnam. Screening for β-thal was studied on approximately 9000 individuals of different ethnic groups and prevalence of α-thal was searched in 1261 subjects. Among Kinh people, they found high prevalence of β^E with gene frequency 0.017, the gene frequencies of --SEA, $-\alpha^{3.7}$, Hb CS and β-thal were 0.017, 0.017, 0.001 and 0.007, respectively [54]. With the high prevalence of α-thal in Vietnam, it is expected that the coinheritance homozygous or compound heterozygous β-thal with α-thal can be a major genetic modifier in TI patients.

Nonetheless, the coinheritance with α-thal was not the major modifier in this study. Among patients shared the same genotype β^E/β^o found in both TI and TM, only 4/43 TI patients had α-thal mutations.

Concomitant α-thal was also not found to be an important factor contributing to TI phenotype in Iran, Iraq and Lebanon. A study in Northern Iraq on 74 TI patients observed that only 4/71 with homozygous or compound heterozygous β-genotype had coinheritance with heterozygous $-\alpha^{3.7}$ mutation [2]. Similarly, a research on 52 TI Iranian patients found only 5/52 (9.6%) overlapped with the $-\alpha^{3.7}/\alpha\alpha$ genotype [51]. The lack of

contribution of α-thal is similar in the study from Lebanon, the α-globin gene mutations were found in 5/73 TI patients [62].

In contrast, TI patients in Indians showed highly frequent co-existence with deletional α-globin gene mutations, seen in 19/50 (38%) patients with homozygous β-thal [58]. A multicenter study of TI in different ethnic populations from Iran, India, Pakistan, Thailand, Mauritius and Cyprus had been done by Verma et al. (2007). Among 325 TI patients, they found α-thal mutations were present with higher frequencies than in the general populations, as 45% patients carried α-globin gene mutations. The α-thal were mainly coinherited with the β^+-thal mutations [79].

5.2.2. The role of Xmn-1 $^{G}\gamma$ polymorphism for the increased production of Hb F for ameliorating clinical phenotype

The possible ameliorating effect of the Xmn-1 $^{G}\gamma$ polymorphism at position -158 was also investigated in this work. Sequencing of the promoter region of the $^{G}\gamma$-globin gene of the 57 TI, and of 24 TM clearly indicated that, at least in Vietnamese population, heterozygosity (CT) or homozygosity (TT) for the polymorphism has not a clear effect on the severity of the disease.

This result was similar to the study in Northeast Thailand. Nuntakarn et al. (2009) reported the frequencies of $^{G}\gamma$ -158 genotype CC, CT and TT as [3.9%, 61.8%, 33.4%] and [6.7%, 71.1%, 22.2%] in TM and TI, respectively. No significant difference was observed between two groups [53]. The same finding has been shown previously in Southern Thailand, studied β-thal patients with homozygous or compound heterozygous cds 41/42 (-TTCT) mutation [45]. Similarity, Chen et al. (2010) analyzed 117 TI patients in Southern China, the neighborhood country of Vietnam, and showed that the effect of -158 (C>T) $^{G}\gamma$ polymorphism in increasing Hb F was not as important as other populations [13].

In summary, compound heterozygotes for a mild and a severe β mutations result in variability of clinical phenotype, ranging from mild to severe forms. Therefore, regardless to the role of coinherited α-thal genes, confirmed also in this study, although in a limited number of samples, the presence of a mild β-thal in combination with a

severe one does not allow to predict the evolution of the thalassemia disease [7]. Thus, the mild phenotype in TI patients could be due to coinheritance with other modifying ameliorating genetic factors.

6. CONCLUSION

Twelve different β-globin gene mutations, mostly characterized by the absence of synthesis of β-globin chains of Hb, were identified. As well as in all SEA, the most common allele results the less severe Hb E mutation.

As a consequence, several different combination of β-globin genotype are found, which increase the number of homozygotes and compound-heterozygotes with the severe (transfusion dependent) β-thalassemia. The genotypes β^E/β^E, β^E/β^+ and β^+/β^+ only observed in TI patients and the genotype β^o/β^o belong to TM patients.

The ameliorating effect of coinheritance of α-thalassemia was confirmed also in this study, although in a limited number of samples.

The -158 (C>T) $^G\gamma$ polymorphism as phenotype ameliorating factors were not evident in this study.

From a population genetics point of view, the allelic frequencies of β-thalassemia mutations are quite different with respect to those of other regions of Vietnam, as well as to other Southeast Asia countries.

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