CHAPTER I

INTRODUCTION

1.1 Hemoglobin (1)

Hemoglobin is a protein that is carried by red blood cells. It picks up oxygen in the lungs and delivers it to the peripheral tissues to maintain the viability of cells. Hemoglobin is made from two similar proteins that "stick together". Both proteins must be present for the hemoglobin to pick up and release oxygen normally. One of the component proteins is called α-like globin, the other is β-like globin.

1.1.1 Hemoglobin structure

All normal hemoglobins are tetramers of two pairs of unlike globin chains (Figure 1.1). Four polypeptide globin chains are arranged in pairs forming the tetrameric molecule or globin portion of hemoglobin (2). Each globin chain is covalently attached to a heme moiety. The globin tetramer is globular or ellipsoid in shape, being approximately 550 nm in diameter. The four porphyrin heme moieties lie in four regularly spaced clefts on the tetramer surface. The globin portion of most normal hemoglobins consists of two chains from chromosome 16 and two from chromosome 11. Normal α chains contain 141 amino acids; normal β chains have 146 amino acids (3). The δ-globin chain varies by 10 amino acids; the γ-globin chain by 39 amino acids compared with the β-globin chain. Adult (HbA) and fetal (HbF) hemoglobins have α-globin chains that combine with β-(HbA, $\alpha_2\beta_2$), δ-(HbA_2, $\alpha_2\delta_2$) or γ-globin chains (HbF, $\alpha_2\gamma_2$), whereas in the embryo, α-like globin chains called ζ-globin chains combine with γ-(Hb Portland, $\zeta_2\gamma_2$) or ε-globin chains (Hb Gower 1, $\zeta_2\varepsilon_2$) and α- and ε-globin chains form Hb Gower 2($\alpha_2\varepsilon_2$) (4). Embryonic hemoglobin is confined to the york-sac stage of development and thereafter is replaced by HbA and HbA_2 over the first year of life, although in normal adults small amounts of HbF, constituting ~1% of the total hemoglobin, continue to be produced (Figure 1.2).

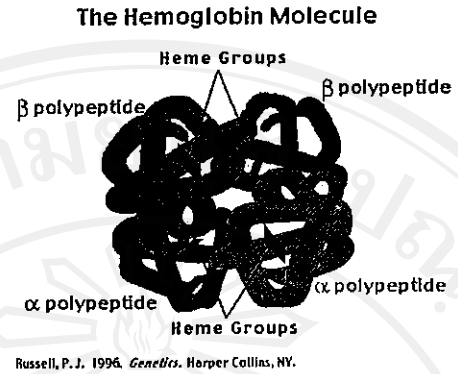

Figure 1.1 The hemoglobin molecule

(http://ntri.tamuk.edu/homepagentri/lectures/protein/hemoglobin/hba.jpg; accessed July 28, 2004)

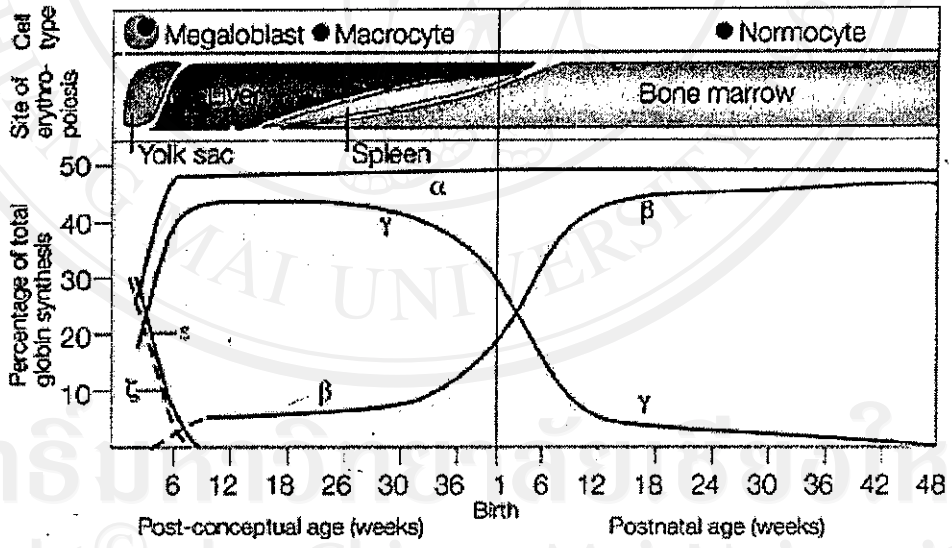

Figure 1.2 Changes in biosynthesis of globin chains during development (5)

Table 1.1 Normal hemoglobin types in human at different developmental stages (4)

Hemoglobins	Embryo	Fetus	Adult
Gower 1 ($\zeta_2\varepsilon_2$)	42%-	-	-
Gower 2 ($\alpha_2\varepsilon_2$)	24%	-	-
Portland ($\zeta_2\gamma_2$)	Present*	-	-
F ($\alpha_2\gamma_2$)	Present*	90%	<1%
A($\alpha_2\beta_2$)	-	10%	97%
A2 ($\alpha_2\delta_2$)	-	-	2.50%

* HbF and Portland have very similar mobility on cellulose acetate electrophoreresis at pH 8.5. Thus this two hemoglobins account for about 34% of total hemoglobins when blood samples from early embryo are examined by this technique.

1.1.2 Hemoglobin synthesis

Hemoglobin synthesis requires the coordinated production of heme and globin. Heme is the prosthetic group that mediates reversible binding of oxygen by hemoglobin. Globin is the protein that surrounds and protects the heme molecule. To deal with hemoglobin synthesis, the productions of heme and globin are considered separately.

1.1.2.1 Heme Synthesis

Heme is synthesized in a complex series of steps involving enzymes in the mitochondrion and in the cytosol of the cell (Figure 1.3). The first step in heme synthesis takes place in the mitochondria, with the condensation of succinyl CoA and glycine by ALA synthase to form δ-aminolevulic acid (δ-ALA) (6). This molecule is transported to the cytosol where a series of reactions produce a ring structure called coproporphyrinogen III. This molecule returns to the

mitochondria where an addition reaction produces protoporhyrin IX. The enzyme ferrochelatase inserts iron into the ring structure of protoporphyrin IX to produce heme. (6)

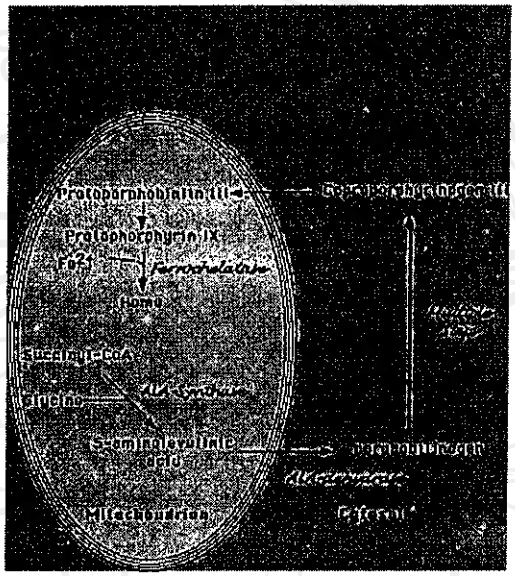

Figure 1.3 Heme biosynthetic pathway (http://sickle.bwh.harvard.edu/hbsynthesis.thml; accessed July 28, 2004)

1.1.2.2 Globin synthesis

Globin chains are synthesized in the cytoplasm by the interaction of mRNA, ribosomes, transfer RNAs and various cofactors in a series of reactions proceeding through three separate phases: initiation, elongation and termination (7).

Initiation: The initial step involves the formation of a tertiary complex between the initiation factor eIF-2, GTP and the initiation transfer RNA, tRNAmet, which subsequently binds to the small 40S ribosomal subunit. With the help of other initiation factors, globin mRNA and the large ribosomal subunit are added to this complex so that, in its final configuration, the mRNA is aligned with the ribosome with the AUG initiation codon opposite the UAC anticodon of the tRNAmet. AUG is the only codon for the amino acid methionine. Thus, in all mammalian systems, just as in bacteria, the first amino acid incoporated into a protein chain is methionine; in bacterial systems the

methionine has a blocked, formylated amino group but this is not found in mammalian cells. Once translation starts the initiation factors dissociate to participate in another initiation cycle.

Elongation: Protein synthesis now proceeds by stepwise addition of amino acids from the N-terminal end to form the growing peptide chain. This cycle involves at least three distinct stages. The first is a codon-directed binding of an aminoacyl-tRNA to a ribosome site next to that occupied by the initiator tRNAmet. Peptide transfer then takes place between the newly bound aminocyl-tRNA and the tRNAmet. Lastly, the newly made peptidyl-tRNA and the mRNA are both shift from the acceptor to the donor site on the ribosome. In this way the acceptor site is freed and the process can be repeated. At the same time the tRNA that donated the growing peptide chain is released. Two protein factors, EF-1 and EF-2 are involved in the elongation process. EF-1 participates in a GTP-dependent reaction by which aminocyl-tRNAs bind to ribosomes, while EF-2 or translocase catalyses the translocation step in another GTP-dependent reaction. The N-terminal methionine incoporated during initiation is removed enzymatically from the globin chain after the addition of about 20 amino acids.

Termination: Globin synthesis stops when the ribosome reaches a chain termination codon (UAA, UAG, UGA) in the message. At this point, the polypeptide chain is splitted from the tRNA in another GTP-dependent hydrolytic reaction, which involves at least one protein cofactor. The ribosome is released from the mRNA and dissociates into subunits, which are then free to participate in another round of synthesis.

1.1.3 Globin genes

The genes that encode the α-like globin chains are on the short arm of chromosome 16 (2) whereas those that encode the β-like globin chains are on the short arm of chromosome 11 (8) (Figure 4). Multiple individual genes are expressed at each site. Pseudogenes are also present at each location. The α-globin complex is called the " α-globin gene cluster", while the β- globin complex is called the "β- globin gene cluster". The expression of the α-like and β-like globin genes is closely balanced

by an unknown mechanism. However, the imbalance was observed in mRNA levels. It could be explained by the less translational efficiency of α-globin mRNA due to the instability of the α-globin mRNA. Balanced gene expression is required for normal red cell function. Disruption of the balance produces a disorder called thalassemia.

1.1.3.1 Globin genes cluster

Alpha (α) globin gene cluster

The α-globin gene cluster (Fig. 4) arranged in the order of 5'-ζ-Ψζ-Ψα2–Ψα1-α2-α1-θ-3' on each chromosome 16 (16p13.3) (9). Since each individual has 2 chromosomes 16, there are usually a total of 4 functional α-globin genes. The CAP site of ζ-globin gene is designated 0, the α_2-globin gene, at +20 kb, lies 20 kb downstream of the ζ-globin gene, and the α_1-globin gene lies a furture 3.7 kb downstream, at +24 kb. About 40 Kb upstream of the α-globin gene cluster is a region known as HS-40, corresponding to a series of DNase hypersensitive sites and binding sites for transcription factors (10). Its integrity is essential for α-globin gene expression, as simply demonstrated by its removal in several natural deletions that effectively silence the expression of α-globin genes downstream (11). In such cases these people present as α-thalassemia carriers. In addition to the α-globin genes and their *cis* regulatory sequences, there are important transcriptional factors encoded by genes unlinked to the globin gene clusters. These factors are pivotal in regulating gene expression by binding to the α-globin gene promoter or HS-40 sequences, or both, interacting with other DNA-binding proteins, or altering chromatin structure. A good example is the ATRX gene on chromosome Xq13.3. Mutations of this gene cause marked down-regulation of α-globin gene expression, plus severe mental disability (12).

Beta (β) globin gene cluster

The β-globin gene cluster is on the short arm of chromosome 11 (11p15.5) (Figure 4). The genes in the cluster are arranged sequentially from 5' to 3' direction beginning with the gene expressed in embryonic development (the first 12 weeks after conception; called episolon: ε) and

ends with the adult β-globin gene. The sequence of the genes is: 5'-ε-^{G}γ-^{A}γ-ψβ-δ-β-3' (13). There are two copies of the γ-gene on each chromosome 11. The others are present in single copies. The two fetal γ -globin genes lie 15 and 20 kb downstream from the embryonic ε gene, while the δ and β-globin genes are 35 and 43 kb further downstream. Upstream of the ε-globin gene, lies the locus-control region (LCR), the regulatory region that is essential for expression of all the genes in the complex. It spans ~15 kb and contains four elements (HS1 to HS5) which are marked by erythroid-cell-specific DNase1-hypersensitive sites (14). There are two other hypersensitive sites, one 5' to the LCR and one ~20 kb 3' downstream of the β-globin gene. It has been suggested that they mark the boundaries of a β-globin gene domain. The β-globin gene clusters of man and mouse (and possibly chicken) are embedded in an array of olfactory receptor genes (15).

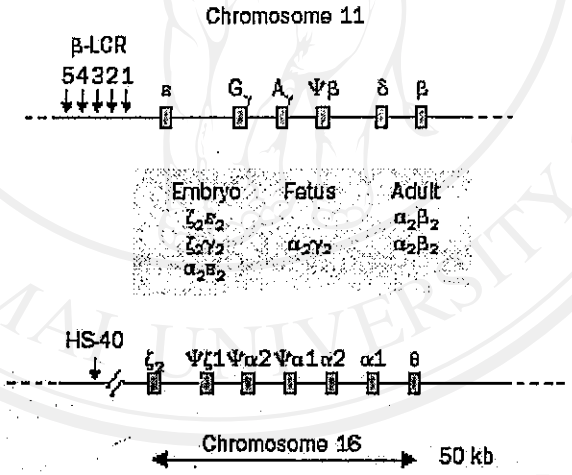

Figure 1.4 Schematic representation of α and β-globin gene clusters and combinations of globin genes to form functional hemoglobin molecules. Vertical arrows represent DNA hypersensitive sites which mark major regulatory regions (locus control region [LCR] and hypersensitive site HS-40) for the β-globin and α-globin gene clusters, respectively (16).

1.1.4 Thalassemia and hemoglobinopathies (8, 17)

In 1925, Thomas Cooley and Pearl Lee described a form of severe anemia, occurring in children of Italian origin and associated with splenomegaly and characteristic bone changes. Over the next decade, a milder form was described independently by several Italian investigators. Because all early cases were reported in children of Mediterranean origin, the disease was later termed thalassemia, from the Greek word for sea (thalassa). Over the next 20 years, it became apparent that Cooley and Lee had described the homozygous or compound heterozygous state for a recessive Mendelian disorder not confined to the Mediterranean, but occurring widely throughout tropical countries. In the past 20 years, the two important forms of this disorder, α- and β-thalassemia, resulting from the defective synthesis of the α- and β-globin chains of hemoglobin, respectively, have become recognized as the most common monogenic diseases in human.

1.1.5 Distribution of thalassemia and hemoglobinopathies across the world

The β-thalassemia is widespread throughout the Mediterranean region, Africa, the Middle East, the Indian subcontinent and Burma, Southeast Asia including Southern China, the Malay Peninsula, and Indonesia. Estimates of gene frequencies range from 3 to 10 percent in some areas (18).

Within each population at risk for β-thalassemia, a small number of common mutations are found, as well as rarer ones; each mutation is in strong linkage disequilibrium with specific arrangements of restriction-fragment–length polymorphisms, or haplotypes, within the β-globin gene cluster. A limited number of haplotypes are found in each population, so that 80 percent of the mutations are associated with only 20 different haplotypes. This observation has helped demonstrate the independent origin of β-thalassemia in several populations. There is evidence that the high frequency of β-thalassemia throughout the tropics reflects an advantage of heterozygotes against *Plasmodium falciparum* malaria, as has already been demonstrated in α-thalassemia (19).

1.1.6 Alpha (α) thalassemia (17)

Alpha thalassemia occurs when one or more of the four α-globin chain genes fails to function (i.e.deletion or malfunction). α-globin chain production, for practical purposes, is evenly divided among the four genes. With α-thalassemia, the "failed" genes are almost invariably lost from the cell due to a genetic accident.

(i) The loss of one gene diminishes the production of the alpha protein only slightly. This condition is so close to normal that it can be detected only by specialized laboratory techniques that, until recently, were confined to research laboratories. A person with this condition is called a "silent carrier" because of the difficulty in detection. Traditionally, this condition is called α-thalassemia 2 or α^{+}-thalassemia heterozygote.

(ii) The loss of two genes (two-gene deletion α thalassemia) produces a condition with small red blood cells, and at most a mild anemia. People with this condition look and feel normal. The condition can be detected by routine blood testing, however. This condition is generally termed α-thalassemia 1 or α^{0}-thalassemia heterozygote.

(iii) The loss of three α-globin genes produces a serious hematological problem (three-gene deletion alpha thalassemia). Patients with this condition have a severe anemia, and often require blood transfusions to survive. The severe imbalance between the α-globin chain production (now powered by one gene, instead of four) and β-globin chain production (which is normal) causes an accumulation of beta chains inside the red blood cells. Normally, β-globin chains pair only with α-globin chains. With three-gene deletion α- thalassemia, however, β-globin chains begin to associate in groups of four, producing an abnormal hemoglobin, called"hemoglobin H; HbH (β_4)". The condition is called "hemoglobin H disease". Hb H has two problems. First it does not carry oxygen properly, making it functionally useless to the cell. Second, hemoglobin H protein damages the membrane that surrounds the red cell, accelerating cell destruction. (iv) The loss of all four α-globin genes produces a condition that is incompatible with life. The γ-globin chains produced during fetal life associate in groups of four to form an abnormal hemoglobin called "hemoglobin Barts; Hb Bart's

(γ_4)". Most people with four-gene deletion α-thalassemia die *in utero* or shortly after birth. Rarely, four gene deletion alpha thalassemia has been detected *in utero*, usually in a family where the disorder occured in an earlier child. *In utero* blood transfusions have saved some of these children. These patients require life-long transfusions and other medical support.

1.1.7 Alpha (α)-hemoglobinopathies

Several nucleotide changes in the α-globin gene produce the variants of α-globin chain. This condition is generally termed "α-hemoglobinopathies". α-hemoglobinopathies which is common in Thailand is hemoglobin Constant Spring (HbCS). HbCS is an α-globin chain of with additional 31 amino acids, which is resulted from point mutation of the termination codon of α_2-globin gene. Due to the instability of this elongated α-globin chains and the reduction of the expression of downstream α_1-globin, the clinical phenotype of those having HbCS allele is generally α-thalassemia 2. The percentage of HbCS is usually about 1% in heterozygous carriers, 5 to 7% in heterozygotes and 3 to 5% in hemoglobin H disease from HbCS in *trans* to deletional α-thalassemia. Heterozygotes of HbCS and two normal *trans* α-globin genes are hematologically normal. Homozygotes for HbCS have a mild hemolytic anemia and may have splenomegaly. Individuals with HbH disease from HbCS and two deletional α-thalassemia have a more severe disease with more HbH and Bart's than three-gene-deletional α-thalassemia. HbCS is common in Southeast Asian and found in high frequency in American immigrants from some those area (20).

1.1.8 Beta (β) thalassemia (21)

The fact that there are only two genes for the β-globin chain of hemoglobin makes β-thalassemia a bit simpler to understand than α-thalassemia. Unlike α-thalassemia, β-thalassemia rarely arises from the complete loss of a β-globin gene. The β-globin gene is present, but mutations could occur along the gene and affect the efficiency of β-globin production. The degree of suppression varies. Many causes of suppressed β-globin gene expression have been found. In some

cases, the affected gene makes essentially no β-globin protein (β^0-thalassemia). In other cases, the production of β-globin chain is lower than normal, but not zero (β^+-thalassemia). The severity of β-thalassemia depends in part on the type of β- thalassemic genes or mutations of β-globin gene that a person has inherited.

(i) one-gene β-thalassemia has one β-globin gene that is normal, and a second, affected gene with a variably reduced production of β-globin gene. The degree of imbalance with the α-globin depends on the residual production capacity of the defective β-globin gene. Even when the affected gene produces no β-globin chain, the condition is mild since one β-globin gene functions normally. The red cells are small and a mild anemia may exist. People with this condition generally have no symptoms. The condition can be detected by a routine laboratory blood evaluation. (Note that in many ways, the one-gene β-thalassemia and the two-gene α-thalassemia are very similar, from a clinical point of view. Each results in small red cells and a mild anemia).

(ii) two-gene β-thalassemia produces a severe anemia and a potentially life-threatening condition. The severity of the disorder depends in part on the combination of genes that have been inherited: β^0-thal/ β^0-thal; β^0-thal/ β^+-thal; β^+-thal/ β^+-thal. The β^+-thalassemia genes vary greatly in their ability to produce normal hemoglobin. Consequently, the clinical picture is more complex than might otherwise be the case for three genetic possibilities outlined.

1.1.9 Beta (β)-hemoglobinopathies

Hemoglobin E is the most common hemoglobin variant in the world. In Thailand, it accounts for approximately 13% to 70% (22). G-A substitution at codon 26 of β-globin chain bring about the β -globin structural variant that when forms tetrameric structure with α-globin chain results in HbE. The β -globin chain codon 26 Glu-Lys mutation (GAG-AAG) partially activates a cryptic splice site towards the 3' end of exon 1, resulting in proportion of abnormally splice mRNA (23). Thus, less β^E-globin chain synthesized and mild thalassemia phenotype results (24). The inheritance of HbE produces the phenotype of a mild form or β^+-thalassemia (25).

1.1.10 Molecular defects in β-thalassemia

While the whole gene deletion is the main cause of α-thalassemia, the molecular background of β -thalassemia is different. The majority of the β-thalassemia patients bears intact β-globin gene with minor changes in the nucleotide sequences or deletion of small part of β-globin gene which is generally called point mutations. Very rare cases of β-thalassemia are resulted from the removal of entire β-globin gene.

1.1.10.1 Nondeletional mutants

This defects account for the vast majority of the β-thalassemia alleles. This involves single-base substitutions, small insertions or deletions within the gene or its immediate flanking sequences and affect almost every known stage of gene expression (transcription, mRNA processing and translation). The details of these defects are listed in Table 1.2.

A. Transcriptional mutants

These mutations occur in the promoter region of the β-globin gene, causing decreased rate of β-globin mRNA transcription. These transcriptional mutants emphasize the role of the conserved promoter sequences in the binding of transcription factors involved in expression of the β-globin gene. The examples of this type of mutations include the nt–28: A->G, nt-86: C->G and nt-101: C-> T, which modify the binding affinity of transcriptional proteins, resulting in reduced efficiency of β-mRNA transcription; the general characteristics of β^{+}-thalassemia. Recently, a study has shown that the β-thalasemia mutations at position -87 specifically ablates EKLF binding affinity, causing decline competitive advantage of β-globin gene for β-LCR (26). Table 1.2 demonstrates the detail of this type of molecular defects on β-globin gene.

B. RNA processing mutants

After being transcribed, immature β-globin mRNA is generated and need a process called mRNA processing in which introns are removed, CAP structure attached at the 5'-end and poly A tail added at the 3' end of the β-globin mRNA. This process requires conserved and concensus sequences at or flanking the site to be processed. They include the ACA trinucleotide at the CAP site, the conserved GT dinucleotide of the 5' donor site: the conserved AG dinucleotide at the 3' acceptor site: other concensus flanking sequences, all of which are needed for normal mRNA splicing and AATAAA polynucleotide at the 3' poly A addition site. Mutations of these nucleotide sequences could result in either β^+ or β^0-thalassemia depending on the site where the mutations occur. Mutations that affect either of the variant dinucleotides in the splice junction completely abolish normal splicing and produce the phenotype of β^0-thalassemia. In contrast, mutations that affect CAP site, poly A addition site and other consensus sequences always generate the phenotype of β^+-thalassemia. For example, the IVS-I-1, G->T considerably reduce splicing at the mutated donor site compared with normal and no β-globin chian produced, thus resulting in the β^0-thalassemia (27). On the other hand, the IVS-I-5, G->A only slightly reduce the rate of β-globin mRNA splicing and some β-globin chains are still produced, thus giving rise to the β^+-thalassemia phenotype as well as those mutations that occur at the CAP and poly A addition sites (28). Table 1.2 demonstrates the detail of this type of molecular defects on β-globin gene.

C. Translational mutants

Most of these defects result from the introduction of a premature termination codon owing to framshifts or nonsense mutations. Framshift defects alter the reading frame and cause premature termination further downstream while nonsense mutation interfere directly with translation by creation of a stop codon via a single-base substitution. These mutations all result in premature termination within exon 1 and 2 with couple of exceptions terminating prematurely in exon 3. Mutations that result in premature termination early in the sequence are associated with minimal

steady-state levels of β-globin mRNA compared with normal in erythroid cells (29). In heterozygotes for such cases, no β-globin chain is produced from the mutant allele and only half the normal β-globin is present. In addition, the mutations affecting the initiation codon also been described and all produce β^0-thalassemia. They are single-base substitution, one affecting the first (A), two the second (T) and two the third (G) nucleotide of ATG (30). Table 1.2 demonstrates the detail of this type of molecular defects on β-globin gene.

1.1.10.2 Deletional β-thalassemia

β-thalassemia resulting from deletion of the whole β-globin gene has been rarely reported. However, the deletion of part of the β-globin gene has been demonstrated in some ethnic group. The 619-bp deletion removing the 3' end of the β-globin gene has been demonstrated among Asian Indian patients. In addition, the 3.4-kb and 105-bp deletions have also been reported. All three of these deletions have also been reported in Thailand (31). Most of the deletional β-thalassemia generate the phenotype of β^0-thalassemia. The deletional β-thalassemia mutations that has been reported in Thailand can be found in the table 1.2.

Table 1.2 Point mutations and frameshifts causing (silent) β-thalassemia (http://globin.cse.psu.edu/html/huisman/thals/tables/table01.html)

Mutation	Type	Ethnic Group	Mutation	Type	Ethnic Group
A. Transcriptional Mutants (n=22)					
-101, C->T	β^+	Turkish; Bulgarian; Italian	-31, A->C	β^+	Italian
-92, C->T	β^+	Mediterranean	-31, A->G	β^+	Japanese
-90, C->T	β^+	Portuguese	-30, T->A	β^+	Turkish; Macedonian; Bulgarian
-88, C->A	β^+	Kurds	-30, T->C	β^+	Chinese
-88, C->T	β^+	Black populations	-29, A->G	β^+	American Blacks; Chinese
-87, C->A	β^+	American Blacks	-28, A->C	β^+	Kurds
-87, C->G	β^+	Mediterranean	-28, A->G	β^+	Chinese
-87, C->T	β^+	German/Italian	+10, -T	β^+	Greek
-86, C->A	β^+	Italian	+22, G->A	β^+	Turkish; Bulgarian; Italians
-86, C->G	β^+	Lebanese; Thai	+33. C->G	β^+	Greek Cypriots
-32, C->A	β^+	Taiwanese	+43 to +40, -AAAC	β^+	Chinese

Mutation	Type	Ethnic Groups
B-a. RNA Processing Mutants: Mutations in Splice Junctions (n=22)		
IVS-I (-3), C->T (codon 29; Gly->Gly)	β^+	Lebanese
IVS-I (-2), A->G (codon 30; Arg->Gly)	β^0	Sephardic Jews
IVS-I (-1), G->A (codon 30; Arg->Lys)	β^0	Bulgarian
IVS-I (-1), G->C (codon 30; Arg->Thr)	β^0	American Blacks; Tunisians; UAE; Indian
IVS-I-1, G->A	β^0	Mediterranean; Asian Indian
IVS-I-1, G->T	β^0	Asian Indian
IVS-II-1, G->A	β^0	Mediterranean; Tunisian; American Blacks
IVS-II-1, G->C	β^0	Iranian
IVS-I-2, T->A	β^0	Algerian
IVS-I-2, T->C	β^0	American Blacks
IVS-I-2, T->G	β^0	Tunisian
IVS-II-2,3, +11 bp, -2 bp	β^0	Iranian
IVS-I, -17 nts (3' end)	β^0	Kuwaiti
IVS-I-130, G->A	β^0	Egyptian
IVS-I-130, G->C	β^0	Turkish; Japanese
Codon 30, G->C [IVS-I-130 (+1)]	β^0	Middle East
IVS-II-849, A->C	β^0	American Blacks
IVS-II-849, A->G	β^0	American Blacks

IVS-II-850, -G	β^0	Italian
IVS-II-850, G->A	β^0	English-Scottish
IVS-II-850, G->C	β^0	Yugoslavian
IVS-II-850, G->T	β^0	Japanese
B-b. RNA Processing Mutants: Mutations in the Consensus Sites (n=12)		
IVS-I-5, G->A	β^+	Algerian; Mediterranean
IVS-I-5, G->C	β^+	Asian Indian; Chinese; Melanesian
IVS-I-5, G->T	β^+	Mediterranean; American Blacks
IVS-II-4,5, -AG	β^0	Portuguese
IVS-II-5, G->C	β^+	Chinese
IVS-I-6, T->C	β^+	Mediterranean
IVS-I-128, T->G	β^+	Saudi Arabian
IVS-II-837, T->G	?	Asian Indian
IVS-II-843, T->G	β^+	Algerian
IVS-II-844, C->G	β^+	Italian
IVS-II-848, C->A	β^+	American Blacks; Egyptian; Iranian
IVS-II-848, C->G	β^+	Japanese
B-c. RNA Processing Mutants: Changes in IVS-I or IVS-II (n=5)		
IVS-I-110, G->A	β^+	Mediterranean
IVS-I-116, T->G	β°	Mediterranean

IVS-II-654, C->T	β^+	Chinese
IVS-II-705, T->G	β^+	Mediterranean
IVS-II-745, C->G	β^+	Mediterranean
B-d. RNA Processing Mutants: Changes in Coding Regions (n=5)		
Codon 10, C->A (Ala->Ala)	β^+	Asian Indian
Codon 19, A->G (Hb Malay; Asn->Ser)	β^+	Malay
Codon 24, T->A (Gly->Gly)	β^+	American Blacks; Japanese
Codon 26, G->A (Hb E; Glu->Lys)	β^+	Southeast Asian
Codon 27, G->T (Hb Knossos; Ala->Ser)	β^+	Mediterranean
C-a. RNA Translation Mutants: Nonsense Mutations (n=12)		
Codon 15, T*G*G->T*A*G	β^0	Asian Indian; Turkish
Codon 15, TG*G*->TG*A*	β^0	Portuguese
Codon 17, A->T	β^0	Chinese
Codon 22, G->T	β^0	Reunion Islanders
Codon 26, G->T	β^0	Thai
Codon 35, C->A	β^0	Thai
Codon 37, G->A	β^0	Saudi Arabian; Spanish
Codon 39, C->T	β^0	Mediterranean
Codon 43, G->T	β^0	Chinese
Codon 61, A->T	β^0	Blacks
Codon 90, G->T	β^0	Japanese
Codon 112, T->A	β^0	Slovakian

C-b. RNA Translation Mutants: Frameshift Mutations (n=55)		
Codon 1, -G	β^0	Mediterranean
Codons 2/3/4, -9 bp; +31 bp	β^0	Algerian
Codon 5, -CT	β^0	Mediterranean
Codon 6, -A	β^0	Mediterranean; American Blacks
Codon 8, -AA	β^0	Mediterranean
Codons 8/9, +G	β^0	Asian Indian
Codons 9/10, +T	β^0	Greek
Codon 11, -T	β^0	Mexican
Codons 14/15, +G	β^0	Chinese
Codon 15, -T	β^0	Malay
Codon 16, -C	β^0	Asian Indian
Codons 22/23/24, -AAGTTGG	β^0	Turkish
Codon 24, -G; +CAC	β^0	Egyptian
Codons 25/26, +T	β^0	Tunisian
Codon 26, +T	β^0	Japanese
Codons 27/28, +C	β^0	Chinese
Codon 28, -C	β^0	Egyptian
Codons 28/29, -G	β^0	Japanese; Egyptian
Codon 31, -C	β^0	Chinese
Codon 35, -C	β^0	Malay
Codons 36/37, -T	β^0	Iranian; Kurds
Codons 37/38/39, -GACCCAG	β^0	Turkish
Codons 38/39, -C	β^0	Czech

Codons 38/39, -CC	β^0	Belgians
Codon 40, -G	β^0	Japanese
Codons 40/41, +T	β^0	Chinese
Codon 41, -C	β^0	Thai
Codons 41/42, -TTCT	β^0	Chinese
Codons 42/43, +G	β^0	Japanese
Codons 42/43, +T	β^0	Japanese
Codon 44, -C	β^0	Kurds
Codon 45, -T	β^0	United Arab Emirates
Codon 47, +A	β^0	Surinamese
Codons 47/48, +ATCT	β^0	Punjabi
Codon 51, -C	β^0	Hungarian
Codons 53/54, +G	β^0	Japanese
Codon 54, -T	β^0	Algerian; Swedish
Codons 54/55, +A	β^0	Indian
Codons 56-60, +14 bp	β^0	
Codons 57/58, +C	β^0	Punjabi
Codon 59, -A	β^0	Italian
Codon 64, -G	β^0	Swiss
Codon 67, -TG	β^0	Filipino
Codons 71/72, +A	β^0	Chinese
Codons 71/72, +T	β^0	Chinese
Codons 72/73, -AGTGA; +T	β^0	British
Codons 74/75, -C	β^0	Turkish
Codon 76, -C	β^0	Italian
Codons 82/83, -G	β^0	Azerbaijani; Czech;

		Croatian
Codons 84/85, +C	β^0	Japanese
Codons 84/85/86, +T	β^0	Japanese
Codon 88, +T	β^0	Asian Indian
Codons 89/90, -GT	β^0	Korean
Codon 95, +A	β^0	Thai
Codons 106/107, +G	β^0	American Blacks
D. Dominant Beta-Thal and Highly Unstable Beta Chain Variants (n=30)		
Codons 24/25, -GGT	β^0	Japanese
Codon 28, C*T*G->C*G*G	β^0	(Hb Chesterfield)
Codons 31/32, +CGG	β^0	Spanish
Codon 32, C*T*G->C*A*G (Leu->Gln); Codon 98, *G*TG->*A*TG (Val->Met)	β^0	(Hb Medicine Lake)
Codons 33/34, -GTG	β^0	(Hb Korea) Koreans
Codon 60, G*T*G->G*A*G (Val->Glu)	β^0	(Hb Cagliari) Italians
Codon 94, +TG	β^0	(Hb Agnana) Italian
Codon 100, -CTT, +TCTGAGAACTT	β^0	South African
Codons 108/109/110/111/112, -12 bp	β^0	Swedish
Codon 109, -G	β^0	(Hb Manhattan) Ashkenazi Jews
Codon 110, T->C	β^0	(Hb Showa-Yakushiji) Japanese
Codon 114, -CT; +G	β^0	(Hb Geneva) French Swiss
Codon 114, T->C	β^0	(Hb Durham-N.C.; Hb

		Brescia) Italians
Codon 115, C->A	β^0	[Hb Hradec Kralove (Hb HK)] Czech
Codons 120/121, +A	β^0	Filipino
Codon 121, G->T	β^0	Polish; Swiss; Japanese; English; Czech
Codon 123, -A	β^0	(Hb Makabe) Japanese
Codons 123/124/125, -ACCCCACC	β^0	Thai
Codon 124, -A	β^0	Russian
Codon 124/125/126, +CCA	β^0	Russian
Codon 125, -A	β^0	Japanese
Codon 126, -T	β^0	(Hb Vercelli) Italian
Codon 126, G*T*G->G*G*G	β^0	(Hb Neapolis) Italians; German; Thai
Codons 126/127/128/129/130/131, -17 bp	β^0	Pakistani
Codon 127, *C*AG->*T*AG (Gln->stop codon)	β^0	English
Codon 127, C*A*G->C*C*G (Gln->Pro)	β^0	(Hb Houston) British
Codon 127, C*A*G->C*G*G (Gln->Arg)	β^0	French
Codons 127/128, -AGG (Gln·Ala->Pro)	β^0	(Hb Gunma) Japanese
Codons 128/129, -4 bp, +5 bp and Codons 132/133/134/135, -11 bp	β^0	Irish
Codons 134/135/136/137, -10 bp, +4 bp	β^0	Portuguese
E. Cap Site Mutations (n=1)		
Cap +1, A->C	β^+	Asian Indian

F. Initiation Codon Mutations (n=6)		
*A*TG->*G*TG	β^0	Japanese
A*T*G->A*C*G	β^0	Yugoslavian
A*T*G->A*G*G	β^0	Chinese; Korean; North European
AT*G*->AT*A*	β^0	Italian; Swedish
AT*G*->AT*C*	β^0	Japanese
AT*G*->AT*T*	β^0	Iranian
G. 3'UTR Mutations (n=3)		
3'UTR +6, +1,480; C->G	β^+	Greek (also known as +6, C->G)
3'UTR +1,565 to +1,577; -13 bp	β^+	Turkish
3'UTR +1,570; T->C	β^+	Irish
H. Mutations in the Polyadenylation Site (poly A) (n=6)		
AA*T*AAA->AA*C*AAA	β^+	American Blacks
AAT*A*AA->AAT*G*AA	β^+	Mediterranean
AATA*A*A->AATA*G*A	β^+	Malay
AATAA*A*->AATAA*G*	β^+	Kurds
AATAAA->AAAA (-AT or -TA)	β^+	French
AATAAA->A (-AATAA)	β^+	Arabian

1.1.11 Common β-thalassemia mutations in Thailand

The survey in Thailand for the frequencies of β-thalassemia mutations has clearly shown the unique characteristics of the pattern of β-thalassemia mutations in Thai people. The β^0-thalassemia producing CDs 41/42 (-TTCT) allele combining with the β^0-thalassemia producing CD17 (A-T) allele accounts for the majority of the cases. Table 1.3 demonstrates the β-globin mutations identified among Thai β-thalassemia, in which defects of β-globin gene causing the disease in some of these patients remains to be determined.

Table 1.3 β-thalassemia mutations in Thailand (32)

Mutation	Central (%)	North (%)	Northeast (%)	South (%)
β^0-thalassemia				
cd41/42 (-TTCT)	41.1	39.8	45.3	30.1
cd17 (A-T)	16.5	39.8	25.6	11.3
cd35 (C-A)	1.9			
IVS1 nt 1 (G-T)	1.3		3.4	6.0
cd71/72 (+A)	2.1		10.2	
cd8/9 (+G)				0.4
cd14/15 (+G)	0.3			
cd15 (-T)	0.3			0.4
cd26 (G-T)			0.9	
cd27/28 (+C)	0.5			
cd41 (-C)	0.5			1.4
cd43 (G-T)	0.5			
cd95 (+A)	0.3			
3.4-kb deletion	0.3	0.9		4.3

Mutation	Central (%)	North (%)	Northeast (%)	South (%)
619-bp deletion	1.3			
1.5-bp deletion				0.4
cd123-125 (-ACCCCACC)			0.9	
IVS1 nt 1 (G-A)				0.4
β^+-thalassemia				
nt-86 (C-G)	0.3			
ATA nt-28 (A-G)	9.3	3.5	1.7	5.7
cd19 (A-G)	2.9			15.2
IVS1 nt 5 (G-C)	4.3	1.8	0.9	18.8
IVS2 nt654 (C-T)	7.5	0.9	8.5	2.1
cd26 (HbE)	0.5			
Unknown	7.8	13.2	2.6	3.1

1.1.12 Clinical classification of the β-thalassemias (17, 21)

(i) β-thalassemia minor, or β-thalassemia trait. These terms are used interchangeably for people who have small red cells and mild (or no) anemia due to thalassemia. These patients are clinically well, and are usually only detected through routine blood testing. Physicians often mistakenly diagnose iron deficiency in people with thalassemia trait. Iron replacement does not correct the condition. The primary caution for people with β-thalassemia trait involves the possible problems that their children could inherit if their partner also has β-thalassemia trait. These more severe forms of β-thalassemia trait are outlined below.

(ii) β-thalassemia intermedia. Thalassemia intermedia is a confusing concept. The most important fact to remember is that β-thalassemia intermedia is a description, and not a pathological or genetic

diagnosis. Patients with β-thalassemia intermedia have significant anemia, but are able to survive without blood transfusions. The factors that go into the diagnosis are:

- The degree to which the patient tolerates the anemia.
- The threshold of the physician to transfuse patients with thalassemia.

(iii) Thalassemia major. This is the condition of severe thalassemia in which chronic blood transfusions are needed (33). In some patients the anemia is so severe that death occurs without transfusions. Other patients could survive without transfusions for a while, but would have terrible deformities. While transfusions are life-saving in patients with thalassemia major, transfusions ultimately produce iron overload. Chelation therapy usually with the iron-binding agent, desferrioxamine (Desferal), is needed to prevent death from iron-mediated organ injury.

1.1.13 Pathophysiology

The pathophysiology of β-thalassemia is resonably well understood. The basic defect is a reduction in the output of β chain. This leads to imbalanced globin synthesis and to the production of an excess of a chains. Although HbF synthesis persists after birth to a varying degree in all the severe forms of β -thalassemia, its overall output is insufficient to compensate for the deficiency of HbA. In other words the output of β and γ chain is never sufficient to match that of α chains: unbalanced globin production and an excess of a chain is therefore the hallmark of β-thalassemia. Unbound α chains precipitate in the red cell precursors in the marrow and in there progeny in the peripheral blood, leading to defective erythroid precursor maturation and ineffective erythropoiesis, and a shortended red-cell survival. The resulting anemia causes an intense proliferative drive in the effective bone marrow, which leads to its expansion. This results in skaletal deformities and variety of growth and metabolic abnormalities. The anemia maybe further exacerbated by hemodilution cause by shuting of blood through the vastly expanded marrow, and also by splenomegaly due to entrapment of abnormal red cells in the spleen. The hyperplasia of the bone marrow leads to increased iron absorption and iron loading, often exacerbated by the need for regular blood

transfusion. This leads to progressive iron deposition in the tissues, organ failure and, if the iron is not removed, death.

1.1.14 Complication of β -thalassemia (34)

Complications caused by profound anemia:

- Pallor
- Easy fatigability
- Growth retardation
- High-output congestive heart failure
- Susceptibility to infection

Complications caused by chronic hemolysis:

- Hepatosplenomegaly
- Jaundice
- Premature biliary tract disease (due to bilirubin gallstones)
- Aplastic crisis (due to parvovirus infection)

Complications caused by enhanced ineffective erythropoiesis:

- Bony and growth deformities, including frontal bossing and maxilla-facial abnormalities

Complications caused by chronic iron overload:

- Endocrine dysfunction (e.g. diabetes mellitus)
- Cirrhosis
- Cardiac hemosiderosis

1.1.15 Iron overload

Iron overload of tissue, which is fatal with or without transfusion if not prevented or adequately treated, is the most important complication of β-thalassemia and is a major focus of

management (35). In patients who are not receiving transfusions, abnormally regulated iron absorption results in increases in body iron burden ranging from 2 to 5 g per year, depending on the severity of erythroid expansion (36). Regular transfusions may double this rate of iron accumulation. Although most clinical manifestations of iron loading do not appear until the second decade of life in patients with inadequate chelation, evidence from serial liver biopsies in very young patients indicates that the deleterious effects of iron are initiated much earlier than this. After approximately one year of transfusions, iron begins to be deposited in parenchymal tissues (37), where it may cause substantial toxicity as compared with that within reticuloendothelial cells (38, 39). As iron loading progresses, the capacity of serum transferrin, the main transport protein of iron, to bind and detoxify iron may be exceeded and a non–transferrin-bound fraction of plasma iron may promote the generation of free hydroxyl radicals, propagators of oxygen-related damage (38, 39). Although the body maintains a number of antioxidant mechanisms against damage induced by free radicals, including superoxide dismutases, catalase, and glutathione peroxidase, in patients with large iron burdens these may not prevent oxidative damage (38, 39).

In the absence of chelating therapy the accumulation of iron results in progressive dysfunction of the heart, liver, and endocrine glands (35). Within the heart, changes associated with chronic anemia are usually present in patients who are not receiving transfusions and are aggravated by iron deposition. In response to iron loading, human myocytes *in vitro* increase the transport of non–transferrin-bound iron, possibly thereby aggravating cardiac iron loading. Extensive iron deposits are associated with cardiac hypertrophy and dilatation, degeneration of myocardial fibers, and in rare cases fibrosis. In patients who are receiving transfusions but not chelating therapy, symptomatic cardiac disease has been reported within 10 years after the start of transfusions and may be aggravated by myocarditis and pulmonary hypertension. The survival of patients with β-thalassemia is determined by the magnitude of iron loading within the heart.

Iron-induced liver disease is a common cause of death in older patients and is often aggravated by infection with hepatitis C virus. Within two years after the start of transfusions,

collagen formation and portal fibrosis have been reported; in the absence of chelating therapy, cirrhosis may develop in the first decade of life. The extent of these processes may be underestimated if fewer than three cores of liver are sampled at biopsy. The risk of hepatic fibrosis is augmented at body iron burdens corresponding to hepatic iron concentrations of more than 7 mg per gram of liver, dry weight. As in cultured heart cells, in cultured hepatocytes the transport of non–transferrin-bound iron is increased, possibly aggravating iron loading *in vivo*.

The striking increases in survival in patients with β-thalassemia over the past decade have focused attention on abnormal endocrine function, now the most prevalent iron-induced complication in older patients. Iron loading within the anterior pituitary is the primary cause of disturbed sexual maturation, reported in 50 percent of both boys and girls with the condition. Furthermore, early secondary amenorrhea occurs in approximately one quarter of female patients over the age of 15 years. Even in the modern era of iron-chelating therapy, diabetes mellitus is observed in about 5 percent of adults. As the iron burden increases and iron-related liver dysfunction progresses, hyperinsulinemia occurs as a result of reduced extraction of insulin by the liver, leading to exhaustion of beta cells and reduced circulating insulin concentrations. Studies reporting reduced serum concentrations of trypsin and lipase suggest that the exocrine pancreas is also damaged by iron loading. Over the long term, iron deposition also damages the thyroid, parathyroid, and adrenal glands and may provoke pulmonary hypertension, right ventricular dilatation, and restrictive lung disease.

1.1.16 Hereditary hemochromatosis (HH)

HH is a disorder of iron metabolism characterized by increased iron absorption. Iron is progressively deposited in various tissues, particularly the liver, pancreas, heart, joints, and pituitary gland. Beside HH, there are other genetic causes of iron overload. Phenotypic expression of HH, which is variable, appears to depend on a complex interplay of the severity of the genetic defect, age,

sex, and such environmental influences as dietary iron, the extent of iron losses from other processes, and the presence of other diseases or toxins (e.g., alcohol). The rate of iron accumulation and the frequency and severity of clinical symptoms vary markedly; early complaints may include fatigue, weakness, joint pain, palpitations, and abdominal pain. Because these symptoms are relatively nonspecific, HH is often not diagnosed at this stage. The disease can ultimately lead to hyperpigmentation of the skin, arthritis, cirrhosis, diabetes mellitus, chronic abdominal pain, severe fatigue, hypopituitarism, hypogonadism, cardiomyopathy, primary liver cancer, or an increased risk of certain bacterial infections. Most of these advanced complications are also common primary disorders, and iron overload can be missed at this stage unless looked for specifically. The liver is usually the first organ to be affected and hepatomegaly is one of the most frequent findings at clinical presentation. In one study, noncirrhotic probands at clinical presentation reported weakness, lethargy, and loss of libido more frequently than probands with cirrhosis, but symptoms of abdominal pain were markedly more frequent in the cirrhotic patients. The proportion of patients with cirrhosis at clinical presentation has varied from 22 to 60 percent. Primary hepatocellular carcinoma is 200 times more common in HH patients but it rarely occurs without cirrhosis. Hepatocellular carcinoma has been reported to account for 30 to 45 percent of deaths among the HH patients seen in referral centers. In patients with this kind of cancer, the prevalence of HH ranges from 11 to 15 percent.

Diabetes mellitus is the major endocrine disorder associated with HH. The mechanisms responsible are still obscure, but iron deposition that damages the pancreatic beta cells and insulin resistance have been postulated. Hypogonadism also occurs and is caused primarily by a gonadotropin deficiency resulting from iron deposition at the pituitary or hypothalamic levels. Other endocrine disorders involving an effect of HH on the thyroid, parathyroid, or adrenal glands are rarely seen.

Cardiac manifestations include cardiomyopathy and arrhythmias. Congestive heart failure has been seen in 2 to 35 percent and arrhythmias are present in 7 to 36 percent of HH patients.

Increases in melanin lead to hyperpigmentation in 27 to 85 percent of patients. Loss of body hair, atrophy of the skin and koilonychia (dystrophy of the fingernails) may also occur.

Arthropathies are found in 40 to75 percent of patients and may affect the second and third metacarpal phalanges, wrist, shoulder, knees, or feet.

Symptoms of HH usually appear between ages 40 and 60 years with the onset normally later in women. This difference may relate to loss of iron with menstruation, pregnancy and lactation and to their lower iron intake relative to their iron needs. Men are more likely to develop clinical disease. Presenting signs and symptoms of HH also vary by sex, with women more likely to present with fatigue, arthralgia, and pigmentation changes and men presenting more often with symptoms of liver disease.

About 10% to 12% of people of European background are heterozygous for the condition (40). The number of people who are homozygous for the condition approaches one in three hundred. This makes hereditary hemochromatosis one of the most prevalent genetic conditions in the world. Interestingly, the clinical expression of the disorder is less than frequency calculations predict. Variable penetrance, prerhaps related to secondary genetic or environemental conditions must influence clinical manifestations (40).

After two decades of intensive research the genetic complexity of hereditary hemochromatosis (HH) is still unfolding. More than 20 years ago, HH was described as an autosomal recessive disorder associated with the human leukocyte antigen (HLA)-A3 complex. Subsequently, HH was linked to HLA-A on the short arm of chromosome 6. In 1996, Feder and colleagues identified a 250-kb region located more than 3 Mb telomeric from the major histocompatibility complex (MHC) on chromosome 6 that was identical by descent (IBD) in 85 percent of HH patients. In this region, they identified a gene related to the MHC class I family that they called HLA-H, but was subsequently named HFE (41). Classic hemochromatosis (HFE1), an autosomal recessive disorder, is caused by mutation in a gene designated HFE on chromosome 6p21.3 (42). Feder *et al* described two missense mutations of this gene (C282Y; 845 G→A producing a substitution of

cysteine for a tyrosine at amino acid position 282 and H63D; 187 C→G causing aspartate to substitute for histidine at amino acid position 63 in the HFE protein) that accounted for 88 percent of the 178 HH probands in their study. The HFE gene is located at 6p21.3- approximately 4.6 Mb telomeric from HLA-A, and covers approximately 10 kb. The HFE protein is a 343-residue type I transmembrane protein that associates with class I light chain β_2-microglobulin. The HFE protein binds to the transferrin receptor and reduces its affinity for iron-loaded transferrin by 5- to 10-fold. The C282Y mutation alters the HFE protein structure and β_2-microglobulin association, disrupting its transport to and presentation on the cell surface. The H63D mutation, in contrast, does not appear to prevent β_2-microglobulin association or cell surface expression, indicating that the C282Y mutation results in a greater loss of protein function than does H63D. The localization of the HFE protein in the crypt cells of the duodenum (the site of dietary iron absorption) and its association with transferrin receptor in those cells are consistent with a role in regulating iron absorption. The observation that HFE-deficient mice (HFE gene knockout model) develop iron overload similar to that seen in human HH provides evidence that the HFE protein is involved in regulating iron homeostasis (43). To date, 37 allelic variants of the HFE gene have been reported, but this study will focus on the C282Y and H63D mutations. In addition to C282Y and H63D, nine other missense mutations causing amino acid substitutions have been documented. In one, a substitution of a cysteine for serine at amino acid position 65 (S65C) has been implicated in a mild form of HH. A number of intronic polymorphisms have also been found. One polymorphism occurs within the intron 4 (5569 G→A) of the HFE gene in the binding region of the primer originally described by Feder *et al* (41). Nearly 90% of people who have hereditary hemochromatosis have the C282Y mutation in HFE (44). Only recently have investigators gained insight into the mechanism by which the mutation in HFE alters cellular iron metabolism. Iron in the circulation is bound to the protein, transferrin, which maintains it in a non-toxic state. Cells contain receptors for transferrin on their plasma membranes which mediate cellular iron uptake. Transferrin receptors bind iron-transferrin complexes which are taken into endosomes. Iron is separated from transferrin in the endosome, and is shuttled into the interior of the cell. The

iron-free transferrin (apotransferrin) is recycled into the circulation and is free to bind and transport additional iron atoms. The HFE protein associates with the transferrin receptor and prevents internalization of iron-transferrin complex into cells (45). The HFE protein, in effect, acts as a brake on cellular iron uptake. The C282Y mutation in HFE disrupts the folding of the protein (46). The mutant protein does not associate with the transferrin receptor and does not dampen iron uptake by cells.

The clinical features of hemochromatosis include cirrhosis of the liver, diabetes, hypermelanotic pigmentation of the skin, and heart failure. Primary hepatocellular carcinoma, complicating cirrhosis, is responsible for about one-third of deaths in affected homozygotes. Since hemochromatosis is a relatively easily treated disorder if diagnosed, this is a form of preventable cancer.

Juvenile hemochromatosis, or hemochromatosis type 2 (HFE2), is also autosomal recessive. One form, designated HFE2A, is caused by mutation in the gene encoding hemojuvelin, which maps to 1q21. A second form, designated HFE2B, is caused by mutation in the gene encoding hepcidin antimicrobial peptide (HAMP), which maps to 19q13 (42).

Hemochromatosis type 3 (HFE3), an autosomal recessive disorder, is caused by mutation in the gene encoding transferrin receptor-2 (TFR2), which maps to 7q22 (42).

Hemochromatosis type 4 (HFE4), an autosomal dominant disorder, is caused by mutation in the SLC11A3 gene, which encodes ferroportin and maps to 2q32 (42).

1.1.17 Zinc protoporphyrin (ZPP)

Zinc protoporphyrin (ZPP) is a normal metabolite that is formed in trace amount during heme biosynthesis. The final reaction in the biosynthetic pathway of heme is the chelation of iron with protoporphyrin. During periods of iron insufficiency or impaired iron utilization, zinc becomes an alternative metal substrate for ferrochelatase, leading to increased ZPP formation. Evidence suggests that this metal substitution is one of the first biochemical responses to iron depletion,

causing increased ZPP to appear in circulating erythrocytes (47). Because this zinc-for-iron substitution occurs predominantly within the bone marrow, the ZPP/heme ratio in erythrocytes reflects iron status in the bone marrow.

When there is not sufficient iron for erythropoiesis, zinc substitutes for iron in the structure of zinc protoporphyrin. The high levels of ZPP do not only help to recognize erythropoiesis under the circumstances of iron deficiency, but also demonstrate its clinical severity. A ZPP level of 60-80 μ mol/mol heme suggests a mild iron deficiency. Anemia and its clinical symptoms usually emerge when the levels of ZPP are >80 μmol/mol heme, and the level of ZPP is frequently higher than 100 μ mol ZPP/mole heme in the severe iron deficiency. Elevated ZPP levels may be also observed in lead intoxication (always higher than 100 μmol ZPP/mol heme), inflammatory diseases, myelodysplastic syndrome, thalassaemia carriers and haemoglobin E disease (47-49).

1.1.18 ZPP, Hereditary Hemochromatosis (HH) and iron overload in β-thalassemia (review literature)

Iron overload is a major clinical complication in β-thalassemia syndrome. It causes varieties of consequences including diabetes mullitus, growth retardation, myocardial heart disease, skin pigmentation and increased susceptability to bacterial infections. Several investigators had drawn their attentions to the mechanisms and outcome of iron overload in β-thalassemia. Pippard and co-workers observed the iron absorption and iron loading in β-thalassemia intermedia and found that iron absorption was strikingly increased with age-related progressive iron loading (36). They emphasized that by the time many of these patients reach the third or fourth decades of life, their total iron loads may be of a similar magnitude to those of transfusion-dependent β-thalassæmia homozygotes. They also suggested that if these patients were to be protected from cardiac, hepatic, and endocrine complications of iron overload in middle life, it would be necessary to reduce gastrointestinal iron absorption, starting from early childhood. In the study performed by Celeda and colleagues among non-transfused patients with thalassaemia intermedia, the evidence of increased

erythropoiesis as an etiology of iron overloading condion in thalassemia intermedia was revealed, confirming the previous observations (50). The patients they analysed did not receive any transfusions or iron therapy, but the iron absorption and the plasma iron turnover (PIT) were increased. After being transfused, the erythropoiesis and PIT were back to normal as well as the intestinal iron absorption. In the survey carried out by Olivieri *et al* (51) among the patients with HbE/β-thalassemia in Sri Lanka, variable but accelerated gastrointestinal iron absorption was implicated. They also pointed out that the iron loading condition associated with chronic transfusions in patients with HbE/β- thalassemia is similar to that observed in patients with homozygous β-thalassemia.

In Thailand, Torcharus and co-workers studied patients with β-thalassemia/HbE with age between 2-13 years old (52). In this study, they found that heavy transfusion led to iron overloading condition and desferrioxamine administering could improve the clinical well being of the patients. In addition, Sirithorn also observed that iron overloading condition could be seen in some cases of β-thalassemia major (53).

In the past, iron status could be evaluated by using erythrocyte protoporphyrin (EP) level determined by fluorometric technique. Pootrakul *et al* investigated the EP levels in normal control subjects, iron-responsive anemic subjects, and in patients with thalassemic diseases (54). They showed that normal subjects had EP of less than 80 micrograms/dl red blood cells, whereas all iron-deficiency subjects had EP of more than 80 micrograms/dl red blood cells. Among patients with β-thalassemia/HbE disease and among patients with HbH disease the elevated EP levels were also seen. Collectively, EP levels has been shown to correlate well with ZPP (55). Thus, Graham and co-workers studied the ZPP levels in thalassemia and found that the α-, β-thalassemia and HbE trait have higher ZPP levels than normal people. Interestingly, they found that α-thalassemia has ZPP higher than β-thalassemia and HbE trait (56). In Thailand, in the survey among blood donors, Tatu *et al* found positive correlation among ZPP, FEP and RDW. However, negative correlation was seen when ZPP levels were compared with that of Hb, Hct, MCV and MCH. They also observed that no

correlation was shown when ZPP was compared with SI, TIBC, TS, ferritin, RBC count and MCHC (55). The ZPP levels among thalassemic patients in Thailand has never been determined.

In the light of hereditary hemochromatosis (HH), the association of body iron status and HFE polymorphisms has been reported. Longo *et al* found no difference in serum ferritin, liver ion content (LIC) and age at the first chelation among patients with and without HFE mutations(57). However, they found that the higher frequency of H63D than the C282Y polymorphism in their series. More importantly, Melis *et al* found that β-thalassmia carriers who were homozygotes for H63D mutation had higher ferritin levels than β-thalassmia carriers with wild type (58).

The ZPP levels, HFE genetype and their associations with other iron parameters in β-thalassemia has never been explored at Maharaj Nakorn Chiang Mai Hospital.

1.1.19 Objectives

1. To determine HFE genotypes in northern Thai individuals
2. To determine zinc protoporphyrin (ZPP) levels, iron status in β-thalassemia patients at Maharaj Nakorn Chiang Mai Hospital
3. To evaluate correlation of ZPP and iron status in β-thalassemia patients at Maharaj Nakorn Chiang Mai Hospital