CHAPTER II
LITERATURE REVIEWS

1. Thalassemia

The thalassemias are genetic disorders of hemoglobin synthesis characterized by a reduction in the synthesis of particular globin chains. They constitute one of the most common single gene disorders occurring in the Mediterranean populations, the Middle East, the Indian subcontinent, Burma and in a line stretching from southern China through Thailand and the Malay peninsula into the island populations of the Pacific. They are also seen commonly in countries in which there has been immigration from these high-frequency populations.

1.1 α-Thalassemia

The α-globin genes are found on the distal segment (p13.1pter) of the short arm of chromosome 16. The gene cluster is made up of one embryonic α-globin gene, two α-globin genes, and four pseudogenes. Unlike α-thalassemia, which results most frequently from single nucleotide substitutions, α-thalassemia is predominantly caused by large deletions within the α-globin cluster. Common deletional mutations that cause α^0-thalassemia and α-thalassemia are seen in Figures 1 and 2, respectively [33]. The mild types α^+-thalassemia carriers have three out of four functional α-globin genes while homozygotes have two. In regions where both types of α-thalassemia are common, individuals can be found with the compound genotype of α^+-thalassemia /α^0- thalassemia , i.e. with only one functional α-globin gene. Such individuals have Hb H disease and although with a moderately severe anemia they have a relative normal life and only occasionally require blood transfusion [4, 34]. Prenatal diagnosis is therefore only usually required when both partners of a couple are carriers of α^0-thalassemia.

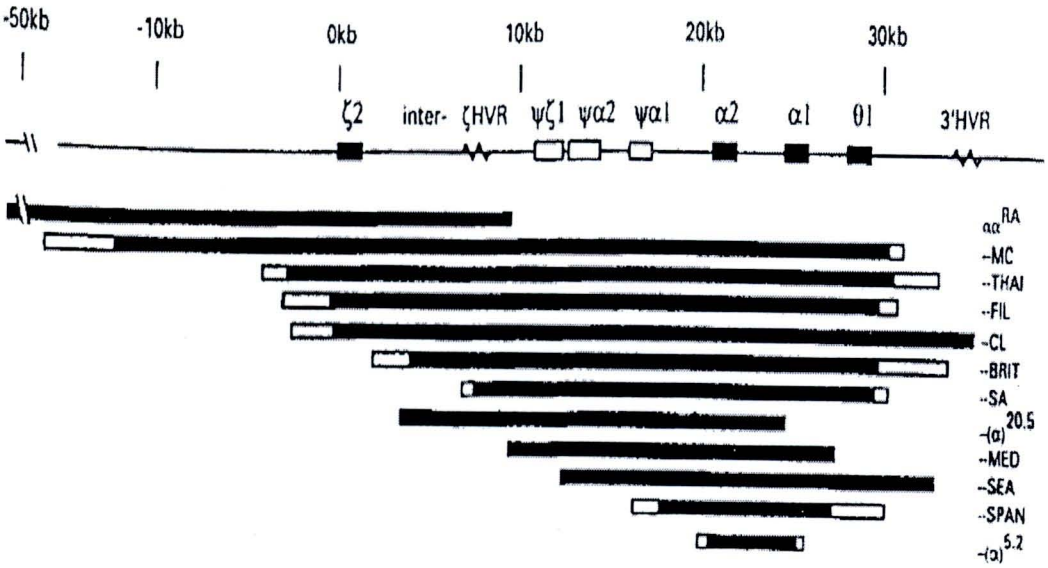

Figure 1 Mutations causing α^0-thalassemia. Above: the α-gene complex is shown. Below: the extent of each deletion is shown by a black bar. Regions of uncertainty for each breakpoint are shown by white boxes [33].

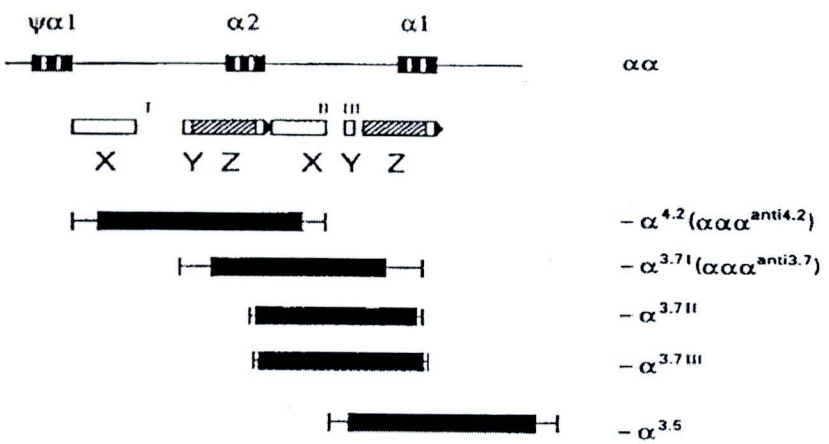

Figure 2 Mutations causing α^+-thalassemia. Above: the α-globin genes are shown with the duplication units divided into X, Y and Z boxes with regions of non-homology (I, II, III). Below: the extent of each deletion, represented by a black bar. The thin bar at the end of each thick bar denote the regions of uncertainty for the deletion breakpoints [33].

In Southeast Asia, the three common α-thalassemia mutations are the $--^{SEA}$, $--^{FIL(Filipino)}$, and $--^{THAI}$ deletions, which are all heterozygous α^0-thalassemia in the *cis* pattern. The $--^{SEA}$ (Southeast Asian type) deletion is 20.5 kb in length; both α-globin genes are deleted, but the embryonic α-globin gene is spared. It is commonly found in Hong Kong [35], Taiwan [36], Southern China [37], and Thailand [7, 38]. Homozygosity for this deletion is the most common cause of the Hb Bart's hydrops fetalis syndrome [39] and was the first discovery of the molecular basis for a human genetic disease [40]. $--^{FIL}$ and $--^{Thai}$ mutations are large α-thalassemia deletions in which all the ζ- and α-globin genes are deleted [41]. (Figure 1)

1.2 Hb Bart's hydrops fetalis

In normal fetal erythropoiesis (Figure 3), three embryonic hemoglobins predominate during embryogenesis: Hb Gower 1 (ζ2ε2), Hb Gower 2 (α2ε2), and Hb Portland 1 (ζ2γ2). As the production of embryonic ζ- and ε-globin chains switches to α-globin chains, the embryonic globin chains become almost undetectable by 6 to 7 weeks [42], and HbF (α2γ2) predominates as the major hemoglobin in the fetus from then on [43].

In fetuses with homozygous $--^{SEA}$ deletions usually survive into the third trimester of gestation, Hb Portland 1 (ζ2γ2) remains the major hemoglobin present; it is expressed at very low levels after the switch to α-globin synthesis in the fetus, with Hb Portland 2 (ζ2β2) being produced to a much lesser extent later. Although these Hb Portlands 1 and 2 can delivery oxygen to fetal tissues, the amounts of these hemoglobins are insufficient to keep pace with the remarkable growth and development of the fetus, especially during the third trimester of gestation. Ultimately the fetus would succumb to hypoxia and heart failure either in utero or shortly after birth [3, 33, 44].

In fetuses with homozygous $--^{FIL}$ or $--^{Thai}$ deletions where lacking the entire ζ-α-globin gene clusters, the hemoglobins present would be homotetrameric ε_4 and Hb Bart's (γ_4). As these hemoglobins have very high oxygen affinity, they are incapable of oxygen delivery to tissues in the rapidly growing embryo. These concept uses generally miscarry after succumbing to severe hypoxia early in gestation. Hence, they

do not present with the Hb Bart's hydrops fetalis syndrome [45]. However, fetuses that are compound heterozygous for $--^{SEA}$ and $--^{FIL}$ deletions, or $--^{SEA}$ and $--^{Thai}$ deletions may still develop the Hb Bart's hydrops fetalis syndrome.

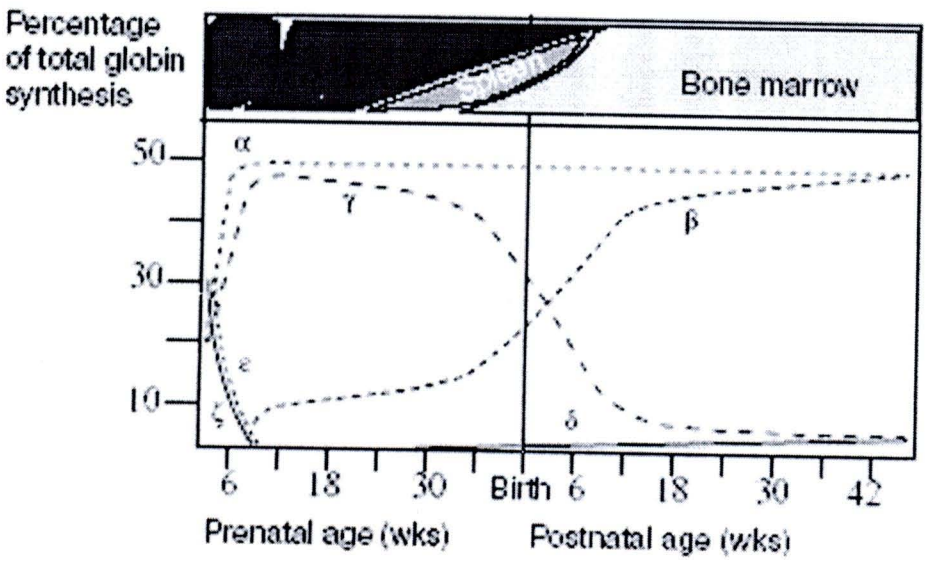

Figure 3 Normal fetal erythropoiesis. The sites of erythropoiesis during development and the different globins produced at each stage [46].

1.2.1 Fetal abnormallities

The affected fetuses have decreased hemoglobin synthesis because of the absence of α-globin genes. Most of the hemoglobin present, Hb Bart's (γ4) which is unable to deliver oxygen to tissues, is relatively unstable, and can precipitate and cause shortened red cell survival and possibly ineffective erythropoiesis [44, 47]. These fetuses are usually severely anemic. The circulating erythrocytes are markedly hypochromic and anisopoikiocytic. There are many nucleated erythroblasts in the peripheral blood, indicative of erythropoietic stress. Extensive extramedullary erythropoiesis is found in many organs and sites. This is the cause for the massive hepatomegaly. The spleen can also be enlarged.

The change from ζ- to α-globin synthesis in a fetus normally is completed by the sixth to seventh week postconception [42]. Thereafter, in fetuses lacking α-globin genes, severe anemia and fetal hypoxia would occur, which can

adversely affect subsequent organogenesis and fetal development. One effect of anemia and hypoxia, ie, placentomegly, can be detected by ultrasonography in some fetuses as early as the 10th week of gestation [48].

1.2.2 Maternal complications

There is an increased incidence of serious maternal complications in pregnancies. It is likely that the placentomegaly is one important causative factor. It was estimated that half of these women could die from complications resulting from these pregnancies if there was no medical care [3]. In a study of 46 women who were pregnant with affected fetuses, 61% developed hypertension during pregnancy, of whom half developed severe pre-eclamsia [18]. Polyhydramnios was present in 59% of the cases. Eleven percent suffered antepartum hemorrhage as a result of either unknown cause or placenta previa. Other less common complications included disseminated intravascular coagulation, renal failure, and pleural effusion. Oligohydramnios, abruption placenta, premature labor, and congestive heart failure have also been reported [19, 21, 49].

1.2.2.1 Placental pathophysiology in pre-eclampsia

The fetal part of the placenta and the fetal membranes separate the fetus from the endometrium of the uterus. There is interchange of substances such as nutrients and oxygen between the maternal and fetal blood streams through the placenta. The vessels in the umbilical cord connect the placental circulation with the fetal circulation.

The placenta, its function is to provide a place where the bloodstreams of the developing embryo and its mother can meet, separated only by thin membranes, for the exchange of nutrition, oxygen, and wastes. The placenta is a fetomaternal organ that has two components:

(1) a fetal portion that develops from the chorionic sac

(2) a maternal portion that is derived from the endometrium

The precise origin of pre-eclampsia remains elusive, but it is believed to be likely multifactorial. A certainty is the central role played by the placenta in its pathology [50-52]. A long standing hypothesis has been that pre-eclampsia develops as a consequence of some kind of immune maladaptation between

the mother and the fetus during the very first weeks of pregnancy, leading to a 2-step disorder progression that can be summarized as following:

In a first–asymptomatic–step, local aberrant feto-maternal immune interactions within the uterine wall lead to impaired tissue and arterial invasion by trophoblast cells. This results in failed transformation of the uterine spiral arteries and subsequently worsened placental perfusion. Chronic hypoxia or alternate periods of hypoxia/re-oxygenation within the intervillous space is expected to trigger tissue oxidative stress and increase placental apoptosis and necrosis [53, 54].

In a second step, the clinical disorder arises, when the maternal vascular and immune systems cannot handle any longer the increased shedding of placentally-produced debris and the aberrant expression of pro-inflammatory, anti-angiogenic and angiogenic factors, leading to a systemic endothelial cell dysfunction and an exaggerated inflammatory response [55-57].

However, the origin of pre-eclampsia might not be restricted to an alteration of trophoblast differentiation, but may also in some cases depend on an underlying maternal constitutional factors such as genetic, obesity, dysfunctional maternal clearance or inflammatory systems [58].

1.2.2.2 Detection of pregnancies at risk for pre-eclampsia

Many studies have demonstrated that there is a five-fold increased in the amount of fetal DNA in samples obtained from women with symptomatic pre-eclampsia; 381 genome-equivalents (GE)/ml, compared with 76 GE/ml in maternal plasma [31]. Fetal DNA levels are also elevated in women who will eventually develop pre-eclampsia [15]; thus, there is much interest in using measurement of cell-free fetal DNA as a predictive marker for the development of pre-eclampsia.

Lavine et al. [59] demonstrated that fetal DNA levels were significantly elevated in cases vs control as early as 17 weeks of gestation, long before the development of clinical symptoms of pre-eclampsia. Furthermore, in cases, a second phase of DNA elevation occurred approximately 3 weeks prior to the development of symptoms. They hypothesize that the initial elevation of DNA represents placental necrosis or apoptosis, whereas the later elevation may be due to subtle maternal end-organ dysfunction, resulting in impaired fetal DNA elimination.

The two-stage elevation suggests the possibility of measurement of fetal DNA both to screen for pre-eclampsia and to indicate impending disease.

1.3 Prenatal diagnosis of Hb Bart's hydrop fetalis

Prenatal diagnosis of Hb Bart's hydrop fetalis early in gestation is critical in order to avoid serious maternal complications later in pregnancy [45]. Usually, it is done by DNA-based diagnosis using amninocytes from amninocentesis or from chorionic villi sampling. In experience clinics, ultrasonography to measure cardiothoracic ratio or placental thickness is a reliable diagnosis tool.

In a previous reported, it was shown that in pregnancies with Hb Bart's hydrops fetalis syndrome, there are fetal erythrocytes in maternal circulation early in gestation that contain only embryonic ζ-globin chains, but not adult α-globin chains, that can be detected by immunofluorescence [60]. Development of various noninvasive prenatal diagnostic assays of globin gene mutations by examining circulating fetal DNA in maternal peripheral blood have been reported [29, 61, 62].

2. Circulating DNA in maternal peripheral blood

During pregnancy, the fetal and maternal circulation are separated by the placental membrane. However, a variety of evidence has pointed toward the incompleteness of this barrier to cellular trafficking. The presence of fetal nucleated cells have been demonstrated in maternal circulation and have been widely pursued as potential substrates for noninvasive prenatal diagnosis [63]. However, the rarity of such fetal cells in maternal blood has been a major obstacle to the routine application of this concept.

Although fetal DNA in maternal plasma has been shown to have many potential clinical applications, little is known about its biology. The possible origins of fetal DNA in the maternal circulation are from fetal blood cells and trophoblast [64]. The presumed source of the fetal blood cells is a continuous leakage of fetal cell across the placenta that is rapidly destroyed by maternal immune system, leaving fetal DNA remaining in the plasma. Fetal blood cells are formed initially in the yolk sac from 4 weeks' gestation [65], but do not circulate until the heart begins to beat at 5 weeks' gestation [66]. Fetal vessels are not present within the chorionic villous stroma until 8 weeks. It is unlikely that fetal blood elements would be able to escape into the

maternal circulation as early as 5 weeks' gestation. The most likely source of the Y-chromosome DNA sequence detected during weeks 5-8 is trophoblast. Trophoblast development in the early embryo is very rapid, and by 4 weeks, the lacunae of the trophoblast are filled with maternal blood into which knots of syncitial trophoblast may be shed directly. After 8 weeks' gestation, the relative contribution of fetal blood and trophoblast cells to the fetal DNA detected in maternal blood is unknown; it may change as the pregnancy advances. Bianchi et al. presented data indicating that there were no detectable fetal nucleated red blood cells at 16-20 weeks' gestation, compared with 11-12 weeks when analyzed for Y-chromosome DNA sequences [67].

Recently, it is observed that a large number of fetal cells undergo apoptosis in the maternal circulation [68]. Indeed, these apoptotic fetal cells are also observed in maternal plasma. These findings strongly suggest that disintegration of circulating fetal cells may liberate DNA from fetal nucleated cells into the maternal plasma. This might also explain the observations that levels of fetal cells and cell-free DNA are both elevated in women with pre-eclampsia and aneuploid pregnancies. However, one should also note that the half-life of maternal plasma fetal DNA is of the order of minutes after delivery [69]. The rapid turnover of maternal plasma fetal DNA clearly suggests that a large amount of fetal DNA is continuously liberated into the maternal plasma during pregnancy. Thus, with regard to the low frequency of nucleated fetal cells in maternal blood (0.0035-0.008%) [70], it is hard to explain how these rare fetal cells can contribute to such a high level of cell-free fetal DNA into maternal plasma (2.33-11.4% of total cell-free plasma DNA). This leads to the second hypothesis that cell-free fetal DNA might be directly released from fetal tissues to maternal plasma via the fetomaternal interface [71]. High levels of fetal DNA have been found in the plasma of women with advanced gestation age or pre-eclampsia. Interestingly, an increased level of apoptotic cells in fetal tissues has also been observed in these cases, suggesting the presence of fetal DNA in maternal plasma is a result of cell death in the fetal compartment. Alternatively, it is possible that maternal plasma fetal DNA is liberated from both fetal and maternal compartments. Indeed, Lo et al. [69] demonstrated that cell-free fetal DNA is cleared in distinct phases, suggesting cell-free DNA might be released by more than one mechanisms or sources. Recently, Zhong et al. [72] reported an interesting finding that the release of fetal DNA is not

coupled with the release of maternal DNA. Further work will be required to elucidate the detailed liberation mechanisms of cell-free fetal DNA in maternal plasma.

However, a possible use of fetal DNA is limited by the fact that only male (Y-linked) sequences can be clearly detected and, so far for such a limit, no randomized studies involving a broad number of subjects have been ever performed. The fetal component is a small fraction of the overall DNA concentration because the majority is of maternal origin [73], and so far only few papers reported the evidence of higher values of total DNA in pre-eclamsia patients [74] and a positive correlation with gestational age [75] expressed by means of ubiquitous GAPDH (Glyceraldehyde 3-phosohate dehydrogenase) genes.

Glyceraldehyde 3-phosphate dehydrogenase (abbreviated as GAPDH or less commonly as G3PDH) is an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. GAPDH is a key glycolytic enzyme that plays a pivotal role in energy production [76]. Indeed, GAPDH is present in both cytoplasm and nucleus indicating that it may shuttle between the two compartments. The nuclear-GAPDH appears to be involved in the initiation of one or more apoptotic cascades [77], play a role in DNA transcription/replication [78], and assist in maintenance of telomeres [79]. Additionally, GAPDH gene is often stably and constitutively expressed at high levels in most tissues and cells, it is considered a housekeeping gene. For this reason, GAPDH is commonly used by biological researchers as a loading control for western blot and as internal standards in quantitative polymerase chain reaction since it is generally assumed that their expression is unaffected by experimental conditions.

3. Real-time PCR

Real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e.

Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. The technique of real-time PCR have been applied to quantification of the PCR/DNA product as well as quantitation of cell-free DNA in maternal plasma.

In a real time PCR protocol, a fluorescent reporter molecule is used to monitor the PCR as it progresses. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification.

3.1 Real-Time PCR chemistries

3.1.1 TaqMan Probes

TaqMan probes depend on the 5'- nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moeity coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage [80].

3.1.2 Molecular Beacons

Like TaqMan probes, Molecular Beacons also use FRET to detect and quantitate the synthesized PCR product via a fluor coupled to the 5' end and a quench attached to the 3' end of an oligonucleotide substrate. Unlike TaqMan probes, Molecular Beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement. Molecular Beacons

form a stem-loop structure when free in solution. Thus, the close proximity of the fluor and quench molecules prevents the probe from fluorescing. When a Molecular Beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation [80].

3.1.3 Scorpions

With Scorpion probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed [80].

3.1.4 SYBR Green

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. SYBR Green is the most economical choice for real-time PCR product detection. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. However, detection by SYBR Green requires extensive optimization. Since the dye cannot distinguish between specific and non-specific product accumulated during PCR, follow up assays are

needed to validate results [80]. This SYBR Green method has been applied to the detection α^0-thalassemia in maternal plasma [30].

4. Abnormal hemoglobins

Abnormal hemoglobins or hemoglobin variants are a heterogeneous group of inherited disorders characterized by structurally abnormal Hb molecule. The genetic mutations including point mutations, deletions or insertions of the globin genes causing change in the amino acid structure of the polypeptide chains, result in conformational changes of the hemoglobin protein molecule [5]. In contrast, the thalassemias are reduced or absent synthesis of normal globin chains. The mutations of hemoglobin variants properties to various degrees. The properties altered include oxygen binding, molecular stability, easy of auto-oxidation, clinical or hematological effects. In heterozygotes, the β chain variant usually constitute about half of the total hemoglobin in the red cells and gives rise to significant change in the function of the red cell. In contrast, normal individuals have four α-globin genes. Accordingly, α-globin mutants usually compose about 25% of the hemolysate and the red cell function consequence to be milder than those produce by β chain variants.

4.1 Genetic basis of the hemoglobin variants

Abnormal hemoglobins result from mutations which change the sequence or number of nucleotides within the globin gene or from mispairing during meiosis. Genetic mutations including point mutations, deletions or insertions of the globin genes causing changes in the amino acid structure of the polypeptide chains, result in conformational changes of the hemoglobin protein molecule [5].

4.1.1 Single base substitution

The vast majority of hemoglobin variants are caused by single amino acid substitution in one of the globin molecules and are derived from single nucleotide substitutions in the corresponding gene. For example, Hb E results from a substitution of glutamic acid to lysine, due to a GAG to AAG mutation in codon 26 of the β-globin gene leading to partially activates a cryptic splice site towards the 3' end of exon 1, resulting in a proportion of abnormally spliced mRNA [81].

4.1.2 Elongated subunits: Termination errors

Rarely, elongated chain result when the terminator codon undergoes a mutation to a codon for an amino acid such as Hb Constant Spring ($\alpha_2^{171aa}\beta_2$). This variant has abnormally long α-chain carrying 31 extra amino acid at the C-terminal end of α-chain. Mutation of the UAA to CAA would result in a codon which codes for glutamine, and glutamine is precisely the amino acid in position 142 of the α^{CS} chain. The other elongated chain abnormalities include Hb Icaria (UAA→AAA), Hb Seal Rock (UAA→ GAA), Hb Koya Dora (UAA→ UCA) and Hb Pakse' (TAA→TAT) are synthesized in extreamly small quantities and play an important role in the pathology of the α-thalassemia syndrome [5].

4.1.3 Frame-shift mutations

Some mutations produce deletion or insertion of one or more amino acid residues. The globin chain being shortened in the former and elongated in the latter. The deletion arises from frameshift mutagenesis due to mis-pairing of two DNA strands within the same chromosome. For example, Hb Tak which is caused by dinucleotide (AC) insertion at termination codon of β-globin gene leading to the synthesis of an elongated β-globin chain with 157 instead of 146 amino acid residues. Hb Tak has increased oxygen affinity, heterozygosity for this variant is characterized by normal hemoglobin concentration and the absence of clinical manifestations [82, 83].

4.1.4 Fusion subunits

Small deletion, large deletion and insertions can result from non-homologous crossing over between a pair of chromosomes during meiosis. These are usually in frame. The fused globin arises through non-homologous crossing over between part of one chain on one chromosome and part of the other chain on the complementary chromosome. Hb Lepore, for example, contains normal α-chains and abnormal non-α chain. These non-α chains have a normal length but an abnormal sequence. The Lepore chain is a fusion or hybrid NH_2-δβ-COOH chain. Three different Lepore chains have been described, in which the switch from δ to β sequences occurs at different points of the sequence [84, 85]. Heterozygous Hb

Lepore should have the clinical features of β-thalassemia trait while homologous Hb Lepore syndrome should mimic β-thalassemia major.

4.2 Clinical outcome of hemoglobin variants

Hemoglobin variants may or may not cause clinical symptom depending on the site where the mutation occurs [86].

4.2.1 Changes in surface amino acids

Nearly all substitutions on the surface of the hemoglobin molecule, except Hb S, are harmless because most of these residues have no specific functional role. For example, Hb E [β26 (Glu→Lys)] the most common human hemoglobin variant in Southeast Asia, has no clinical manifestation either in heterozygotes or homozygotes. About one half of the known hemoglobin mutations are of this type and were only discovered accidentally or through surveys of large populations.

4.2.2 Changes in internally located amino acids

Changing an internal residue often destabilized the hemoglobin molecule. This can occur through the weakening of the heme-globin association or as a consequence of other conformational changes. Some abnormal globin chain that does not form a tetramer may aggregate with each other (known as "Heinz bodies") and adsorb hydrophobically to the erythrocyte cell membrane. The membrane permeability is thereby decreased causing premature cell lysis. Carriers of unstable hemoglobin therefore suffer from "hemolytic anemia" of various degree of severity. Some hemoglobin variants are rapidly degraded by proteolytic enzyme.

4.2.3 Changes in active site

Some defective subunit cannot bind oxygen because of a structural change near the heme group directly affects oxygen binding. For example, substitution of tyrosine for the histidine proximal or distal to heme stabilizes heme in ferric form (Fe^{3+}), which can no longer bind oxygen. The tyrosine side chain is ionized in this complex with ferric ion of the heme. Mutant hemoglobin characterized by a permanent ferric state of two of the heme are called "hemoglobin M". The letter M signifies that the altered chains are in the methemoglobin (ferrihemoglobin) form. These patients are usually cyanotic.

4.2.4 Changes at the $\alpha_1\beta_2$ contact

Mutations at the $\alpha_1\beta_2$ contact often interfere with hemoglobin quaternary structural changes. Some abnormalities at this subunit contact lead to the abnormal oxygen affinities. Most such hemoglobins have an increased O_2 affinity so that they release less than normal amount of O_2 in to the tissues. Individuals with such defects compensate for it by increasing the concentration of erythrocytes in their blood leading to secondary polycythemia. Some amino acid substitution at the $\alpha_1\beta_2$ interface results in a decreased oxygen affinity. Individuals carrying such hemoglobins are cyanotic.

4.3 Classification of Abnormal hemoglobins

Abnormal hemoglobin can be classified according to phenotypic expression into four overlapping group (Table 1). Most carriers are symptomless whereas the clinical important variants are rare and fall into two major groups; those that alter oxygen affinity with resulting polycythemia or methemoglobinemia and unstable hemoglobins that cause hemolytic anemia. There are a few variants which are synthesized ineffectively and have a thalassemia phenotype.

Table1 Diseases resulting from structural abnormal hemoglobin [81].

Hemolytic anemia
Hb S, Hb C
Unstable hemoglobins
Hereditary polycythemia
High-oxygen-affinity hemoglobins
Hereditary cyanosis
M hemoglobins
Low-oxygen-affinity hemoglobins
Thalassemia phenotype
Highly unstable hemoglobins
Chain-termination hemoglobin variants
Fusion-chain hemoglobin variants

4.3.1 Sickle cell disease

An adenine to thymidine substitution in codon 6 of the β-globin gene (GAG→GTG) specifies the synthesis of the sickle β-globin chain (β^S). Hb S ($\alpha_2\beta_2^S$) has the unique property of polymerizing when deoxygenated. Sufficient Hb S polymer within the erythrocyte evokes the cellular injury responsible for the phenotype of sickle cell disease. The result in reduced deformability of the red cell and seriously affect its passage through the microcirculation. In addition, the altered structural properties of sickle red cells lead to a chronic hemolytic anemia due to a shortened red cell life span.

4.3.2 Unstable hemoglobin (Heinz body anemia)

Certain abnormalities in the globin chain sequence produce a hemoglobin that is intrinsically unstable. When the hemoglobin destabilizes, it forms up into erythrocyte inclusions called Heinz bodies. These inclusions attach to the internal aspect of the red blood cell membrane and reduce the deformability of the cell and basically turn it into spleenfodder. The result is hemolytic anemia. All of these hemoglobins are rare; inheritance is autosomal dominant. Homozygotes have not been described. Examples of unstable hemoglobins are Hb Gun Hill, Hb Leiden, and Hb Köln.

4.3.3 High O_2 affinity

These hemoglobins tend to result when mutations affect the portions of the amino acid sequence that compose 1) the regions of contact between α and β chains, 2) the C-terminal regions, and 3) the regions that form the pocket which binds 2,3-DPG. The hemoglobin eagerly scarfs up the O_2 from the alveoli but then only stingily gives it up to the peripheral tissues. The kidney, always compulsively vigilant for hypoxia, cranks out the erythropoietin thinking that a few extra red cells might help out matters. Erythropoiesis then is stimulated, even though there is no anemia, and erythrocytosis (increased total body rbc mass, increased blood hemoglobin concentration, increased hematocrit) is the result. Examples of these include Hb Chesapeake and Hb J Capetown.

4.3.4 Low O_2 affinity

These hemoglobins are reluctant to pick up O_2 from the lung. The result is a decreased proportion of hemoglobin that is oxygenated at a given PO_2. The

remainder of the hemoglobin is, of course, deoxygenated and is blue. If the level of blue hemoglobin exceeds 5 g/dL in capillary blood, the clinical result is cyanosis, a bluish discoloration of skin and mucous membranes.

4.3.5 Methemoglobinemia

These hemoglobins are a special class of low O_2 affinity hemoglobin variants that are characterized by the presence of heme that contains iron in the ferric (Fe^{+++}) oxidation state, rather than the normal ferrous (Fe^{++}) state. These methemoglobins are all designated "Hb M" and further divided by the geographic site of their discovery, e.g., Hb $M_{Saskatoon}$ and Hb $M_{Kankakee}$. The affected patients have cyanosis, since the methemoglobin is useless in O_2 binding.

4.4 Abnormal hemoglobins found in routine services at Khon Kaen Univarsity

Eight hundred and thirty-two charaterized Hb variants have been described [87]. In Thailand, more than 30 hemoglobin variants have been reported. They are summarized in Table 2 [17]. Most of them are rare except Hb E and Hb Constant Spring which are two most common variants with thalassemic effects. Most mutations found in do not result in any abnormalities in properties or clinical manifestation [17], but they require differential diagnosis with other clinically relevant hemoglobinopathies.

4.4.1 α-Globin chain variants

4.4.1.1 Hemoglobin Q-Thailand

Hemoglobin Q-Thailand (Hb Q-T) [α74(EF3)Asp→His] caused by a point mutation at codon 74 (GAC-CAC) of the α1-globin gene on chromosome 16p with a leftward single α-globin gene deletion ($-\alpha^{4.2}$), has been found in individuals from China and Southeast asia [88, 89]. Heterozygous for Hb Q-T usually show slight red cell microcytosis because the mutation is invariably linked to ($-\alpha^{4.2}$). The co-inheritance of Hb Q-T and α-thalassemia 1 (SEA deletion) leads to a clinical phenotype of Hb Q-H disease which can present with chronic anemia associated with jaundice and hepatosplenomegaly. [89-91]. Additionally, this Hb variant is resulting from tetrameric assembly of the $\alpha^{Q\text{-}T}$ with the β^{A} globin chain

($\alpha^{QT}{}_2\beta^{A}{}_2$). Interaction of Hb Q-Thailand with Hb E leads to the formation of Hb QE ($\alpha^{QT}{}_2\beta^{E}{}_2$) [92].

4.4.1.2 Hemoglobin Siam

Hemoglobin Siam [α15(A13)Gly→Arg] (also known as Hb Ottawa) has been described originally in a Chinese male from Thailand [93]. This mutation is non-pathological α-chain variants characterized by base substitution, GGT(Gly)→ CGT(Arg) at codon 15 of the α1-globin gene. This mutation occur at the external surface of hemoglobin structure, they would not be expected to create any major disruption to the structure or alter function properties. Heterozygous Hb Siam is healthy, but interaction with α-thalassemia 1 resulting in moderate microcytosis and hypochromia [94], with a hematological profile typical of an α-thalassemia 1 heterozygote.

4.4.1.3 Hemoglobin Queens

Hemoglobin Queens [α34(B15)Leu→Arg] (also known as Hb Ogi) was first found in a 16 year old Korean girl, encountered at Queens Hospital Center, Queens, New York [95]. This mutation has been characterized by base substitution, CTG(Leu)-CGG(Arg) at codon 34 of the α1-globin gene. Later, it has been found in oriental races: Korean, Japanese, Chinese, Vietnamese and Thai [96-99]. Hb Queens is non-pathological α-chain variants, because the substitution of arginine for leucine would not be expected to create any major disruption to the structure and does not exhibit abnormal functional properties [97].

4.4.1.4 Hemoglobin Hekinan

Hemoglobin Hekinan is a non-pathological α-chain variant characterized by mutation at the α^{27} position of either α1- or α2-globin gene that changes glutamic acid to aspartic acid. Although resulting in the same amino acid substitution, the affected mutation on each α-globin gene is different, α^{27}: GAG-GAT for α1 gene and α^{27}: GAG-GAC for α2 gene [100]. It has been recognized as a rare Hb variant found mainly among the Japanese [101] and Chinese [102]. A sporadic case of Hb Hekinan heterozygote was also reported in a woman with

Chinese-Black descent from French Guyana [103]. The heterozygous form of this abnormal Hb reported so far was not associated with disease. Hb Hekinan is not separated well from Hb A and Hb E on HPLC and co-migrates with them on Hb electrophoresis. Interaction of the Hb Hekinan with the Hb E and α-thalassemia 1 has been reported [104].

4.4.1.5 Hemoglobin Beijing

Hemoglobin Beijing [α16(A14)Lys→Asn] is α-chain variant originally detected in a healthy Chinese adult [105]. It is caused by a mutation at codon 16 (AAG→AAT) of α_2-globin gene, resulting in a substitution of asparagine for lysine. Coinheritance of this abnormal Hb with α-thalassemia 1 and Hb E have a more severe hypochromic microcytosis with reduced MCV, MCH, MCHC and Hb levels. This association of Hb E and Hb Beijing in heterozygote state could lead to the formation of a new variant namely the Hb EBeijing ($\alpha^{BJ}{}_2\beta^{E}{}_2$) [106].

4.4.2 β-Globin chain variants

4.4.2.1 Hemoglobin S

Hemoglobin S [β6(A3)Glu→Val] is a hemoglobin variant, commonly known as sickle hemoglobin, carrying an amino acid substitution at position 6 of the β-globin gene (GAG→GTG), which leads to sunstitution of valine for glutamic acid. This Hb variant is one of the most commonly encountered Hb variants worldwide. It is present in the large number of people in sub-Saharan Africa, the Middle East and part of the Indian subcontinent. Heterozygous Hb S are no clinical associations, except in conditions of extreme oxygen deprivation. The homozygous state for the sickle cell gene is characterized by chronic hemolytic anemia, an increased propensity to infection, a number of complications due to chronic vascular occlusion, and acute exacerbations, or crises, which may take several different forms. Compound heterozygotes with hemoglobin sickle C disease result when the gene for sickle hemoglobin is inherited from one parent and the gene for hemoglobin C from the other. In general, the clinical manifestations of hemoglobin SC disease are very similar to homozygous sickle cell disease. In addition, co-inheritance of the gene for hemoglobin S and beta thalassemia, individuals with

sickle/β^0-thalassemia have a very severe disease essentially identical to homozygous sickle cell anemia. Most individuals with sickle/β^+-thalassemia have fewer problems with infections and spleen involvement, fewer pain episodes, and less organ damage [107].

4.4.2.2 Hemoglobin C

Hemoglobin C [β6(A3)Glu→Lys] is a hemoglobin variant carrying an amino acid substitution at position 6 of the β-globin gene (GAG→AAG), which leads to sunstitution of lysine for glutamic acid. It has been found mainly among West Africans and African Americans but rarely in Southeast Asians [108, 109]. Hb C migrates like Hb A2 and Hb E on alkaline electrophoresis. Hb C heterozygous have no anemia but usually have target cells on blood smear and may have a slightly low MCV. There are no other clinical problems. Compound heterozygotes with Hb SC disease have a sickle syndrome which is very similar to sickle cell anemia but generally less severe than in the SS form. Compound heterozygous for Hb C and β-thalassemia have moderate to severe anemia with splenomegaly and sometimes hypersplenism, similar to homozygous Hb C if the β-thalassemia allele is β^+. The clinical picture is more severe if the β-thalassemia allele is β^0. Coinheritance of Hb C and Hb Lepore, δβ-thalassemia, or Hb E leads to clinically mild disease comparable to Cβ-thalassemia [110].

4.4.2.3 Hemoglobin Tak

Hemoglobin Tak [β147 Term→Thr] was first reported in a Thai patient in 1971 by Flatz et al. [111] It is an abnormal Hb caused by the insertion of dinucleotide AC after codon 146, a termination codon of the β-globin gene, leadind to a synthesis of the abnormal β-globin chain with an extended 11 amino acid residue. This mutation is a mutant hemoglobin with high oxygen affinity resulting in erythrocytotic phenotype. Hb Tak heterozygotes are clinically asymptomatic and have 30-40% Hb Tak with normal or slightly high Hb level between 14-17% [111]. Compound heterozygotes between β-thalassemia and Hb Tak, however, with only Hb Tak and Hb F, are characteristically polycythemic which may be explained by the high oxygen affinity of the mutant hemoglobin [112, 113]. Patients are usually

brought to physicians because of looking plethoric or "cyanotic" suspected of other more prevalent polycythemic or cyanotic conditions such as congenital cyanotic heart disease. While compound heterozygous Hb Tak /Hb E were clinically asymptomatic [82].

4.4.2.4 Hemoglobin D-Punjab

Hemoglobin D-Punjab [β121(GH4) Glu→Gln] also know as Hb D-Los Angeles, is a β-globin chain variant resulting from a glutamic acid to glutamine substitution at codon 121 with a GAA→ CAA change at the DNA level. It is one of the most commonly encountered Hb variants worldwide. It is quite prevalent in Pakistan and northwest India [114], China [115], and Middle East countries [116]. Both heterozygotes and homozygotes are clinically and hematologically normal. Typical thalassemic indicies with hypochromic microcytosis were observed in compound Hb D-Punjab/β^{+}-thalassemia and Hb D-Punjab/Hb E [117]. In addition, compound heterozygotes for Hb S and Hb D-Punjab tend to have a severe clinical course and may be indistinguishable from Hb S homozygosity . It has been suggested that Hb S polymerization is facilitated by the presence of Hb D-Punjab [118].

4.4.2.5 Hemoglobin Korle-Bu

Hemoglobin Korle-Bu [β73(E17)Asp→Asn], also known as Hb G-Accra, is a non- pathological β-chain variant characterized by a GAT-AAT mutation at the codon 73 that changes aspartic acid to asparagine [100]. It has been recognized as a rare Hb variant in the population of West Africa [119], Guadeloupe [120], Mexico [121], and Ivory Coast [122]. A high incidence of Hb Korle-Bu was reported only in a rural district of Jamaica [123]. The heterozygous form of this abnormal Hb is not associated with disease, but its compound heterozygosity with Hb C is associated with moderate chronic hemolytic anemia with microcytosis and acceleration of crystal formation [124]. Compound heterozygous Hb Korle-Bu/Hb E with co-inheritance of α-thalassemia 2 ($-\alpha^{3.7}$) were presented with slight hypochromic microcytosis [2].

4.4.2.6 Hemoglobin Hope

Hb Hope [β136(H14)Gly-->Asp] was first identified by Minnichi et al. [125]. It is a β-globin chain variant characterized by GGT-GAT mutation at the β136 position that changes glycine to aspartic acid, which has been reported in diverse geographic location and in individuals of diverse ethnicities. This Hb variant is mildly unstable and has reduced oxygen affinity, but is generally innocuous clinically [126]. Heterozygous form of Hb Hope in Thai individuals showed normal hematological feature similar to several previous reports. This Hb variant constitutes between 40 and 50 % of the total Hb. However, many report showed that Hb Hope association with other hemoglobinopathies and thalassemia can cause clinical conditions [126-135].

4.4.2.6 Hemoglobin Pyrgos

Hemoglobin Pyrgos [β83(EF7)Gly→Asp] has a substitution of a charged amino acid (aspartic acid) for neutral one (glycine) at codon 83 by a GGC-GAC mutation. It was first reported in a three year-old Greek boy with Hb S [β6(A3)Glu→Val] [136], and later found in Japanese [137], Malian [138], Sicilian [139], Chinese [140] and Thai [82]. Heterozygous Hb Pyrgos is not associated with disease but homozygous or compound heterozygous states with other hemoglobinopathies and thalassemia can cause clinical conditions [82, 141, 142].

4.4.2.7 Hemoglobin J-Bangkok

Hemoglobin J-Bangkok [β56(D7)Gly→Asp] (also known as Hb J-Meinung). It is the β-globin chain variant characterized by GGC-GAC mutation at the β56 position that changes glycine to aspartic acid, which was first described in a Chinese-Canadian newborn [143] and found occasionally in Black Americans [144], Japanese [145], Taiwanese [146] and Thai [147]. This Hb variant is a non-pathological β-chain variant with a more negative charge than Hb A due to a substitution of aspartic acid for glycine at codon 56. Heterozygous Hb J-Bangkok is not associated with disease but homozygous or compound heterozygous states with other hemoglobinopathies and thalassemia can cause clinical conditions [146, 147].

5. Hemoglobin analysis

5.1 HPLC

With the advent of HPLC, chromatographic separations of hemoglobins can be achieved rapidly and reproducibily for routine use in screening of abnormal hemoglobins and for quantification of Hb A2, Hb F, Hb A, Hb S and Hb C. The hemoglobins with a net positive charge is separated into its components by their adsorption on to a negative charge stationary phase in the chromatographic column, followed by their elutions by mobile phase. The mobile phase is a liquid with an increasing concentration of caions flowing through the column, thus the adsorbed positive charged hemoglobin molecules are eluted from the stationary phase. The separated hemoglobin can be detected optically in the eluate, identified the type of hemoglobin by their elution time, and quantify them by computing the area of peak in the elution profile. This technique is less labour-intensive and can be used for quantification of many hemoglobins [17]. The retention times of Hb variants using cation exchange HPLC are shown in Figure 4.

5.2 Capillary Zone Electrophoresis (CE)

Capillary Zone Electrophoresis (Sebia Capillarys system) has been developed for Hb analysis. In free solution, briefly, charged molecules are separated according to their electrophoretic mobility in an alkaline buffer containing electrolytes that create an electro-osmotic flow. This new system is built with eight capillaries (17 cm x 100 μm) in parallel, allowing multiple and simultaneous sample analysis. One capillary can be used at least 3000 times. Sample dilution of 18 μl of whole blood with a hemolyzing solution is automatically carried out by the Capillarys®. The sample is then injected by aspiration at the anodic pole of the capillary. The hemoglobin separation is performed under high voltage. Direct detection is obtained at 415 nm at the cathodic pole of the capillary. Using an alkaline buffer, normal fractions and abnormal variants are detected from the cathode to the anode in the following sequence: C, A2E, S, D, G, F, A, Hope, Bart's, J, N, Baltimore and H [148] (Table 3).

Table 2 Abnormal hemoglobins found in Thailand [17]

Hemoglobin	%	Mutation	Base change	Location	Charge change	Hematology
Hb Anantharaj (Hb J Wuming)	rare	α11 Lys-Gln	AAG-CAG	external	1+decrease	Normal
Hb Siam (Hb Ottawa)	rare	α15 Gly-Arg	GGT-CGT	external	1+increase	Normal
Hb Queens (Hb Ogi)	rare	α34 Leu-Arg	CTG-CGG	α1β1/external	1+increase	Normal
Hb Thailand	rare	α56 Lys-Thr	AAG-ACG	external	1+decrease	Normal
Hb J Buda	rare	α61 Lys-Asn	AAG-AAC	external	1+decrease	Normal
Hb Mahidol (Hb Q-Thailand)	rare	α74 Asp-His	GAC-CAC	external	1+increase	Normal
Hb Suan Dok	rare	α109 Leu-Arg	CTG-CGG	internal	1+increase	Mild α-thal
Hb Constant Spring	1-8%	αC.t. elongation	TAA-CAA			Hb H/α-thal
Hb C	rare	β6 Glu-Lys	GAG-AAG	external	2+increase	Target cells
Hb Siriraj	rare	β7 Glu-Lys	GAG-AAG	external	2+increase	No anemia
Hb Malay	rare	β19 Asn-Ser	AAC-AGC	external	no charge	β-thal-like
Hb G Coushatta (G Hsin Chu)	rare	β22 Glu-Ala	GAA-GCA	external	1-decrease	Normal
Hb E	10-53%	β26 Glu-Lys	GAG-AAG	external	2-decrease	Microcytosis
Hb J Bangkok (J Meinung)	rare	β56 Gly-Asp	GGC-GAC	external	1-increase	Normal
Hb Pyrgos	rare	β83 Gly-Asp	GGC-GAC	external	1-increase	Inc O2 affinity
Hb New York (Kaoshiung)	rare	β113 Val-Glu	GTG-GAG	internal/α1β1	1-increase	No effect
Hb D Punjab (D Los Angeles)	rare	β121 Glu-Gln	GAA-CAA	external	1-decrease	Normal alone
Hb Dhonburi	rare	β126 Val-Gly	GTG-GGG	surface crevice	no charge	β-thal-like
Hb Cook	rare	β132 Lys-Thr	AAA-ACA	ext/nr.cavity	1+increase	unstable
Hb Hope	rare	β136 Gly-Asp	GGT-GAT	central cavity	1-increase	Normal
Hb Tak	rare	βC.t. elongation	+AC			Normal
Hb Lepore Washington-Boston	rare	δ87-β116 fusion	deletion			β-thal-like

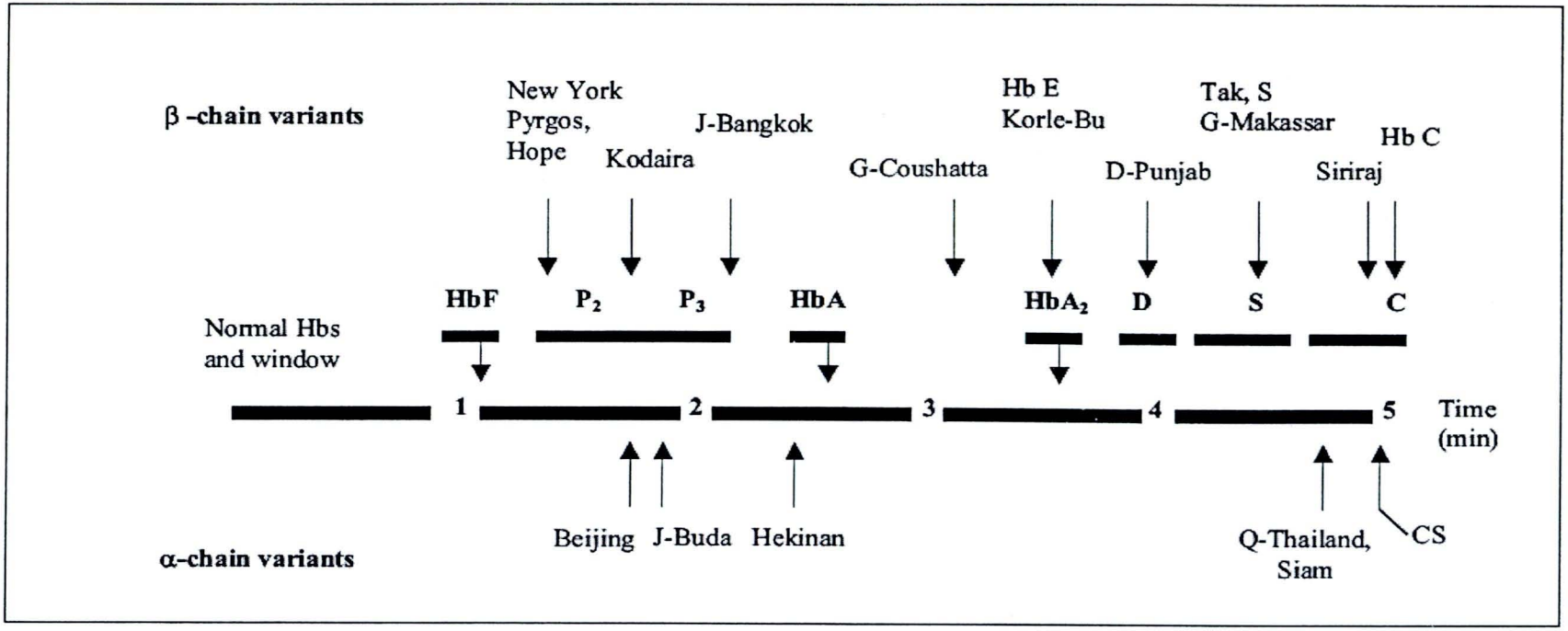

Figure 4 Retention times of Hb variants found in Thailand using cation exchange HPLC (VARIANT™ with the β-thalassemia Short Program System; Bio-Rad Laboratories). The values were normalized using 3.8 minutes as reference for Hb A_2 [149].

Table 3 The different migration zones of hemoglobin variants (called Z1 to Z15)

Zone	**Hemoglobin (Hb)**
Z1	Hb δA'2, Hb αA'2, Hasharon Hb A2 variant, Winnipeg Hb A2 variant, **Q-Thailand Hb A2 variant, other Hb A2 variants**
Z2	**Hb C, Hb Constant Spring**, Set if Hb A2 variant
Z3	**Hb A2**, Hb O-Arab
Z4	**Hb E**, Hb Koln, Hb A2 variants, degraded Hb C
Z5	**Hb S**, Hb Hasharon, degraded Hb O-Arab
Z6	**Hb D-Punjab (D-Los Angeles)**, Hb Osu Chistiansborg, Hb D-Culed Rabah, **Hb Lepore**, Hb G-Philadelphia, **Hb Korle-Bu (G-Accra)**, Hb Koln, Hb G-Taipei, Hb Winnipeg, Hb Setif, J-Toronto Hb A2 variant, J-Rovigo Hb A2 variant, degraded Hb E
Z7	**HbF, Hb Q-Thailand (G-Taichung)**, Hb Richmond, Hb G-San Jose, Hb Porto-Alegre, Hb Presbyterian, **Hb Tak**, degraded Hb S
Z8	Acetylated Hb F, Hb Atlanta
Z9	**Hb A**, Hb Phnom Penh, Hb Toulon, Hb Okayama, Hb Fortainebleau, Hb Raleigh, **Hb Hekinan**, Hb Camperdown
Z10	**Hb Hope**
Z11	Degraded Hb A, Hb J-Kaohsiung
Z12	**Hb Bart's**, Hb J-Providence, Hb J-Broussais, Hb J-Toronto, **Hb J-Meinung (J-Bangkok)**, Hb J-Mexico, Hb J-Baltimore, **Hb Pyrgos**
Z13	Hb J-Rovigo, Hb N-Baltimore
Z14	
Z15	**Hb H**