CHAPTER I

INTRODUCTION

1.1. Statement and significance of the problem

Hemoglobin (Hb) Bart's hydrops fetalis syndrome or homozygous α-thalassemia 1 are the most severe type of thalassemia syndrome caused by the deletion of all four α-globin genes. The total absence of α-globin chain causes the abnormality of hemoglobin production and defective in oxygen delivery. The fetus with this genotype suffers from severe anemia *in utero* which causes hypoxia, heart failure and consequently hydrops fetalis. Approximately 50% of Hb Bart's hydropic fetuses die during pregnancy at the age 23-38 weeks while the stillborn infants die within a few hours after birth (Weatherall & Clegg, 2001). The fetuses with homozygous α-thalassemia 1 have massive edema (hydrops), ascites and a large friable placenta. The hydropic fetuses gross show enlargement of liver with a spleen which may be normal or only slightly enlarged. The peripheral blood demonstrates severe erythroblastosis with accompanying reticulocytosis, target cells, hypochromia with fragmentation and decreased osmotic fragility (OF). The mean corpuscular volume (MCV) is often very high due to the large number of circulating nucleated red cells. Hemoglobin electrophoresis demonstrates a high levels of Hb Bart's (γ_4) with variable levels of Hb Portland ($\zeta_2\gamma_2$), and trace levels of Hb H (β_4) (Liebhaber, 1989). Moreover, this defective genotype is not only effect to baby, but may also adversely affect the health of the mother during pregnancy. Mothers of hydrops

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usually suffer from obstetric complication such as hydramnios, difficult vaginal delivery, pre-eclampsia, dystocia, post-partum hemorrhage due to a large placenta and the psychological burden for carrying a non-viable fetus to term (Wanapirak *et al.*, 1998). It was estimated that half of these women could die from complications resulting from these pregnancies if there is no proper medical care (Chui & Waye, 1998). Thus, Hb Bart's hydrops fetalis is a serious disorder and needs to be controlled.

The couples of α-thalassemia 1 is the most common cause of Hb Bart's hydrops fetalis, accounting for 60% to 90% of the cases (Chui & Waye, 1998). In South-East Asia, approximately 10% of the population are carriers of α-thalassemia 1; the severe two α-globin gene deletion in cis (--/αα). In Thailand, the incidence of α-thalassemia heterozygotes was 20-30% or more than 15 million of Thai population is considered (Wanapirak *et al.*, 1998). If no proper prevention is considered, in the future the thalassemia patients would be produced from these couples. Due to the increase of population migration across the world, the incidence of thalassemia heterozygote out side of the epidemic area expands gradually. Thus, thalassemia syndrome nowadays becomes a global health problem and need to be concerned. To control α-thalassemia disease, the α-thalassemia heterozygotes must be firstly identified, followed by genetic counseling. The risk of the high-risk couples for delivering a baby with Hb Bart's hydrops fetalis as well as fatal complications in the mother have to be informed (Tongsong *et al.*, 2001).

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To date, the detection of mutant gene using polymerase chain reaction (PCR) is the certainly method for diagnosis of α-thalassemia carriers. However, this method are not suitable for screening in large population due to the complexity of the method and high cost. However, Simple and rapid method, such as the immunological test, can overcome these problems.

It was demonstrated that abnormal hemoglobins, such as Hb Bart's and other embryonic ζ−globin chains, can be detected in α-Thalassemia 1 heterozygotes. (Ausavarungnirun *et al.*, 1998; Chui *et al.*, 1989; Chung *et al.*, 1984; Ireland *et al.*, 1993; Tang *et al.*, 1992). Therefore, detection of Hb Bart's or embryonic globin chains can be used as markers for identifying α-thalassemias 1 carriers. The development of classical hybridoma technology (Kohler & Milstein, 1975) leads to the production of monoclonal antibodies (mAbs) directed against proteins of interest. These specific monoclonal antibodies are utilized in many branches of the biological sciences due to its high specificity and infinitive production (Ball & Finlay, 1998; Dhanireddy *et al.*, 2004; Eisenbarth, 1981). Monoclonal antibodies against abnormal hemoglobins or globin chains have also been generated and applied for the detection of hemoglobins in screening of the area for thalassemia heterozygotes. An immunological diagnostic test kit for α-thalassemia has been developed based upon the use of mAb (Luo *et al.*, 1988). However, this diagnostic kit is not widely utilized due to its high cost.

Thalassemia syndromes are serious public health problem commonly seen across the kingdom of Thailand. However, no immunodiagnostic kits for thalassemia screening or diagnosis have so far been generated. In an attempt to develope immunodiagnostic kit for screening of α-thalassemia 1 heterozygotes, we aimed

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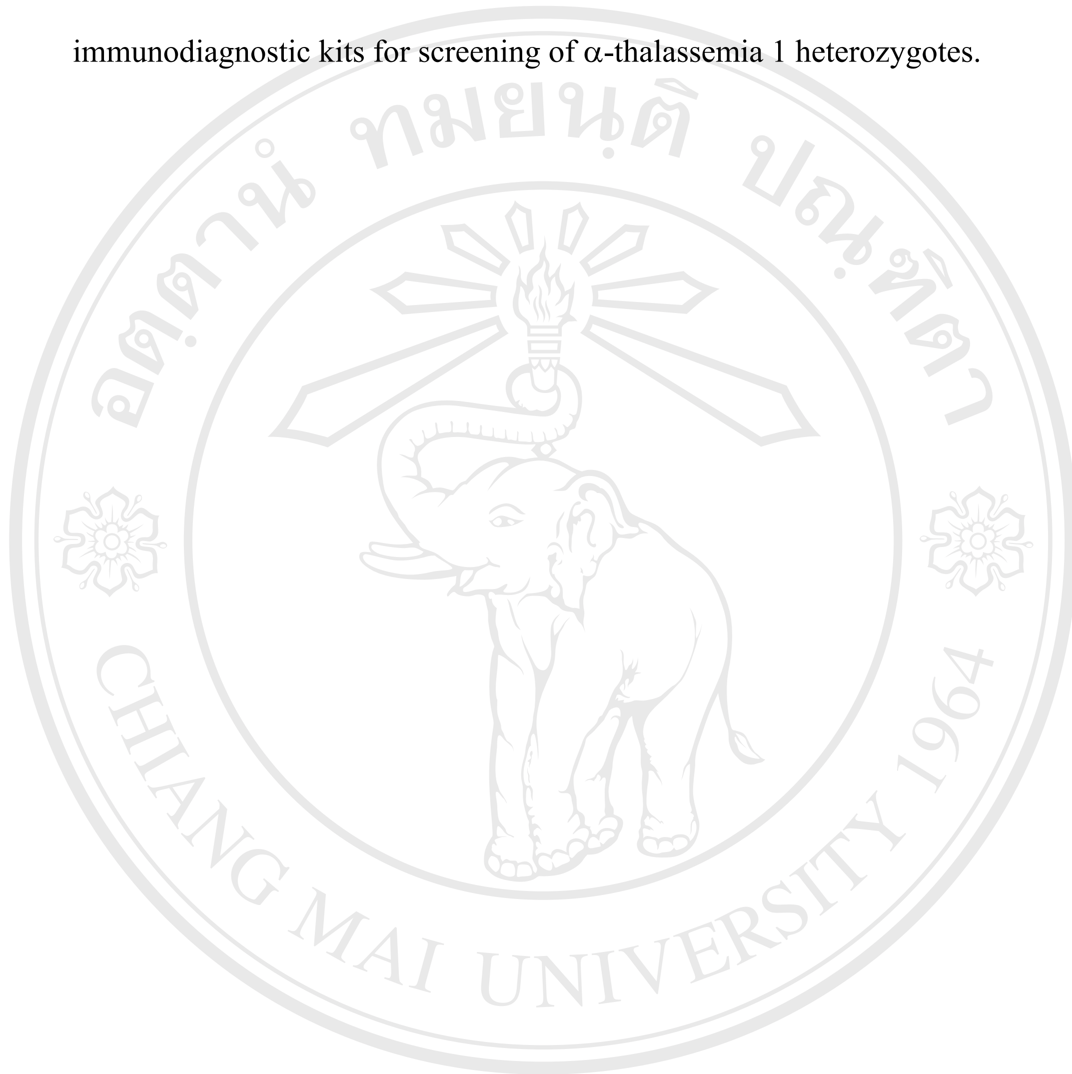
to produce mAbs against abnormal hemoglobins presenting in Hb Bart's hydrops fetalis. The generated mAbs can be applied for the development of simple immunodiagnostic kits for screening of α-thalassemia 1 heterozygotes.



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1.2. Literature reviews

1.2.1. Structure and function of hemoglobin

The erythrocytes in systemic arterial blood carry oxygen from lungs to body's tissues and return in venous blood with carbon dioxide to the lungs (Figure 1.1). Hemoglobin (Hb) is an iron-rich protein with the molecular mass of 64 kDa (Weatherall & Clegg, 2001), contained in all erythrocytes. It has been known for many years that Hb is the oxygen-carrier. The main function of Hb is to carry oxygen to body's tissue. In addition, Hb is also responsible for transport of some carbondioxide (approximately 10%) in blood by combining of carbon dioxide to N-terminal amino group of hemoglobin to form a carbamate ion (Kilmartin & Rossi-Bernardi, 1969).

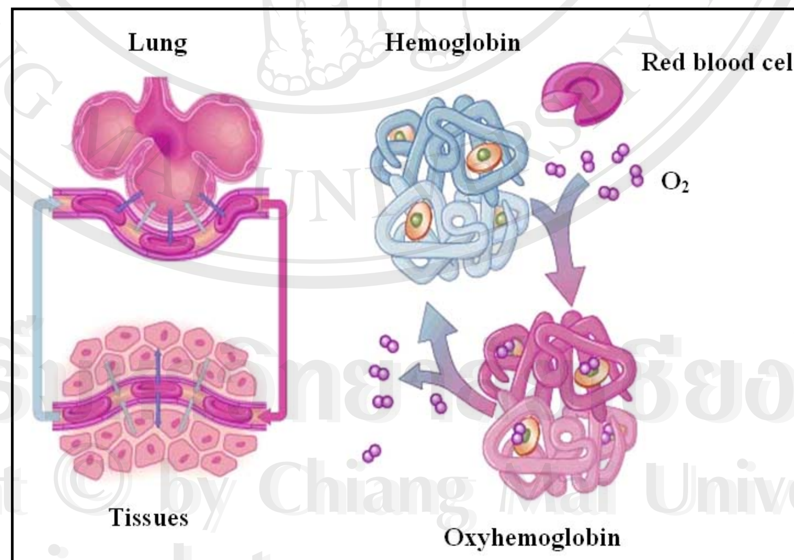

Figure 1.1. Function of hemoglobin. Hemoglobins is the body oxygen-carrier. It carries oxygen from lung and release oxygen to peripheral tissues.

(http://www.il.mahidol.ac.th/course/respiration/heme.jpg accessed 28 August 2006)

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Hb is polypeptide tetramer with globular structure. Hb molecule is composed of two pairs of globin chains, i.e. two α-like chain, α or ζ chains, and two β-like chains, β, δ, γ, δ or ε chains (Figure 1.2) (Rifkind *et al.*, 1984). The γ globin chain has two variants, $^{A}\gamma$ and $^{G}\gamma$, which differ by a single amino acid either alanine (A) or glycine (G) at position 136. All globin chains are arranged in a series of straight stretches in the α-helical regions configuration, joined by the short nonhelical regions. The interior spaces of the hemoglobin tetramer contain only non-polar amino acids, preserving a non-aqueous (hydrophobic) internal environment. Amino acids with polar side chains are exclusively directed at the external surface of the molecule. Each globin chain bind prosthetic heme group. The heme group and their Fe^{2+} atom are embedded within the hydrophobic interior space of the globin molecule. This nonaqueous environment is essential to preserve the heme in its biologically active Fe^{2+} form.

The α-globin gene, which is encoded in duplicate, and ζ-globin gene are located on the short arm of chromosome 16. The non-α globin genes including β, δ, γ and ε genes are located as a cluster on the short arm of chromosome 11 (Figure 1.3). Therefore, a diploid cell has four α-globin genes and two β-globin genes (Figure 1.4). The α-like and β-like globin chains consist of 141 and 146 amino acid residues, respectively. There is some sequence homology between the two chains (64 individual amino acid residues in identical positions), and the β chain differs from the δ and γ chains by 39 and 10 residues, respectively (Clarke & Higgins, 2000).

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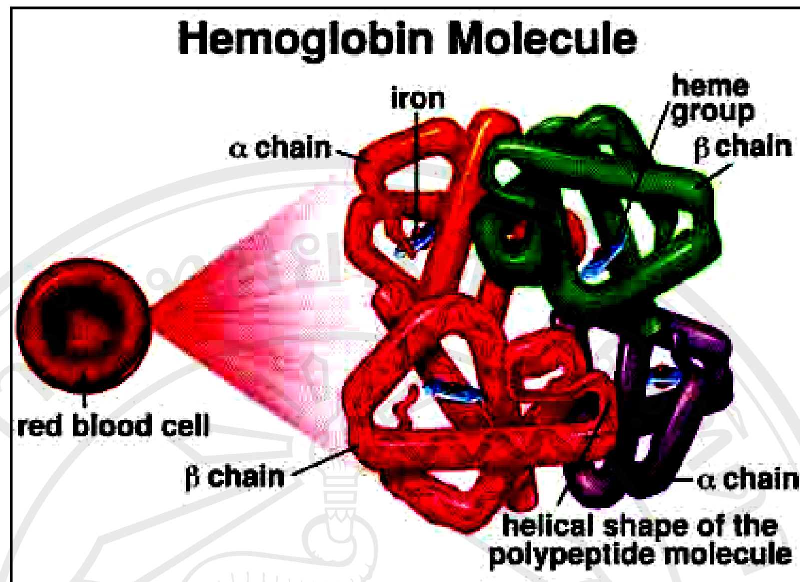

Figure 1.2. Molecular structure of hemoglobin. The molecule of Hb composes of four globin chains and prosthetic heme group within each molecule of the globins. *(http://www.kacr.or.kr/img/gene_expression/hemoglobin.jpg accessed 12 July 2006)*

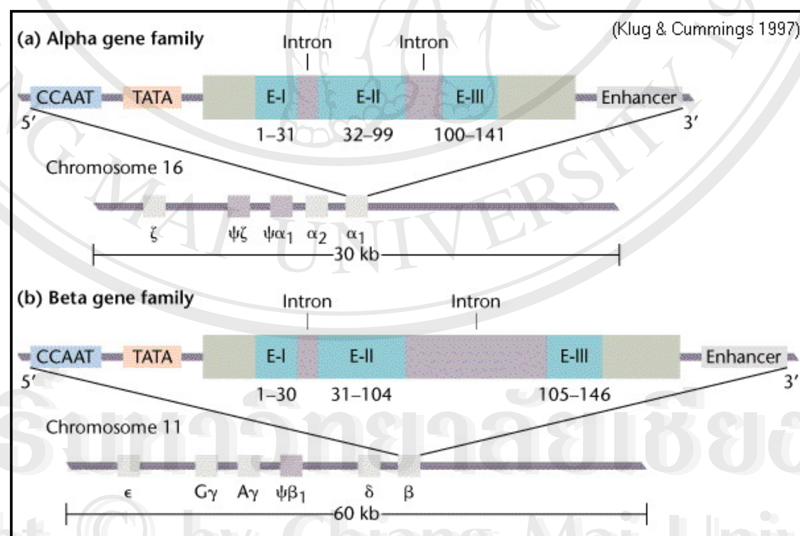

Figure 1.3. The chromosome of α and β globin genes family. (a) The α globin gene family including α and ζ are located on chromosome 16. (b) The β globin gene family including β, δ, $^{A}\gamma$, $^{G}\gamma$ and ε are located on chromosome 11.

(http://www.mun.ca/biology/scarr/Fg17_19.gif accessed 15 July 2006)

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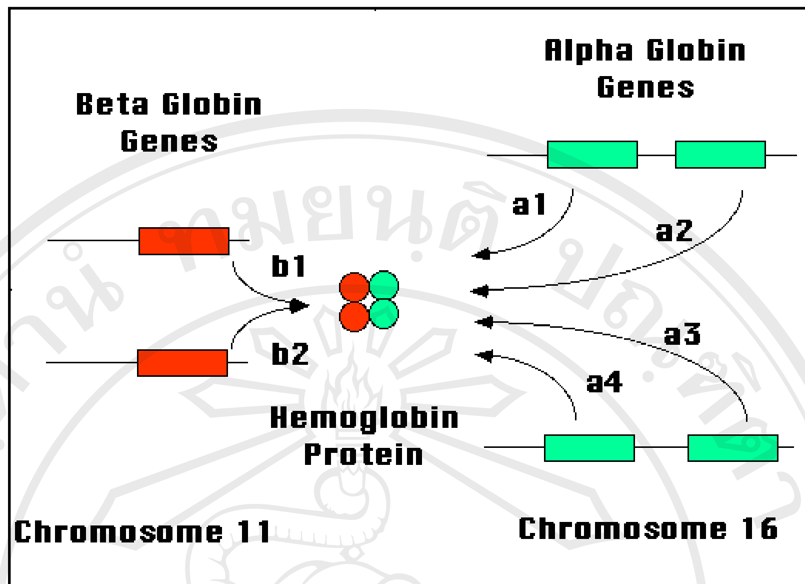

Figure 1.4. α and β globin genes in diploid cell. The diploid cell has four α-globin genes in chromosome 16 and two β-globin genes in chromosome 11. *(http://sickle.bwh.harvard.edu/globin_genes.gif accessed 15 July 2006)*

1.2.2. Hemoglobin synthesis and oxygen binding

Sixty-five percent of hemoglobin is synthesized in the erythroblast and 35% at the reticulocyte stage. Heme synthesis occurs largely in the mitochondria by a series of biochemical reactions beginning with the condensation of glycine and succinyl coenzyme A under the catalytic reaction of a rate-limiting enzyme δ-amino levulinic acid (ALA)-synthetase (Figure 1.5.). Pyridoxal phosphate (vitamin B_6) is a coenzyme for this reaction which is stimulated by erythropoietin and inhibited by heme. In the last step, protoporphyrin combines with iron to form heme (Figure 1.6A.). Each molecule of heme is combined with a globin chain (Figure 1.5.). A tetramer of four globin chains with its own heme group in a 'pocket' is then formed to make up a hemoglobin molecule. The normal Hb molecule possesses a cyclic

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affinity for oxygen bound to iron (Figure 1.6B.). When the first iron atom binds to oxygen, three remaining atoms of iron exhibit an increased affinity as more oxygen is bound. This phenomenon is known as the heme-heme interaction (Simmons & Fimls, 1989).

When fully saturated, each gram of Hb binds 1.34 ml of oxygen, the degree of saturation being related to the blood oxygen tension. At the usual tissue, the oxygen tension is around 40 mmHg and increases to complete saturation (100 mmHg) at the pulmonary alveolar capillaries. The oxygen affinity of Hb is directly influenced by a pH greater than 6.0 to 8.5. This is termed the Bohr effect. This effect is a benefit to the tissues when the decrease in pH resulting from carbondioxide production, the oxygen affinity will be lowers and aids in oxygen release. Oxygen affinity is also dependent upon the concentration of red cell 2,3-diphophoglycerate (2,3-DPG). This phosphate-containing enzyme combines reversibly with deoxygenated hemoglobin, decreasing the affinity of hemoglobin for oxygen without disturbing heme-heme interaction or the Bohr effect. The oxygen-dissociation curve of hemoglobin is sigmoid in configuration (Figure 1.7.).

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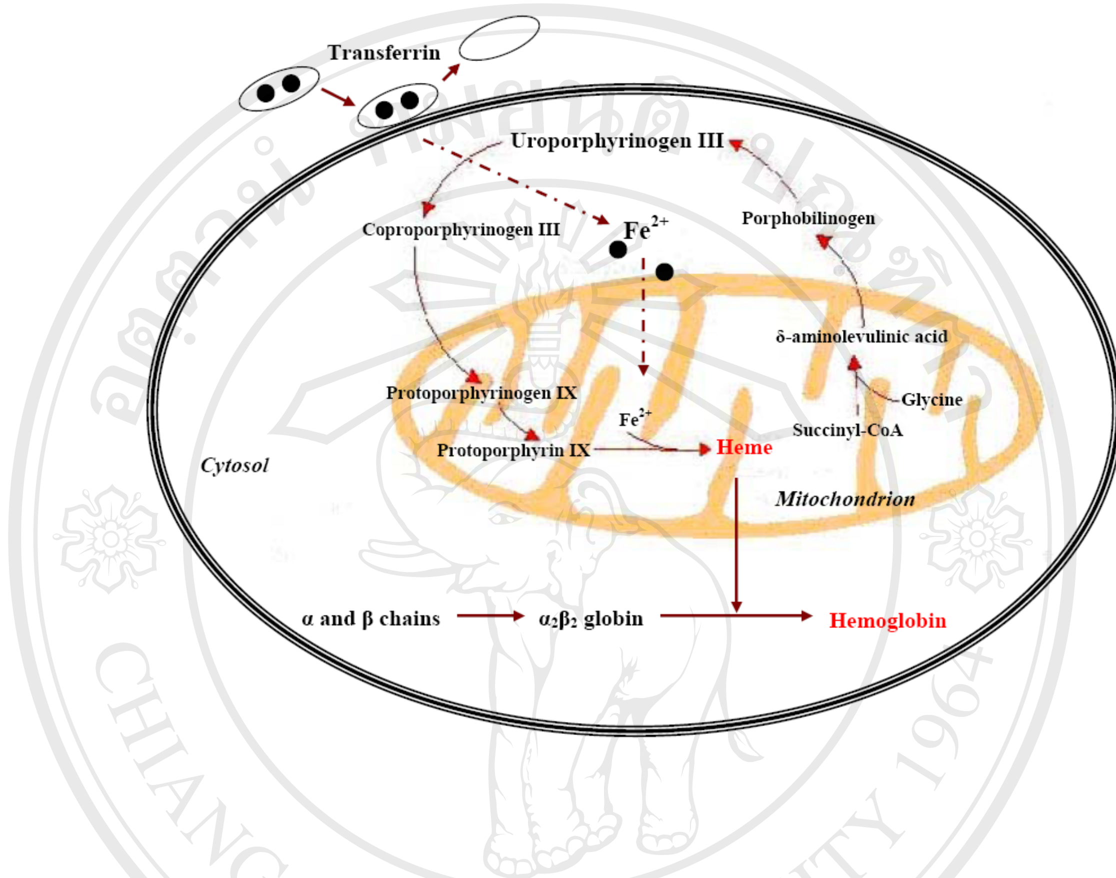

Figure 1.5. Hemoglobin synthesis in the developing red cell. The mitochondria is the main site of heme synthesis, iron is supplied from circulating transferrin and globin chains are synthesized in the cytosol. Four globin chains contained heme group are combines with each other to form the hemoglobin tetramer in cytosol of the red cell. *(Adapted from http://edoc.hu-berlin.de/dissertationen/xie-jing-2003-12-15/HTML /xie_html_3a64873a.gif accessed 4 August 2006)*

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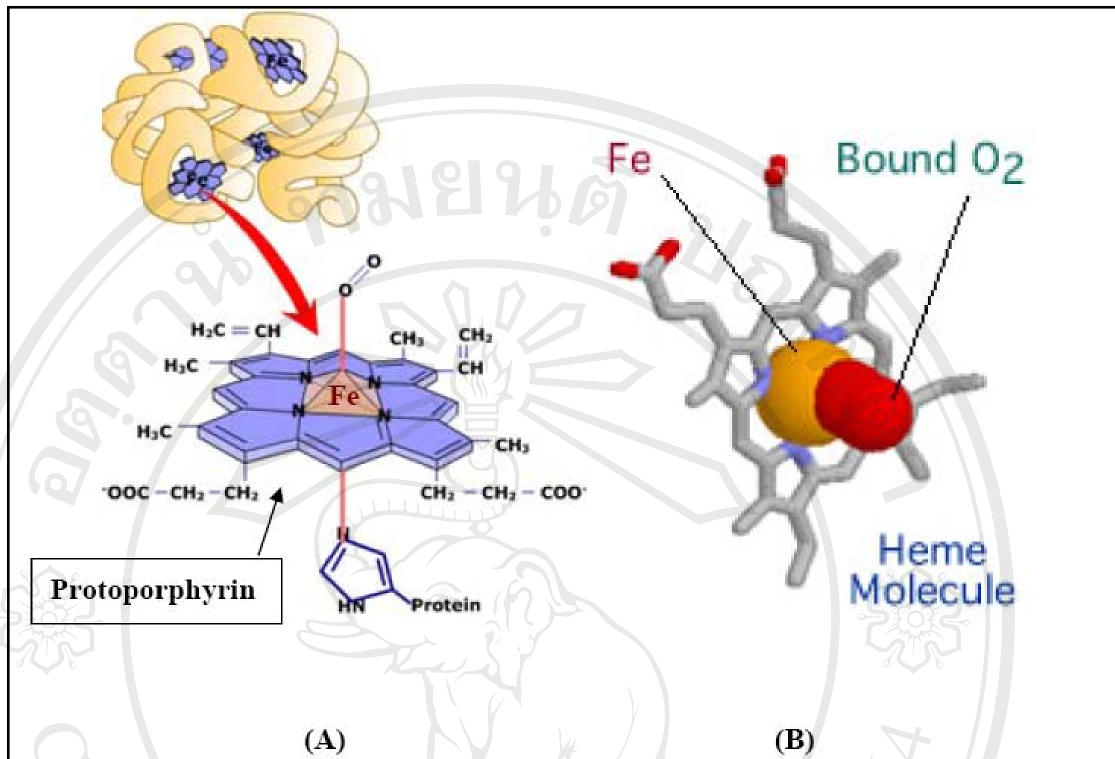

Figure 1.6. Molecular structure of heme. (A) The molecular structure of heme that composes of protoporphyrin and iron atom, (B) Three dimensional structure of heme, the iron atom is played the importance role in oxygen binding.

[(A) http://www.il.mahidol.ac.th/course/ecology/picture/heme.jpg, (B) http://dwb.unl.edu/Teacher/NSF/C05/C05Images/heme-O2.jpg accessed 16 August 2006]

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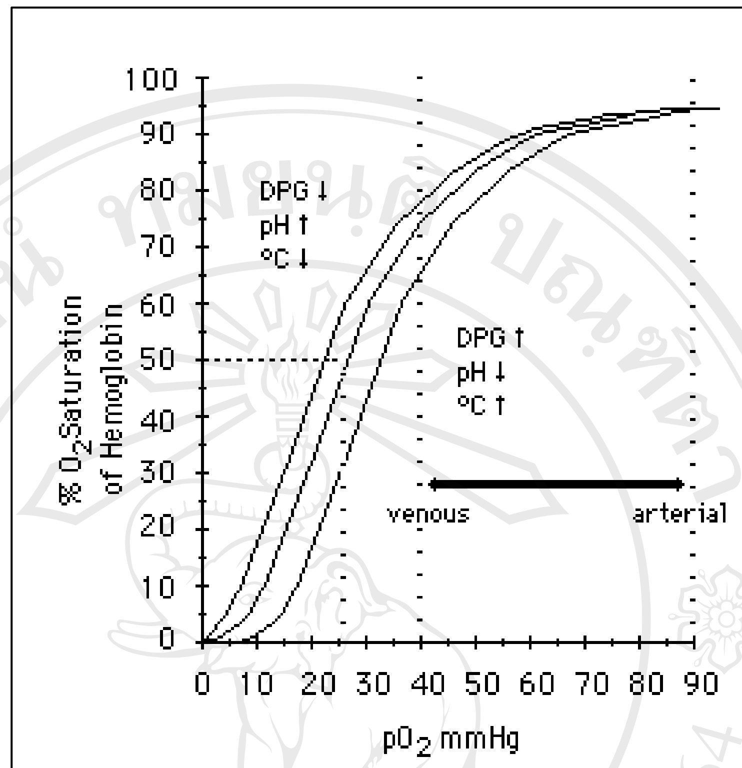

Figure 1.7. Hemoglobin oxygen dissociation curve. The change in pH, temperature and red cell 2,3-DPG level are effects on oxygen affinity of Hb. *(http:// www.med-ed.virginia.edu/courses/path/innes/images/nhgifs/hemoglobin2.gif accessed 25 August 2006)*

1.2.3. Developmental change in hemoglobin

The earliest globin chains in the embryo are the zeta (ζ) which is an α-globin family and the epsilon (ε) which is similar to the gamma (γ) chain. Hb Gower 1 ($\zeta_2\varepsilon_2$) is the major hemoglobin of embryos of less than 5 to 6 weeks of gestation (Figure 1.8.). Hb Gower 2 ($\alpha_2\varepsilon_2$) has been found in embryos with gestation age as young as 4 weeks and is absent in embryos older than 13 weeks. Hb Portland ($\zeta_2\gamma_2$) is found in young embryos but persist in infants with homozygous

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α-thalassemia (Williams *et al.*, 1991). Synthesis of the ζ and ε chains decrease as that of α and γ chains increase. This progression occurs about the time that the liver replaces the yolk sac as the main site of erythropoiesis. Hb F ($\alpha_2\gamma_2$) is also present in very young embryos and is the major hemoglobin of fetal life. The Hb F is constitutes 90 to 95 percent of the total hemoglobin in the fetus until about 34 to 36 weeks of gestation (Figure 1.8.). Synthesis of Hb A can be demonstrated in fetuses as young as 9 weeks of gestation (Kazazian & Woodhead, 1973; Thomas *et al.*, 1960). In fetuses of 9 to 21 weeks of gestation, the amount of Hb A increases from 4 to 13 percent of the total hemoglobin. After 34 to 36 weeks of gestation, the amount of Hb A rise further, while that of Hb F decreases. The amount of Hb F in blood varies in term infants from 53 to 95 percent of total hemoglobin (Armstrong *et al.*, 1963; Kirschbaum, 1962). The mean synthesis of Hb F in term infants was found to be 59 ± 10 percent of total hemoglobin synthesis as assessed by ^{14}C-leucine uptake (Bard, 1974).

The fetal hemoglobin concentration in blood decreases after birth by approximately 3 percent per week and is generally less than 2 to 3 percent of the total hemoglobin by 6 month of age. This rate of decrease in Hb F production is closely related to the gestational age of the infant and does not appear to be affected by the changes in environment and oxygen tension that occur at the time of birth (Bard, 1973).

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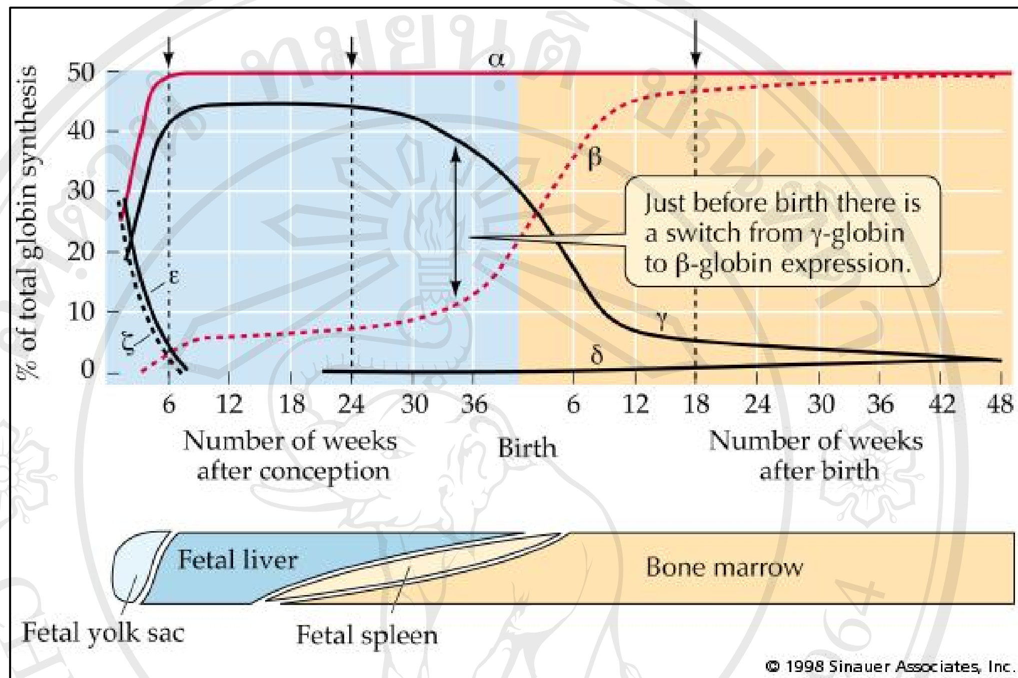

Figure 1.8. Developemental change in globin chains and production organs from fetus to adult. During development of fetus, the globin chains has been switched into several types by various organs and predominately synthesized the α and β chain that produced by bone marrow after birth. *(http://www.mie .utoronto.ca/labs/lcdlab/biopic/fig/14.10.jpg accessed 20 August 2006)*

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1.2.4. Thalassemia

1.2.4.1. Introduction to thalassemia syndromes

Thalassemia syndrome is a group of hereditary disorders of hemoglobin synthesis characterized by the reduction or absence of one or more of the globin chains. All type of thalassemias are considered quantitative hemoglobin diseases (Hartwell *et al.*, 2005). Thalassemia can be categorized into three classes: major, intermediate and minor according to the severity of the symptoms. The two main syndromes (thalassemia major) are α- and β-thalassemia which involve homozygous genetic defects in the α-globin and β-globin chain productions, respectively. β-thalassemia and sickle cell anemia have wide distribution in tropical areas due to natural selection by malaria (Weatherall & Clegg, 2001). α-thalassemia is the most common found in Southeast Asia and Africa. Related thalassemia minors or carriers are α-thalassemia 1 (2 out of 4 globin gene deletion) and α-thalassemia 2 (1 out of 4 globin gene deletion). α-thalassemia 1 has insignificant but observable anemia while α-thalassemia 2 is a silent carrier without any symptoms shown. Compound heterozygtes of α-thalassemia 1 and α-thalassemia 2 result in Hb H disease that is composed of β_4 chains instead of $\alpha_2\beta_2$ chains as in normal Hb A. Hb H disease is a relatively mild form of thalassemia. However, combination of Hb H with Hb CS (Hemoglobin Constant Spring), a type of hemoglobin variant of the globin gene that has an elongated α-globin chain with 31 extra amino acids, is severe and blood transfusion may be necessary. Homozyous α-thalassemia 1 (Hb Bart's hydrops fetalis) produced Hb Bart's which consists of γ_4 chains instead of $\alpha_2\gamma_2$ as in normal Hb F. Unborn infants with Hb Bart's hydrops fetalis normally die just before birth or within a short time after birth, this will be described in detail in 1.2.4.3.

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The incidence of thalassemia syndrome is usually found accountably in malarial epidemic areas (figure 1.9.). To date, the increase in population migration all over the world results in expanding of the thalassemia heterozygotes out side of the epidemic area. Under improper prevention, in the future the thalassemia patients will be increased from these thalassemia heterozygotes (Hofstaetter *et al.*, 1993). Thus, the thalassemia syndrome is increasingly becoming the health problem of the world and great concerns are required.

Figure 1.9. Incidence of thalassemia syndrome worldwide. The global distribution of thalassemia syndrome is are represented in the orange-color areas. *(http://www.abanet.it/fondazioneberloni/images/mondo.gif accessed 6 June 2006)*

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1.2.4.2. Alpha thalassemia

The α-thalassemia is a result of the decrease in production of normal α globin chains. As mentioned in 1.2.1, α globin proteins are synthesized by four α globin genes on chromosome 16 (two α globin genes on each chromosome). Deletion of one or more α genes results in the reduction of α globin chain production. Molecular studies have demonstrated that the most common form of α-thalassemia is cause by the deletions of α-globin gen cluster. Less commonly, point mutation in regulatory genes will also result in a decrease or absence of α globin chains production. About 41 such mutations and small deletions/insertions have been reported (Weatherall & Clegg, 2001). Loss of one α gene (termed α-thalassemia 2) may result in an MCV at the low normal to slightly low range. The amount of reduction in α globin produced will depend upon the specific deletion. For instance, with the $\alpha^{-3.7}$ deletion (the loss of rightward 3.7 kb of DNA), there is a compensatory increase in the production of α globin by the remaining α gene (Liebhaber & Cash, 1985; Tang *et al.*, 1992). This produces a silent carrier. With the $\alpha^{-4.2}$ deletion (the loss of leftward 4.2 kb of DNA), there is a greater loss of α gene production, however, the patient is clinically unaffected. If two in-cis α-globin genes are deleted (α- thalassemia 1), the MCV will almost always be reduced. There also may be a minor reduction in the MCHC, the erythrocyte count may be in the high normal range or slightly increased, while the RDW remains in the normal range (unless there is a co-existing iron deficiency). The deletion of both α globin genes in one chromosome or α-thalassemia 1 are variable in sizes from rather small (5.2 kb) to those which remove the entire cluster ($--^{FIL}$, $--^{THAI}$ and a newly described deletion of >47 kb in a Northern European family). The two most common deletions, ($--^{SEA}$)

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and (--^{MED}), occurs in Southeast Asia and the Mediterranean Basin, respectively. These two types of deletion (--^{SEA} and --^{MED}) are approximately the same size (20-30 kb) and remove both α globin genes (--) but spare the functional ζ2 gene (Figure 1.10) (Wilkie *et al.*, 1990). A neonate with α-thalassemia 1 have small amount of hemoglobin Bart's (γ_4) and may have a minute amount of ζ globin chain if their genotype is --^{SEA} or --^{MED} deletions (Tang *et al.*, 1992).

Alpha thalassemia can be categorized into three classes following to the clinical manifestations: I. α-thalassemia minor or carrier is composed of heterozygous α-thalassemia 1 (--/αα) or α-thalassemia 2 (-α/αα) and homozygous α-thalassemia 2 (-α/-α), II. Hb H disease is composed of compound heterozygous of α-thalassemia 1 and α-thalassemia 2 (--/-α) or heterozygous α-thalassemia 1 with Hb CS (-/αα^{CS}) and III. α-thalassemia major is composed of homozygous α-thalassemia 1 (--/--) or Hb Bart's hydrops fetalis (Cohen *et al.*, 2004), the most severe type of α-thalassemia (Wanapirak *et al.*, 1998).

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(A)

(B)

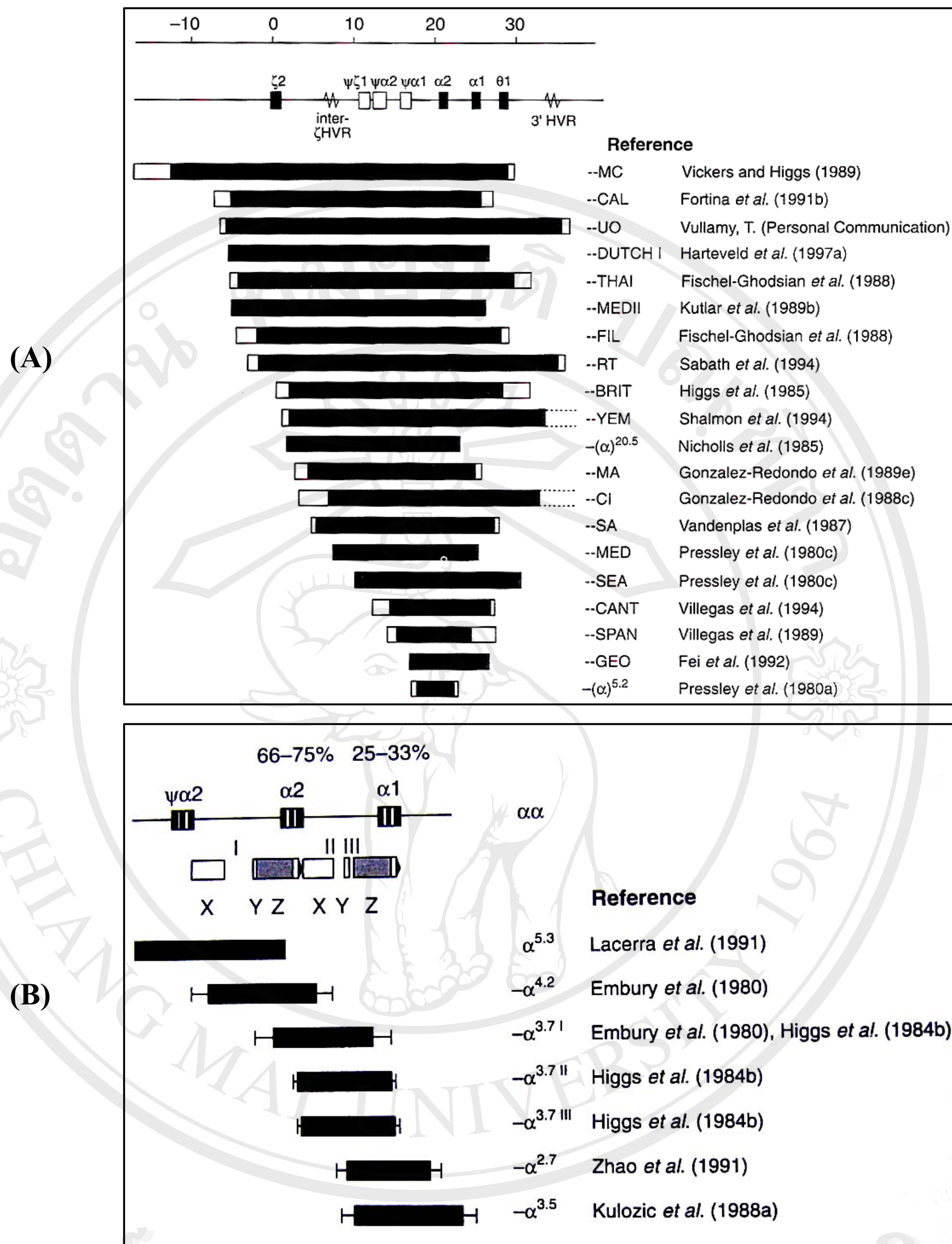

Figure 1.10. A summary of major deletions in the α globin gene cluster. Above: the α-gene complex is shown (scale in kilobases, 0 indecates the ζ2-globin mRNA CAP site). Below: the extent of each deletion is shown by a black bar. Regions of uncertainty for each breakpoint are shown by white boxes. To the right are the shorthand notations, primary references for the characterization and examples of PCR analysis for each deletion. (A) α-thalassemia 1 deletions; (B) α-thalassemia 2 deletions (Weatherall & Clegg, 2001).

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In addition non-deletional types of α-thalassemia (α^T) were first described in 1977 (Kan *et al.*, 1977) and shown to result from a variety of mechanisms (Higgs *et al.*, 1981). At present we know of 41 well defined types of non-deletion α^+-thalassemia; 25 occur in the α2 gene, seven in the α1 gene, and six on a -α chromosome. Unlike the situation in which one α-globin gene is deleted, there does not appear to be a compensatory increase in expression of the remaining functional α-globin gene when its partner is inactivated by a point mutation. Furthermore, some highly unstable variants may have multiple secondary effects on red-cell structure and function, producing a more severe phenotype than would be predicted from the decrease in α-gene expression. At present these ideas are based on a small number of observations and further evaluation of the pathophysiology of each mutation is needed (Table 1.1).

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Table 1.1 Examples of non-deletion mutants that cause α–thalassemia (Weatherall & Clegg, 2001).

Affected gene	Mutation	Alternative notation	Distribution
mRNA processing			
α2	IVS1;5bp del	$\alpha^{Hph}\alpha$	Mediterranean
α2	IVS1;116 A→G		Dutch Caucasian
α1	IVS1;117 G→A		Middle East
mRNA translation			
α2	TER; T→C (Constant Spring)	$\alpha^{CS}\alpha$	South-east-Asian
α2	TER; A→T (Pakse)		Laotian
Post-translational			
-α	CD14; T→G (Evanston)		Black
α1	CD59; G→A (Adana)		China
α2	CD109; T→G (Suan Dok)	$\alpha^{SD}\alpha$	South-east Asia
α2	CD125; T→C (Quong Sze)	$\alpha^{QS}\alpha$	South-east Asia
Uncharacterized			
α	Not determined		Pacific

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1.2.4.3. Hemoglobin Bart's hydrops fetalis

Loss of all four α globin genes is incompatible with life, resulting in mid- to late-gestational stillbirth of a hydropic fetus (Chui & Waye, 1998). This syndrome is almost always deletional in origin (--/--). α-thalassemia is the most common cause of fetal hydrops in Southeast Asia with an estimated frequency of 1:1,550 total birth in Hong Kong. In Thailand, there are approximately 15,000 pregnancies with hydropic fetuses per year. The most common cause of α-thalassemia hydrops fetalis in Southeast Asia and Southern China is homozygosity for the ($--^{SEA}$) deletion which has a gene frequency in this population of approximately 3% (Ausavarungnirun *et al.*, 1998; Liang *et al.*, 1985). The major hemoglobin in these infants, Hb Bart's (γ_4), is nonphysiologic. Residual expression of the ζ globin gene results in synthesis of sufficient functional hemoglobin tetramers, Hb Portland I ($\zeta_2\gamma_2$), to carry the fetus through mid to late gestation. The mean gestational age at delivery is 32 weeks. Approximately 50% of the infants are stillborn while the remainders expire within a few hours after birth (Liang *et al.*, 1985).

The predominant physical findings in the fetuses are generalized and massive edema (hydrops), ascites, gross enlargement of the liver with a spleen which may be normal or only slightly enlarged, and a large friable placenta (Figure 1.11). The peripheral blood demonstrates severe erythroblastosis with accompanying reticulocytosis, target cells, hypochromia with fragmentation and decreased osmotic fragility (Figure 1.12). The MCV is often very high due to the large number of circulating nucleated red cells. Hemoglobin electrophoresis demonstrates high levels of Hb Bart's (γ_4) with variable levels (usually 10-29%) of Hb Portland, and trace levels of Hb H (β_4) (Liebhaber, 1989; Weatherall *et al.*, 1970).

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In addition, there is an increased incidence of serious maternal complications in these pregnancies. It is likely that the placentomegaly is one important causative factor. It was estimated that half of these women die from complications resulting from these pregnancies if there was no proper medical care. In a study of 46 women who were pregnant with affected fetuses, 61% developed hypertension during pregnancy, 50% developed severe pre-eclampsia. Polyhydramnios was presented in 59% of the cases. Eleven percent suffered antepartum hemorrhage as a result of either unknown cause or placenta prevails. 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 (Chui & Waye, 1998).

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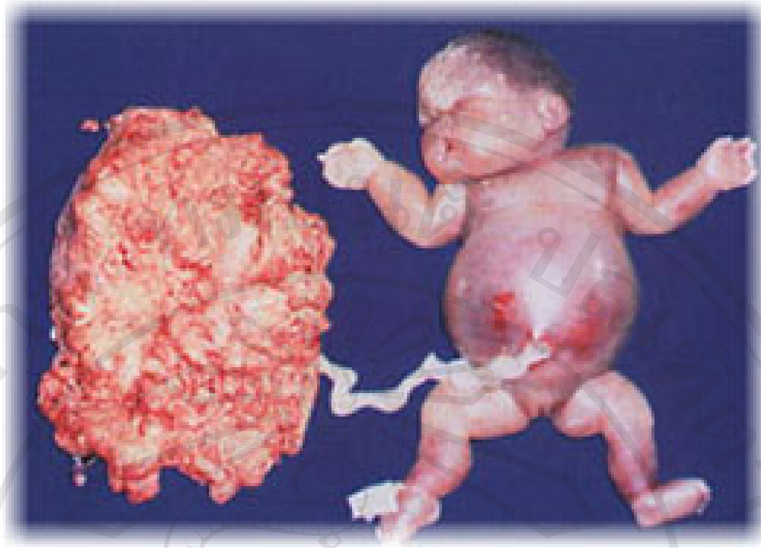

Figure 1.11. Hb Bart's hydrops fetalis. Affected fetus with enlargement of liver and a large friable placenta. *(http://www.thalassemia.or.th/picture/alpha1.jpg accessed 26 August 2006)*

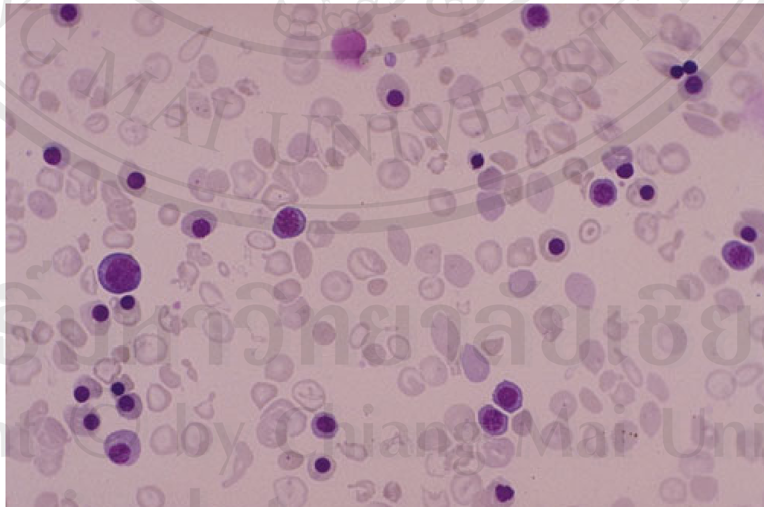

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Figure 1.12. Blood smear of Hb Bart's hydrops fetalis. Severe anemia is present in blood cells morphology of Hb Bart's hydrops fetalis. *(http://www.medlib.med.utah .ed /WebPath/COW/COW011.jpg accessed 26 August 2006)*

1.2.4.4. Laboratory diagnosis of thalassemia

Carrier or heterozygote screening and mutation identification are the best way of prevention program for the hemoglobin disorders including thalassemia. The strategy for carrier screening and mutation analysis is based on that fact that although heterozygotes are symptom free, they present specific hematologic characteristics that are useful for their identification (Figure 1.13). The accurate determination of the carrier phenotype is essential for the selection of the appropriate molecular tests to determine the carrier genotype and exclude from the other acquire anemia disorders such as iron deficiency anemia (Old, 2003). The basic hematological tests required are the measurement of the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) value and the quantification of Hb F by alkaline denaturant test (Betke, 1953) or High performance liquid chromatography (HPLC) and Hb A_2 by ion-exchange chromatography (Moors *et al.*, 1979). In addition, the hemoglobin pattern needs to be examined. The traditionally electrophoresis methods and isoelectric focusing electrophoresis (IFE) have been used for this purpose. HPLCis also used to detect most of the common, clinically relevant hemoglobin variants, such as Hb S, Hb C, Hb D-Punjab, Hb O-Arab, Hb E and abnormal hemoglobin such as Hb Bart's, Hb H at the same time (Old, 2003; Sanguansermsri *et al.*, 2001). Detecting the mutant gene using polymerase chain reaction (PCR) can be identified the majority of the common thalassemia mutations and abnormal hemoglobins also.

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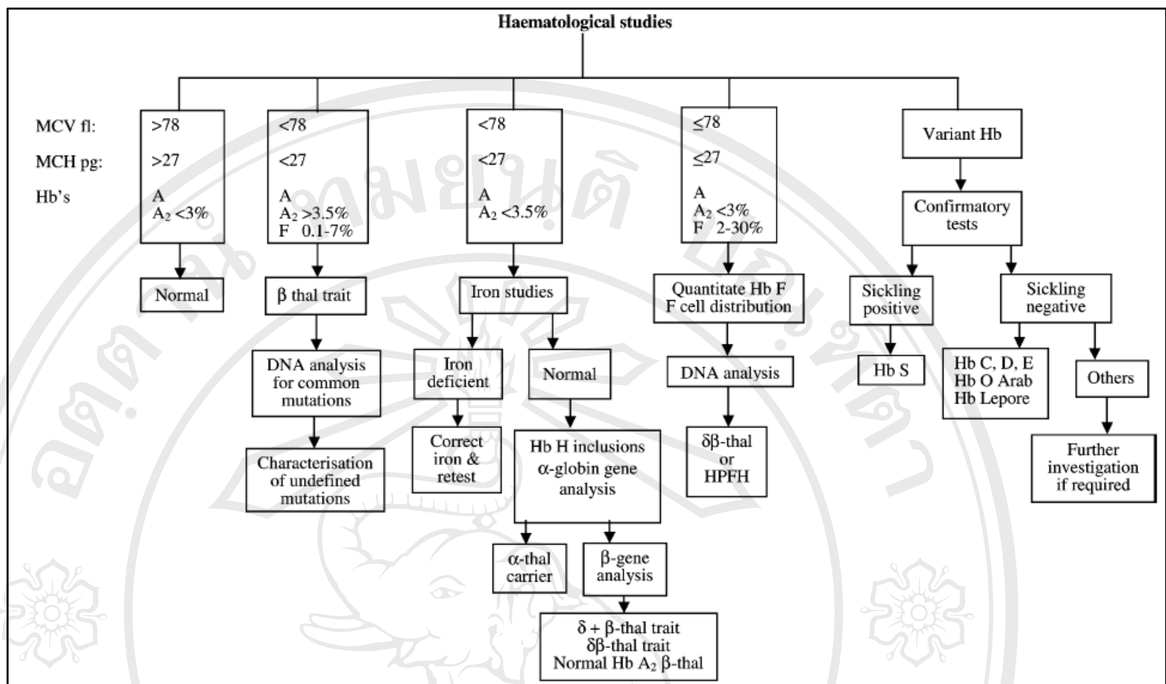

Figure 1.13. Simplified flow chart base on the MCH, Hb A_2, Hb F value and DNA analysis for screening of thalassemia carriers (Old, 2003)

1.2.4.5. Laboratory investigation for α-thalassemia

The primary screen for all form of thalassemia involves red-cell indices, RBC morphology and OF test. The heterozygote states for the deletion ($-\alpha/\alpha\alpha$) and non-deletion ($\alpha^T\alpha/\alpha\alpha$) forms of α-thalassemia usually show minimal hematological changes and no abnormalities of the hemoglobin pattern. In some, but not all, cases there is an elevated level of Hb Bart's in the 1-3% range in the neonatal period. The heterozygote states for α^0-thalassemia ($--/\alpha\alpha$), and the homozygote state for α^+–thalassemia ($-\alpha/-\alpha$) are characterized by significantly reduced levels of the MCV and MCH together with hypochromic red cells. There are no changes in the hemoglobin pattern in adult life but in the neonatal period from 5 to 10%

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Hb Bart's is found. The latter conditions can also be identified by globin synthesis. The secondary screen involves examination of H inclusion bodies and Hb electrophoresis to search for Hb H and to rule out any other structural Hb variants. Although this approach identifies the condition as a type of α-thalassemia it does not allow a definitive diagnosis. There may be a few cells with Hb H inclusion bodies, particularly in heterozygotes for α^0-thalassemia, but their absence does not exclude the diagnosis. In short, the only definite way of identify-homozygote states for α^+–thalassemia is by DNA analysis (Weatherall & Clegg, 2001).

However, these methods are not suitable for screening in large population due to the low sensitivity and specificity. The high-sensitivity and high-specificity-immunological tests could overcome this problem. The immunological technique is the new trend for screening of thalassemia including α-thalassemia. It is band on simple techniques requiring no high-tech equipment and inexpensive. At present, there is only one company that has successfully developed the commercial immunodiagnostic kit for screening of α-thalassemia 1 carriers (Unitedbiotech, 2006).

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1.2.5. Monoclonal antibody

1.2.5.1. Hybridoma technique

Monoclonal antibodies (mAb) are antibodies produced by a single clone of hybridoma cells. These cells are derived from the fusion of B-cell (antibody producing cell) and myeloma cell (immortal gene contained cell) by hybridoma technique (Kohler & Milstein, 1975). All mouse myeloma cells commonly used for hybridoma production are of BALB/c origin, and it is generally easiest to use BALB/c mice as the spleen donor. In 1959, it was accidentally discovered that peritoneal irritants could cause development of myelomas in BALB/c mice (Merwin & Redmon, 1963). Subsequently, it was found that mineral oil or pristane were potent inducers of myeloma in BALB/c mice (Potter & Boyce, 1962). Approximately 40% of BALB/c mice will develop myeloma within one year of a series of three intra-peritoneal injections of mineral oil (Warner, 1975). Until now, several myeloma cell lines have been produced including Sp 2/0-Ag-14 (Shulman *et al.*, 1978), X63-Ag8.653 (Kearney *et al.*, 1979) and NSO/1 (Galfre & Milstein, 1981). The X63-Ag8.653 is probably the cell of choice, because it losses both immunoglobulin heavy and light chains,

is widely available, has a high fusion frequency and is easy to grow.

When cells are treated with Sendai virus or high concentration of polyethylene glycol (PEG), their membranes fuse and multinucleate cells called heterokaryons are formed (Ringertz & Savage, 1976). At the next cell division, the nuclei of heterokaryons fuse, and the daughter cells possess a more or less equal share of the genetic material. So far, the fusion events are poorly controlled. In addition to myeloma (M) and spleenocyte (S) fusion, it is to be expected that many

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fusions will be M-S, M-M or S-S, or even higher multiples of these. Thus, if it is desired to produce a long-term hybrid cell from two cell types, a selection procedure is required. By far the most common selection procedure is that devised by Littlefield in 1964 (Littlefield, 1964). Littlefield's procedure depends on the fact that when the main biosynthetic pathway for guanosine, *de novo* pathway, is blocked by the folic acid antagonist aminopterin, there is an alternative "salvage" pathway in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate via the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT; Figure 1.14). Cells lacking HGPRT die in medium containing hypoxanthine, aminopterin and thymidine (HAT medium), because both the main and the salvage pathways are blocked. However, an HGPRT$^-$ cell can be made to grow in HAT medium if it is provided the missing enzyme by fusion with an HGPRT$^+$ cells.

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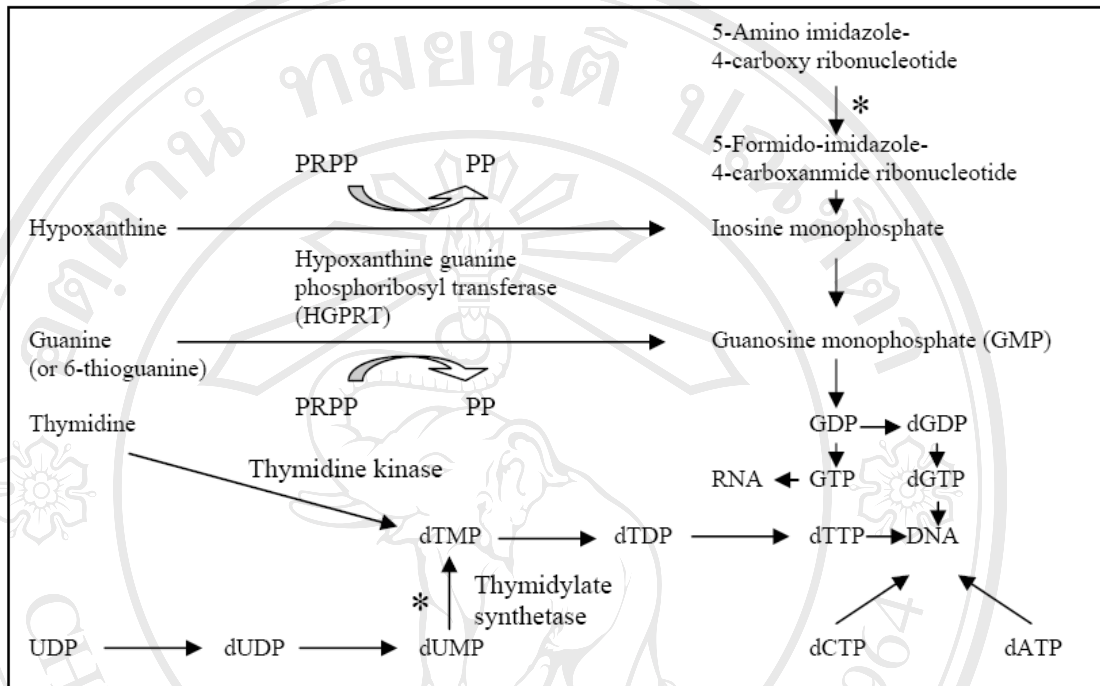

Figure 1.14. Metabolic pathway relevant to hybridoma selection in medium containing hypoxanthine, aminopterin and thymidine (HAT medium). When the main synthetic pathways are blocked with the folicacid analogue aminopterin (*), the cell must depend on the "salvage" enzymes HGPRT and thymidine kinase. HGPRT$^-$ cells cannot grow in HAT medium unless they are fused with HGPRT$^+$ cells (Goding, 1986).

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In 1975, Kohler and Milstein has developed the Hybridoma technique by fusing a HAT-sensitive variant of MOPC-21 myeloma cells with spleen cells from mice immunized with sheep red cells. The fusion was mediated by Sendai virus, and hybrids were selected by growth in HAT medium. It was known that normal spleen cells could only survive a few days in culture, but it was hoped that they would "support" the missing HGPRT in the myeloma cells, and that the myeloma cells would provide the "immortality" needed for continuous culture (Figure 1.15.). The experiment worked exactly as planed, and a number of cloned hybrid lines secreting anti-sheep erythrocyte antibodies were produced. The tumours are now known as "hybridomas". The serum of hybridoma-bearing mice contains large amounts of homogeneous antibody. The use of Sendai virus has been superseded by polyethylene glycol (Galfre & Milstein, 1981; Pontecorvo, 1976), but apart from this modification, the basic procedure is essentially unchanged.

Because of the high probability of chromosome loss in the generated hybrids, and to ensure that the antibodies were indeed monoclonal, it was essential to clone the hybridoma cell lines. Hybridoma cell line should be cloned at least twice to make absolutely certain that each is a true clone, and also because of the relatively high probability of growth of nonproducer variants due to chromosome loss. After two cycles of cloning, the rate of chromosome loss is small, although the risk of over-growth by nonproducer cells never ceases completely. Two method are available for single cell cloning of hybridomas, i.e. soft agar cloning and limiting dilution technique. Cloning in soft agar has the disadvantages that unless an overlay technique is available, colonies need to be plucked out and regrown in liquid culture prior to

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testing for antibody production. For this reason, many workers now prefer to clone by limiting dilution.

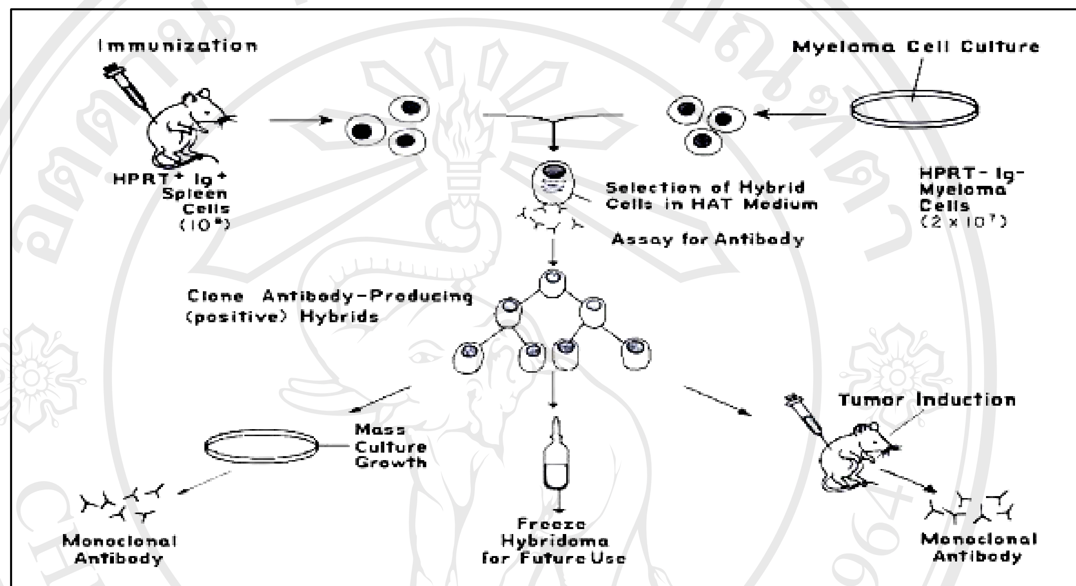

Figure 1.15. Production of hybridomas. Spleen cells from immune mice are fused with $HGPRT^-$ myeloma (plamacytoma) cells using polyethylene glycol. The binucleate fusion products are known as heterokaryons. At the next division, the nuclei fuse, generating hybrid cells, which grow in HAT medium. Unfused myeloma cells die in HAT medium, and unfused spleen cells can only survived a few day in culture. Hybrids are test for production of antibody of the desired specificity, and cloned by limiting dilution. *(http://www.aecom.yu.edu/cancer/new/Assetts/images /new%20navbar/cores /image1.jpg accessed 4 September 2006)*

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1.2.5.2. Thalassemia diagnosis by immunological method

Antibody specifically binds to their specific antigen and can be applied for detection or purification of the antigen. Recently, antibody has become an important tool in biochemistry, molecular biology and biomedical technology (Wikipedia, 2006). Immunological methods are currently utilized in several approaches based on antigen-antibody reactions, including measurement of proteins and drug levels in serum, tissue typing and blood grouping, identifying infectious agents, identifying cell surface molecules, classification and follow-up therapy of leukemias, identifying tumor antigens and auto-antibodies, and quantifying hormones (Osburn *et al.*, 1996). In addition, several immunoagnostics are also developed for disease diagnosis and forecasting.

The immunological tests have been applied for diagnosis of α-thalassemia. Most of these methods are base on the use of specific mAbs to the globin chains or Hb such as mAb agaist ζ globin chain or Hb F. The principles of thalassemia immunodiagnosis are based on the fact that imbalance of globin chain production in thalassemic red cells results in lower quantities of the affected globin and increase in the amount of the unaffected or related globin chains in terms of compensation. For example, in α-thalassemia 1 of Southeast Asian type ($--^{SEA}$), the loss of two α-globin genes results the body compensates by produce the ζ globin chain instead which can be detected in the hemolysate. Thus mAbs against ζ chain were utilized in diagnostic the α-thalassemia 1 ($--^{SEA}$) carriers (Ausavarungnirun *et al.*, 1998; Ma *et al.*, 2002; Panyasai *et al.*, 2002). The accumulation of unaffected β-like globin chain such as Hb Bart's (γ_4) in α-thalassemia 1 is also the biological marker for diagnosis the α-thalassemia 1 carriers by using anti-Hb Bart's antibody (Makonkawkeyoon *et*

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al., 1992; Wasi *et al.*, 1979). In addition, the mAbs anti-γ chains was used to monitor the level of fetal hemoglobin containing in red cells (F cells) that is a parameter for monitoring the sickle cell anaemia (SS) patients undergoing treatment with Hb F modulating drugs (Mundee *et al.*, 2001). The mAbs anti-β^E chain was developed for detection of Hb E in general population purpose (Makonkawkeyoon *et al.*, 2006). The mAbs anti-δ chain was also use to detect and quantify the amount of Hb A_2 in the hemolysate (Shyamala *et al.*, 1991).

1.3. Objectives

1. To produce monoclonal antibodies against hemoglobins in Hb Bart's hydrops fetalis hemolysate
2. To characterize the produced monoclonal antibodies

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