CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

1.1.1 Thalassemia and hemoglobinopathies

Thalassemia is a type of anemia caused by reduction or absence of globin chain synthesis which results in imbalanced-globin chain synthesis; the major pathogenesis of the disease. The unaffected globin chains continued to be synthesised at the normal synthetic rate tend to form homotetramers that can harm the red blood cells both at young and mature stages. α-globin chain homotetramer (α_4) generated in β-thalassemia causes premature destruction of young erythrocytes in the bone marrow, leading a so-called "ineffective erythropoiesis". γ- and β-globin chain homotetramer (γ_4, β_4) founded in α-thalassemia harm mature erythrocytes. γ_4 possesses very high oxygen affinity and inhibit oxygen release from erythrocytes which, in turn, results in tissue anoxia. β_4 is an unstable hemoglobin. It precipitates easily under the stress that might occur in the patients' body. Once precipitates, the erythrocytes are removed by the RE system and anemia resulted. In severe cases, the patients suffer from chronic and marked anemia with life depending solely on blood transfusion. They also suffer from several complications which can be fatal if the management is inadequate. Anemia causes

expansion of bone marrow, leading to osteoporosis and changes of bone structure. Blood transfusion causes iron over loading state. Excess iron accumulates in many vital organs such as heart, endocrine grands and deteriorates their normal functions.

Thalassemia is considered as the most common autosomal single-gene disorder worldwide. It can be found in more than 150 countries with estimated carrier frequency of about 7 %. The Mediterranean region, certain parts of North and West Africa, Middle East, Indian subcontinent, Southern Far East and South East Asia have the highest prevalence of the disease (Weatherall and Clegg 2001).

In contrast to the thalassemia, hemoglobinopathies, is an inherited disorder of hemoglobin productions characterized by production of abnormal hemoglobins or hemoglobin structural variants occuring from genetic alterations including point mutations, deletions or insertion of the normal globin genes.

1.1.2 Common types and prevalence of thalassemia and hemoglobinopathies commonly found in Thais

The thalassemia syndromes are usually classified according to the type of globin chain that is absent or present in decreased amount. The major categories consist of α and β -thalassemia. These two thalassemia types are also common in Thailand. Several surveys has shown that 30-40% of Thai population are carriers for thalassemia and hemoglobinothies. Carriers of α-thalassemia account for about 20-30% (approximately 3.5% α-thalassemia 1 and 16% α-thalassemia 2), while carriers of β-thalassemia are found in about 3-9% of Thai population (Fucharoen, *et al* 1998). Two types of

hemoglobinopathies also commonly found in Thailand, by globin chain classification, are alpha (α) and beta (β) hemoglobinopathies. To date, 1,358 hemoglobin variants have been described (http://globin.bx.psu.edu: accessed 25/02/09), over 90% of which are single amino-acid substitutions in the α, β, γ or δ-globin chains and over 60% involve the α-globin chain. The most important hemoglobin structural variants commonly seen in Thailand are hemoglobins Constant Spring (CS) and E. HbCS has been shown to be 1-6% common in Thais, whereas Hb E is found in approximately 13-17% on the population, especially in the Thai-Laos-Combodian boundary or "Hb E triangle" where more than 32-60% of the people carry HbE gene (Fucharoen 2529).

Alpha-Thalassemia (α-thalassemia)

α-Thalassemia is a syndrome caused by a deletion of one or more of the four α-chain genes. Therefore, excess γ and β-globin chains that result from defective α-chains production are able to form homotetramers which are γ_4 (Hb Bart's) and β_4 (Hb H), respectively. Hb Bart's has very high oxygen affinity that could lead hypoxemia in those severely affected. HbH, in contrast, is unstable and tend to be denatured under the stress condition and precipitates inside the erythrocytes. Severe hypoxemic state caused by Hb Bart's and hemolysis caused by precipitated HbH are harmful to the patients. Thus the clinical features of the severe forms of α-thalassemia are reflection of the properties of Hb Bart's and H and their effects on erythrocytes, and in particular on red cell survival (Higgs and Weatherall 2008).

α-thalassemia is genarally divided into 2 groups, α-thalassemia 1 and α-thalassemia 2, depending on the α-globin gene(s) that is (are) absent or inactivated. At molecular level, the α-thalassemia may be caused by either deletion or point mutations of the α-globin genes and results in both types of α-thalassemia stated earlier.

Deletional types of α-thalassemia

α-thalassemia 1 (α^0-thalassemia) is characterized by deletion of both α-globin gene *in-cis* or otherwise inactivated (--/αα in heterozygote). It is sometimes called α^0-thalassemia because there is no output of α-globin from the one affected chromosome. The α-thalassemia-1 of Southeast Asian (SEA) type is the most frequently found α-globin deletion in Thailand. The SEA deletion removes about 19.304 kb of α-globin gene cluster including α_1 and α_2 globin genes (Weatherall and Clegg 2001).

α-thalassemia 2 (α^+-thalassemia) is characterized by loss or deactivation of one of two α-globin gene. The remaining 3 intact α-globin genes still working well and the amount of α-globin chain produced depends upon these intact α-globin genes. Thus, this type of α-thalassemia results in milder phenotype than the α-thalassemia 1. The common types of α-thalassemia 2 worldwide include α-thalassemia 2 (3.7-kb deletion, $\alpha^{-3.7}$) and α-thalassemia 2 (4.2-kb deletion, $\alpha^{-4.2}$). The $\alpha^{-3.7}$ causes by the rightward deletion of region on α-globin gene cluster spanning 3.7 kb, resulting in deletion of α2-globin gene and the remaining α-globin gene is the α2/α1 fused globin genes. In the $\alpha^{-4.2}$ deletion (the loss of leftward 4.2 kb of DNA), α2-globin gene is also removed

leaving α1-globin gene intact. Although only one α-globin gene remains, the patient is clinically unaffected, especially in heterozygous state (Higgs, *et al* 1990).

Non-deletional types of α-thalassemia

In addition to deletional types, non-deletional types of α-thalassemia (α^T) were first described in 1977 and shown to result from a variety of mechanisms. Phenotypically,this type of α-thalassemia always present as the α-thalassemia 2. At present, 32 well-defined types of non-deletion α-thalassemia (25 occur in the α2 globin gene, 7 in the α1 globin gene) were identified. (Kan, *et al* 1977). 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 *in-cis* when its partner is inactivated by a point mutation (Higgs, *et al* 1981). 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-globin gene expression.

α-hemoglobinopathies

α-hemoglobinopathy is characterized by production of abnormal α-globin chains. In Thailand, α-hemoglobinopathies are also found, including Hb Suandok ($\alpha_2^{CD109:\ Leu\text{-}Arg}\beta_2$), Hb Mahidol or Hb Q-Thailand ($\alpha_2^{CD74:\ Asp\text{-}His}\beta_2$), Hb Paksè ($\alpha_2^{CD142:\ TAA\text{-}TAT}\beta_2$), Hb Constant Spring ($\alpha_2^{+31aa}\beta_2$), Hb Thailand ($\alpha_2^{CD56:\ Lys\text{-}Thr}\beta_2$) and Hb Pak Num Po ($\alpha_2^{CD132:\ +T}\beta_2$). In particular, Hb CS is common in Thailand, about 1%

heterozygous carrier, 5 to 7% homozygotes and 3 to 5% in hemoglobin H disease form which HbCS allele is *in-trans* to the deletional α-thalassemia 1 allele. HbCS is an α-globin chain variant which is elongated by 31 amino acid residues at its C-terminal end. Heterozygotes for HbCS have a mild anemia and may have splenomegaly. The patients with the form of HbH associated with HbCS are more anemic than those of patients with other forms of HbH disease (Fucharoen 2529, Sanchaisuriya*, et al* 2002, Viprakasit*, et al* 2004). Then, it was severe type of HbH disease.

β-thalassemia

The β-thalassemia are a diverse group of disorders of hemoglobin synthesis which is characterized by reduced or absent β-globin chain synthesis. There are two main varieties of β-thalassemia, β^{o}-thalassemea in which no β-globin chain is produced, and β^{+}-thalassemia in which some β-globin is produced but less than normal. The majority of β-thalassemia are caused by point mutations and small deletions or insertion within the β-globin genes (Loasombat 2541, Thein 1993, Wasi*, et al* 1969, Wasi*, et al* 1980, Weatherall and Clegg 2001, Winichagoon*, et al* 1995).

β^{0}-thalassemia is severe β-thalassemia with no production of β-globin chain. It is mainly caused by point mutations in coding region (exon) or exon-intron junction of β-globin gene which lead to premature stop codon or genaration of abnormal β-globin mRNA. The end results of these abnormalities are a absence of the β-globin chain production. In Thailand, at least 3 common mutations in the β-globin gene are of this category. They comprise A-T substution at codon 17 (CD17: A-T) which creates

premature stop codon, the TTCT-deletion at codons 41/42 (CD41/42: -TTCT) which causes reading frameshift and premature stop condon at codon 59 instead of codon 147, the G-T substitution at IVSI-nt1 which leads to abnormal splicing of immature β-mRNA and results in no production of normal β-mRNA. In general, thus, genotype of heterozygote is written as β^0/β^A and that for homozygote as β^0/β^0.

β^+-thalassemia is a milder form of β-thalassemia in which some β-globin chains are still produced. The majority of cases possess point mutations outside exons, especially in the promoter region. The mutations of β-globin gene leading to the β^+-thalassemia include mutations at ATA box (nt-28, nt-29 or nt-30 from cap site), CACCC element (about nt-86 to nt-90 from cap site) and mutations in intron or exon of gene to produce new splice site to race in RNA splicing process, as if mutation in IVS2-nt654 (C-T), mutation of IVS1-nt5 (G-C). The genotype were β^+/β^A and β^+/β^+ for heterozygote and homozygote consecutively.

β-hemoglobinopathies

β-hemoglobinopathies is characterized by the production of abnormal β-globin chains due to changes or mutations (missense mutations) on the β-globin gene. Two abnormal β-globin chains then complex with 2 normal α-globin chains to form abnormal hemoglobin or β-structural variants. These abnormal hemoglobins generally have different electrophysical properties from their normal counterparts; i.e. due to the molecular conformational alteration. Theoritically, synthetic rate of the abnormal β-globin chain should be normal. However, some are produced in reduced rate, thus

producing a phenotype resembling the β-thalassemia. Todate, there are more than 737 β-globin variants reports across the world (http://globin.cse.psu.edu/). In Thailand, HbE is the most common β-globin structure variant accounting for approximately 54% (8-70%) of population (Fucharoen*, et al* 1988). In HbE, the G-A substitution at codon 26 partially activates a cryptic splice site towards the 3' end of exon 1, resulting in a proportion of abnormally splice mRNA. Thus, less β^E globin is synthesized and a mild thalassemia phenotype results. HbE heterozygotes are clinically normal and have only minor hematological changes. Homozygote have a very mild anemia, but are otherwise well; their hematological changes are similar to those of heterozygote β thalassemia. It is the compound heterozygous state between HbE and β thalassemia that gives rise to really serious clinical disease, with a phenotype ranging from mild anemia to the most severe from like β thalassemia major. Other β-structural variants found in Thai were HbD-Punjab ($\alpha_2\beta_2^{121Glu\text{-}Gln}$), Hb J-Bangkok ($\alpha_2\beta_2^{56Gly\text{-}Asp}$), Hb Siriraj ($\alpha_2\beta_2^{7Glu\text{-}Lys}$), and Hb Tak ($\alpha_2\beta_2^{+11AA}$) (Fucharoen*, et al* 1989, Thein 1993, Thein*, et al* 1990).

1.1.3 Laboratory diagnosis of thalassemia and hemoglobinopathies

1.1.3.1 Screening techniques for thalassemia and hemoglobinopathies

Initial screening techniques are defined as techniques that are simple and relatively low cost, which can indicate the possibility of having thalassemia. These techniques should involve the least sample pretreatment and is rapid sample preparation, and may not need special instrumentation. This would lead to low cost and high sample throughput analysis. They provide a "*yes/no*" type answer. Positive samples need further

confirmatory test while negative samples can be eliminated from further complicated and expensive testing. The red blood cell indices generated by automated complete blood count (CBC) or the alternative one-tube osmotic fragility test (OF test) can be used to screen for α-thalassemia. The negative result eliminates the possibility of having thalassemia. These screening techniques, however, cannot provide the information on the exact type of thalassemia of the positive persons.

Complete blood count (CBC): Complete blood count, a screening test, involving the measurement of important characteristics of the blood, has an impact to the diagnosis of thalassemia. The main features of the blood normally tested in the CBC are the total white blood cell (WBC) count, red blood cell (RBC) count, hematocrit (Hct), hemoglobin (Hb), red cell distribution width (RDW), peripheral blood smear and other important erythrocyte indices (EI), namely mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Among these parameters, MCV and MCH are the most important. Individual with hypochromic microcytic red blood cell indices that is with an MCH below 27 pg or an MCV below 80 fL should be investigated further (Bernadette F. Rodak 2007).

One-tube osmotic fragility test (OF test): This simple test utilizes osmosis, the movement of water from lower to higher salt concentration region, to test for the osmotic resistance of the red blood cells. Whole blood is thoroughly mixed with 0.36% buffered saline solution. In a hypotonic condition, the concentration of salt on the outside of a cell is lower than that on the inside, resulting in net water movement into cells. Normal red

blood cells are lysed within 1-2 minutes and the mixture then turns clear and reddish. Abnormal red blood cells have deviated osmotic resistances as compared to normal red cells. Red blood cells of thalassemia have higher osmotic resistance and thus have slower rupture rate, therefore the mixture remains turbid even after 1-2 hr. This technique can be carried out in one test tube and it is therefore called "One-Tube" method. Different laboratories may be using slightly different recipes for preparation of hypotonic salt solution, but all are normally based on the same concept of kinetic osmotic fragility (Winichagoon, *et al* 2002). Although the OF test is a quick preliminary and very economic test before performing further studies of the red blood cells and carrying out thalassemia in large populations. However, the specificity of OF test is not so high (74.9%) and its false positive rate is rather high (25%). This means increase the workload of the later conformational PCR analysis. Recently, the use of a 0.34% modified OF test was suggested for reducing the workload of PCR by about 70% (Sirichotiyakul, *et al* 2004).

Dichlorophenol-indolphenol (DCIP) test: 2,6-dichiorophenol indophenols (DCIP) were oxidizing chemicals, whereas, normally used in redox reaction and indicator in amount of ascorbic acid measurement. Hemoglobin E is resulted from amino acid change at codon 26 of β-globin from glutamic acid to lysine. This alternation makes contact of α-globin chain and β^E-globin chain less stable. Thus, in DCIP solution, molecule of HbE changes from tetramer to monomer, sulhydryl group of amino acid becomes free and oxidized by DCIP. Finally, HbE is denature and precipitates (Chapple, *et al* 2006).

Hb H inclusion body test : Hb H arises in the setting of α-thalassemia where the decreased production of α-globin chains lead to β-globin excess. Oxidation of these tetramers provokes intra-erythrocytic precipitation, which can be visualized microscopically and called "Hb H inclusion bodies". Diagnosis of α-thalassemia carrier relies on the test for Hb H inclusion bodies (α-thalassemia 1 about 1:10,000 and α-thalassemia 2 occasionally found). Generation of Hb H inclusion in the Hb H inclusion body test is accomplished by incubating unfixed cells with an oxidative dye such as New Methylene Blue or Brilliant Cresyl Blue. Blood film examination is undertaken with a search for cells with typical "golf ball" inclusions. Approximately, 30-100% of red cell containing Hb H inclusions was found in Hb H disease (α-/--). However, as few as Hb H inclusion bodies-containing cell in 1,000-10,000 red blood cells are found in α-thalassemia heterozygote, particularly of α-thalassemia 1 type. The search for inclusions is laborious, observer–dependent and reported significantly lower sensitivity for the detection of the ($--^{SEA}$) deletion and all α-thalassemia genotype when compared with the multiplex PCR because Hb H test is seldom positive in α-thalassemia 2. The absence of Hb H inclusions therefore does not exclude α-thalassemia trait. In addition, a brisk reticulocytosis can make identification of a rare Hb H inclusion-containing cell difficult. The use of a low MCV plus the Hb H inclusion body test is sufficiently sensitive for the diagnosis of α-thalassemia 1 ($--^{SEA}$) carriers in area with a high prevalence of ($--^{SEA}$) deletion and laboratories have no molecular capabilities (Clarke and Higgins 2000, Pan, *et al* 2005, Trent 2006).

1.1.3.2 Confirmatory method for thalassemia and hemoglobinopathies

Cellulose acetate electrophoresis: Electrophoresis is one of the widely used techniques for analyzing hemoglobin variants based on the movement of different hemoglobin, which contain different charges, in the electric field. At an alkaline pH, Hb is negatively charged and move toward the anode (positively charged). Electrophoresis is labor-intensive, and inaccurate in quantification of low concentration Hb variants such as Hb A_2 or in the detection of fast Hb variants such as Hb H, Hb Bart's. Detection of Hb Bart's in α-thalassemia carriers using electrophoresis can be carried out only from newborn blood samples (Clarke and Higgins 2000).

Microcolumn chromatography: Microcolumn chromatography is an anion-exchange chromatography-based method. Microcolumns are prepared containing a suspension of an anion –exchange, such as DEAE-cellulose or sephadex resin, in buffer. The resin is composed of small particles of cellulose covalently bound to small positively charged molecules. A hemoglobin solution is applied to the column and is adsorbed on to the resin. There is then an interchange of charged groups between the positive charged resin and the negative charged hemoglobin molecules, which retards the passage of hemoglobin through the column. The strength of the association of various types of hemoglobin molecule to the matrix can be altered by alterations in the pH or ionic strength of an eluting solution applied to the column. It is therefore possible to elute different hemoglobins selectively by using different eluting solution. When this method is used for the quantification of hemoglobin A_2, there is elution of hemoglobin A_2 first

and then, using a second eluting solution, of hemoglobin A and other hemoglobins. The two fraction are collected separately and the absorbance of the eluate is read on a spectrophotometer, permitting the expression of the amount of hemoglobin A_2 present as a percent age of total hemoglobin. Alternatively, it is possible to elute only hemoglobin A_2 and measure total hemoglobin in a second tube (Bain 2005).

Alkaline denaturation test: Fetal hemoglobin (HbF) is more resistant to denaturation in alkaline solution than adult hemoglobin (HbA) and other hemoglobins. Alkali converts HbA to alkaline hematin. Alkaline hematin is insoluble and precipitates. HbF is quantitated by measuring the hemoglobin concentration before and after denaturation (Bernadette F. Rodak 2007).

High performance liquid chromatography (HPLC): In high performance liquid chromatography, particles size of the stationary phase packed in the column is quite small. High pressure is required to force the mobile phase to continuously flow through the column. As the samples solution flows with the liquid mobile phase through the stationary phase, the components of the sample will migrate according to the non-covalent interactions of the compounds with the stationary phase. The degree of interactions determines the degree of migration and separation of the components (i.e., the component with a stronger interaction with the mobile phase than with the stationary phase will have a shorter retention time and thus will be eluted from the column first and *vice versa*). HPLC has become a very important tool for thalassemia and Hb variants diagnosis because of its ability to accurately, rapidly and quantitatively differentiate types

of hemoglobins. In addition to typing and quantifying hemoglobin pattern in adult blood, HPLC can also detect and quantify small amount of HbA in case of prenatal diagnosis of the *in utero* fetus at risk of β-thalassemia major.

DNA analysis: As the knowledge of molecular defects of thalassemia and hemoglobinopathies has dramatically increased during the past decade, the molecular diagnostic techniques have also been invented to make the determination of those molecular defects more feasible in diagnostic laboratories. Polymerase chain reaction (PCR) technique is one among those molecular techniques widely employed at the present time(Gu and Zeng 2002). PCR allows a small amount of DNA to be amplified *in vitro*. The process is composed of cycles of the three following steps: perform heat denaturing to separate the DNA sequence target in to two strands, anneal each strand to the specific primers and then extend the polymerase chain from the primer termini. Then, agarose gel electrophoresis is commonly done following the PCR to separate different DNA fragments. The PCR technique has been increasingly used to identify deletions of the α-globin gene and point mutations of the β-globin gene in many ethnic groups. Both single PCR and multiplex PCR have been developed to diagnose both α-thalassemia breakpoints as well as β-thalassemia mutations. This PCR technique, however, is practicable only in highly specialized laboratories.

1.1.4 Silent form of thalassemia

The silent thalassemia is characterized by a very mild type of thalassemia, particularly in heterozygous form. It manifests barely on hematologic parameters, such as the MCV and MCH; thus making the values of these indeces not different from the normal ones. As a result, detection of this type of thalassemia is completely impossible by relying on only the red cell indeces. In practice, screening of this condition is accomplished by using the information from both the red cell indices and that from the α/β synthetic ratio (Cappellini 1997). Moreover, availability of family that has (a) obligate-carrier member(s) certainly helps diagnosis of this type of thalassemia. Both α- and β-globin gene defects can cause the silent thalassemia in many ethnic groups. In α-globin gene defect, for instance, the $-\alpha^{3.7}$, $-\alpha^{4.2}$, α^{NcoI}, α^{HphI} and $\alpha^{Constant\ Spring}$ have been shown to be responsible for the silent phenotypes of α-thalassemia. In silent β-thalassemia, the $\beta^{-101\ (C-T)}$, $\beta^{IVS2nt844}$, $\beta^{-90\ (C-T)}$, $\beta^{-28\ (A-G)}$, $\beta^{-86\ (C-G)}$ and Hb Knossos (β27 Ala-Ser) have also been reported to be involved. In addition to the defects stated above, the double heterozygous state for α/β-thalassemia, homozygous α-thalassemia 2 with β-thalassemia hetrozygote has also been shown to cause silent thalassemia in some individuals, presenting no alteration of initial screening results. Because synthetic rates of globin genes change very slightly, the degree of imbalanced globin chain synthetic ratio is almost negligible and α/β-ratio is approaching 1.00. Based of this situation, ineffective erythropoiesis is minimal and, as a result, hematologic phenotype remain unaltered. Thus, in almost all cases of silent thalassemia, the MCV value is normal or

low normal (around 80 fL or more than 80 fL). Employing MCV in the screening protocol could certainly lead to mis-diagnosis.

1.1.5 Double heterozygous α/β-thalassemia

Double heterozygote of α and β-thalassemia is characterized by the reductions of synthetic rate of both α- and β-globin chains in the same degree. As a result, the α- and β-synthetic ratio approached 1.0 (Weatherall and Clegg 2001). This seemingly balanced globin chain ration could then alleviate the severity of the disorder. The affected individuals have no clinical symptoms and various hematologic pictures with some resembling silent thalassemia and some identical to the β-thalassemia heterozygote. These situation could lead to the wrong diagnosis by mis-interpreting as being normal or heterozygote for only one type of thalassemia. Thus, consideration for this phenomenon must be taken seriously when carrying out heterozygote screening in the area where α- and β-thalassemia are common as discusses previously. The initial laboratory screening approach for this combination of thalassemia should include the use of red blood cell indices (MCV) in combination with HbA_2 levels and DNA analysis.

Double heterozygous α/β-thalassemia is highly prevalent in the region where α- and β-thalassemia are endemic. Surveys performed by many centers has revealed that this phenomenon is fairly common, particularly in Asia. Wee and colleagues (Wee, *et al* 2008) has found that 12.7% of Malaysian have this disorder, while Li, et al (Li, *et al* 2006) observed that 3.2% of Chinese people are affected by the double α/β-thalassemia. In Thailand, surveys at Maharaj Nakorn Chiang Mai Hospital carried out by Dr. Chanane

Wanapirak and co-workers of PND clinic reported the incidence of double α/β-thalassemia to be approximately 1.2% (Wanapirak, *et al* 2004). Recently, the survey performed by Dr. Thanusak Tatu and colleagues using blood samples from the Out Patient Department (OPD) of Maharaj Nakorn Chiang Mai Hospital has also indicated that double heterozygous α-thalassemia 1 (SEA type)/β-thalassemia and double heterozygous α-thalassemia 1 (SEA type)/HbE possessed the incidence of 4.3% and 4.3%, respectively (Tatu, *et al* 2006). Based on these data, the DNA-based diagnosis is required in these areas.

1.2 Literature review

Both silent α- and silent β-thalassemia are frequently found across the world. In the silent α-thalassemia, defects on the α-globin gene spanning from 5' promoter region to the 3' untranslated region (UTR), including the poly A addition site (AATAAA) have been indentified. The defects included both deletions and point mutations of part of the gene, particularly in the regions outside the coding sequences or exons. The survey in Italy by Bianco, *et al* demonstrated that the $-\alpha^{-3.7}$, $-\alpha^{NcoI}$, $-\alpha^{HphI}$ were invloved in the silent α-thalassemia in which the hematological phenotypes were completely normal in heterozygous state (Bianco, *et al* 1997). Moreover, the study in Arabians revealed that the A-G substitution at poly A site 3' to the α2-globin structural gene was associated with the phenotype of silent form of α-thalassemia (Fei, *et al* 1992). The mutation affecting initial codon of α1-globin gene was also shown to be involved in silent α-thalassemia. This phenomenon (ATG changs to GTG) was observed among Sardinians in the survey

carried out by Moi and co-worker in 1987 (Moi, *et al* 1987). Apart from those stated defects, silent α-thalassemia was found to be caused by the A-G substitution in IVS1-nt116 of α2-globin gene. This defect was seen in Dutch by the study undertaken by Harteveld et al in 1996 (Harteveld, *et al* 1996).

In Thailand, apart from the common $-\alpha^{-3.7}$ causing the α-thalassemia 2, other α-structural variants have also been shown to cause the silent α-thalassemia. These α-structural variants include Hb Suan Dok (α2-globin gene with CD109: T-G; Leu-Arg) (Sanguansermsri, *et al* 1979), Hb Constant Spring (α2-globin gene with CD 142: T-C; Stop-Gln) (Thonglairoam, *et al* 1991) and Hb Paksè (α2-globin gene with CD 142: A-T; Stop-Tyr) (Viprakasit, *et al* 2002). Among these abnormal hemoglobin, Hb Constant Spring possesses more frequency in Thai population than other two abnormal hemoglobins.

In the side of double α/β-thalassemia, Thailand is said to be rich in α-, β-thalassemia as well as HbE. Based on this fact, chances of occuring of double form of α/β-thalassemia heterozygote and α/HbE heterozygote are rather high, the frequencies of which were mentioned earlier. As also discussed earlier that only initial screening protocol was not sufficient to correctly identify these condition and that DNA–based diagnosis is required. Thus, to make things simpler, several authors have developed the so-called "multiplex allele specific PCR"; i.e. to simultaneously detect the causative mutaions on α- and β-globin gene. This approach was initiated by Nirut Siriratnaneewong and co-workers in 2001 to detect β-thalassemia mutations and α-thalassemia 1 (SEA type) (Siriratmanawong, *et al* 2001). In this study, DNA preparation was accomplished by

standard method (phenol/chloroform extraction) and only one type of β-globin gene mutation was detected simultaneously with the α-thalassemia 1 (SEA type).

1.3 Rationale and hypothesis of the study

As mentioned previously, silent α-thalassemia express very mild hematologic alteration, particularly the MCV which is always more than 80 fL. In practice, thus, diagnosis of this disorders is made by only determining the α/β-globin synthetic ratio in addition to the information from family history. Hb Bart's (γ_4) is the well-recognized marker for α-thalassemia, the level of which correlates positively to the severity of the disease. In heterozygous state, cord blood collected during delivery is the only reliable source of sample for Hb Bart's detection, to make the diagnosis, using the techniques routinely employed such as cellulose acetate electrophoresis at alkaline condition (CAE), high performance liquid chromatography (HPLC) and low pressure liquid chromatography (LPLC). However, in the adult life, the production rate of Hb Bart's reduced drastically, making its level fall below the detection limit of those routine methodologies. In this case, high-sensitivity detection technique is thus required.

In the survey performed by Ms. Sumontida Sayachak (Former MS student at Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University), four adult blood samples with normal preliminary thalassemia screening results were observed. However, the determination of Hb Bart's levels using ELISA technique revealed that their Hb Bart's levels were higher than normal (Table 1.1) (Sayachak 2006). Thus, these four samples

should be affected by α-thalassemia and, with the normal phenotype, fell into the category of silent α-thalassemia based on the definition stated earlier. The determination of underlying molecular backgrounds would then help understand the phenomenon observed in these blood samples. It was hypothesised that if the molecular backgrounds are revealed, then Hb Bart's levels would be applicable as the marker for the silent α-thalassemia.

Moreover, this study also found higher Hb Bart's than normal values in nine and five samples initially diagnosed as β-thalassemia and HbE traits, respectively (Table 1.1). Hence, it might be possible that α-thalassemia determinants co-segregated with β-thalassemia and HbE alleles in these particular samples (double α/β-thalassemia heterozygote and double α/HbE-heterozygote). This observation prompted the development of simple detection technique for detecting these double form of thalassemia heterozygotes in the population by setting up the multiplex allele-specific PCR utilizing the target DNA which was directly prepared from whole blood by Chelex™ extraction. Thus it was hypothesis that the simple detecting system for double form of thalassemia heterozygotes is successfully set up and that the levels of Hb Bart's could also be used as the marker for this condition as well.

1.4 Objectives

1. To determine the molecular background of silent α-thalassemia in 4 blood samples with normal hematological parameters and with detectable Hb Bart's level

2. To develop the multiplex allele-specific PCR to detect double heterozygotes of α/$\beta^{41/42}$- thalassemia, α/β^{17}-thalassemia and α-thalassemia/HbE

Table 1.1 Hematological parameters, Hb identification and Hb Bart's level in 18 samples analyzed in this study. Samples numbers 1-4 (N and R/O AH) are those suspected to be silent α-thalassemia and samples numbers 5-18 are those suspected to be double heterozygous α/β-thalassemia (5-13, BH) and double heterozygous α-thalassemia/HbE (14-18, EH)

No.	Diag.	RBC	Hb	Hct	MCV	MCH	MCHC	OF	HPLC	% A_2	Bart's.(ng/ml.)
1	N	3.87	11	33.9	87.6	28.4	32.4	neg	A_2A	1.8	91,790.40
2	N	4.44	11.4	37.3	84	25.7	30.6	neg	A_2A	3.0	24,413.79
3	N	4.58	13.2	40.3	88	28.8	32.8	neg	A_2A	2.4	5,924.29
4	R/O AH	5.25	12.8	39.9	76	24.4	32.1	neg	A_2A	2.8	100,997.49
5	BH	6.91	14.4	46	66.6	20.8	31.3	pos	A_2A	6.5	21,619.53
6	BH	5.94	11.5	39.6	66.7	19.4	29	pos	A_2A	5.8	4,550.63
7	BH	6.42	12.1	40.8	63.6	18.8	29.7	pos	A_2A	5.1	9,852.20
8	BH	5.87	12.6	41.2	70.2	21.5	30.6	pos	A_2A	5.2	40,258.01
9	BH	7.01	14.1	46.2	65.9	20.1	30.5	pos	A_2A	5.1	6,636.61
10	BH	5.62	12.1	39.5	70.3	21.5	30.6	pos	A_2A	6.8	188,619.61
11	BH	6.19	12	38.9	62.8	19.4	30.8	pos	A_2A	4.8	17,342.73
12	BH	6.36	12.5	41.1	64.6	19.7	30.4	pos	A_2A	3.9	6,121.44
13	BH	5.57	12.5	40.8	73.2	22.4	30.6	pos	A_2A	5.5	7,039.09
14	EH	4.91	12.4	38.9	79.2	25.3	31.9	pos	AE	20.0	33,311.73
15	EH	5	12.2	38.4	76.8	24.4	31.8	neg	AE	22.8	16,209.35
16	EH	5.09	12.4	39.2	77	24.4	31.6	neg	AE	25.6	15,965.43
17	EH	5.94	15.2	47.2	79.5	25.6	32.2	pos	AE	22.3	16,564.74
18	EH	5.79	14	43.8	75.6	24.2	32	pos	AE	26.8	4,816.33