CHAPTER I
INTRODUCTION

1. Rationale and Background

1.1 Thalassemia and abnormal hemoglobin

The thalassemia are genetic disorders of hemoglobin synthesis characterized by a reduction in the synthesis of particular globin chains. There are two main classes of thalassemia, α or β, in which the α- and β-globin genes are involved, and rare forms due to abnormalities of other globin genes. These conditions all have in common an imbalance rate of production of the globin chains of adult's hemoglobin, α-chains in β-thalassemia and β-chains in α-thalassemia. Several hundred different mutations at the α- and β-globin loci have been defined as the cause of the reduced or absent output of α or β chains [1]. In contrast, 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 [3].

1.1.1 α-Thalassemia

The synthesis of α-globin chains is directed by the duplicated α-globin genes (α2 and α1) located within the α-globin gene cluster on the short arm of chromosome 16. The α-thalassemia are the most common hemoglobin disorder, most frequently α-thalassemia is caused by deletions within the α-globin gene cluster that either reduce (α^+ or α-thalassemia 2) or completely abolish (α^0 or α-thalassemia 1) the production of α-globin chains by the affected allele. Less commonly, non-deletion α-thalassemia determinants ($\alpha^T\alpha$ or $\alpha\alpha^T$) involve nucleotide changes, usually within the dominantly expressed α2-globin gene [4].

There are potentially several hundred different interactions that could take place between the large number of α-thalassemia determinants that have been described. Phenotypically these interactions result in one of three broad categories; α-thalassemia trait, in which there are mild hematologic changes but no major clinical abnormalities, Hb H-disease, and the Hb Bart's hydrops fetalis syndrome. Four clinical syndromes are associated with α-thalassemia [5]:

1.1.1.1 Heterozygous α-thalassemia 1

1.1.1.2 a. Mild heterozygous α-thalassemia 2, or "silent carrier."

b. Hb Constant Spring trait ($\alpha\alpha/\alpha^{CS}\alpha$).

1.1.1.3 Hb H disease: the result of combination of (1) and (2a) or (2b).

1.1.1.4 Hydrops fetalis associated with Hb Bart's: homozygosity for the α- thalassemia 1 gene.

1.1.2 β-Thalassemia

The synthesis of β-globin chains is directed by the β-globin genes on the chromosome 11. There are diverse group of disorders of hemoglobin synthesis. Most mutations are small nucleotide substitutions within the β-globin gene cluster but the other mutations, including deletions, also occur. All mutations result in either the absence of β-globin chain synthesis (β^0-thalassemia) or a reduction in synthesis (β^+-thalassemia). β^0- alleles are truncating mutations, wheareas β^+ alleles alter the transcription or mRNA processing. β^0 and the majority of β^+ alleles give rise to thalassemia major, however a small number of β^+ alleles give milder thalassemia disease.

The β-thalassemias are heterogeneous clinical conditions. Hypochromia and microcytosis red blood cells with slightly low hemoglobin concentration, are characterizes in all forms of β-thalassemia [6]. Most homozygous states suffer from a severe syndrome and require regular blood transfusions to survive. This makes β-thalassemia one of the most significant single genes disorders globally.

1.1.3 Abnormal hemoglobin

The majority of Hb variants described are due to mutations in the α or β chain of the major adult component, Hb A. 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. The mutations of hemoglobin variants alter properties of Hb to various degrees. The properties altered include oxygen binding, molecular stability, easy of auto-oxidation, clinical or hematological effects.

Most mutations found in Thailand do not result in any abnormalities in properties or clinical manifestations. However, Hb E and Hb Constant Spring, the most common variants in Thailand have thalassemic effects [7].

1.1.3.1 Hb E

Hb E is the most common β-thalassemic hemoglobinopathy among Asian populations particularly in the Northeast of Thailand where its frequency is between 20-30% [4, 8]. Hb E results from the substitution of lysine for glutamic acid in the β-chain of hemoglobin, which acts as a mild β^+-thalassemia. In the heterozygous state, patients suffer from a mild microcytic anemia with decreased erythrocyte survival. Target cells are visible on peripheral blood smears. Hb E is slightly unstable and there is an associated thalassemic component. It is relatively harmless in the homozygous state Hb E, but also results in a severe hemoglobinopathy when combined with a β-thalassemia gene.

1.1.3.2 Hb Constant Spring

Hb Constant Spring (CS), an α chain C-terminal extension, is the next most frequent variant in Thailand. This Hb variant is very unstable and is very difficult to detect on electrophoresis. Hb CS represents 1 to 2 % of total hemoglobin in heterozygotes state which are clinically and hematologically normal. Homozygosity for Hb CS shows a clinical picture of mild thalassemia intermedia with mild anemia, jaundice and hepatosplenomegaly. When combined with deletional α-thalassemia 1, Hb H disease results [8].

Thalassemia and hemoglobinopathies are common genetic disorders in Asian population. In Thailand, the prevention and control program of thalassemia has now been implemented throughout the country with the aim of reducing new case with the following diseases:

(1) Homozygous α-thalassemia 1 or Hb Bart's hydrops fetalis

(2) Homozygous β^0-thalassemia

(3) β^0-thalassemia / Hb E

Strategies include 1. providing appropriate treatment for the existing patients and 2. prevention of a new case with severe thalassemia. These important steps are carrier screening, genetic counseling and prenatal diagnosis.

1.2 Prenatal diagnosis of thalassemia

Several programs, based on carrier screening and counseling of couples at marriage, preconception or early pregnancy, are operating in several at-risk populations. These programs have been very effective, as indicated by increasing knowledge on thalassemia and its prevention by the target population and by the marked decline of the incidence of thalassemia major.

Prenatal diagnosis of thalassemia syndromes and hemoglobinopathy can be performed directly by analysis of globin gene structure and composition in fetal DNA obtained from invasive procedures such as amniotic fluid, chorionic villus sampling (CVS) or fetal blood sampling. However, the main disadvantage is that the use of invasive procedures can not be carried out well until at the second trimester of pregnancy. Hence, if the fetus is affected with a form of severe thalassemia, the pregnancy has to be termination toward the end of the second trimester, which may be extremely distressing for the mother. On the other hand, fetal cells in maternal blood are a source of fetal genetic material for noninvasive prenatal diagnosis. Very small number of nucleated fetal cells can be isolated from maternal peripheral blood [9]. Enrichment of fetal cells in therefore essential. Most enrichment techniques are time-consuming, and require expensive equipment to distinguish maternal cells and fetal erythroblasts. However, it has been demonstrated that a large amount of cell-free fetal DNA is present in maternal plasma [10]. Fetal analysis in maternal plasma has been reported to be applicable to the prenatal diagnosis such as sex-linked disorders [11],

fetal rhesus D status [12], single-gene disorders [13], fetal aneuploidy and paternally inherited disorders [14]. In addition to the prenatal assessment of fetal genetic traits, the quantitative assessment of fetal DNA concentrations in maternal plasma have been demonstrated to be potentially useful for the monitoring of some pregnancy-associated complications. Aberrations in the fetal DNA concentrations in maternal plasma have been reported for preeclampsia, fetal aneuploidy, fetomaternal hemorrhage, preterm labor, polyhydramnios, and invasive placentation. For preeclampsia in particular, it has been shown that the elevation in fetal DNA concentration predates the development of signs of preeclampsia [15]. Furthermore, the extent of elevation appears to correlate with the clinical severity [16].

2. Problem and Research Rationale

In Thailand and other Asian countries, both thalassemia and Hb variants are very common. α-Thalassemia has a frequency of 20-30 % whereas that of β-thalassemia ranges from 3%-9% [4]. The average frequency of Hb E is approximately 13% (but rises to near 50% in the north-east) and that of the Hb Constant Spring varies between 1 and 8% [8]. Other abnormal Hbs caused by both α-chain and β-chain variants are occasionally reported [17]. Heterozygous individuals are easily identifiable by hematologic and molecular analysis, permitting the control of the serious hemoglobinopathies by a program of carrier screening, counseling, and prenatal diagnosis by fetal DNA analysis. In Thailand, the most important disorders which prenatal diagnosis are homozygous α^0-thalassemia, homozygous β^0-thalassemia and β-thalassemia / Hb E.

α^0-Thalassemia is the most severe type which results in the Hb Bart's hydrops fetalis syndrome in homozygous state ($--^{SEA}/--^{SEA}$), is common in Thailand. Mothers of infant with this disorder often give a history of previous stillbirths or neonatal deaths. All reports point to an increased incidence of serious maternal complications [18-20]. Common complications include anemia, pre-eclampsia characterized by hypertension and fluid retention with or without proteinuria, polyhydramnios (excessive accumulation of amniotic fluid), oligohydramnios (decreased amniotic fluid), antepartum haemorhage and the premature onset of labour

[18-21]. Diagnosis of Hb Bart's hydrops fetalis syndrome is made when fetal hydrops is present and Hb Bart's constitutes the major hemoglobin in fetal blood. With better and earlier prenatal diagnosis of fetuses affected by this condition, homozygous α^0-thalassemia is often detected when the fetus has not shown any signs of hydrops on ultrasound. Mostly invasive method such as chorionic villus sampling (CVS), amniocentesis and fetal blood sampling are used for prenatal diagnosis of severe thalassemia syndromes. However, depending on invasive technique employed, the procedure-related miscarriage risk ranges between 0.5-2% [22]. The presence of fetal DNA in maternal plasma and serum has offered new approaches to non-invasive prenatal diagnosis [23]. Since fetal DNA in maternal plasma circulates in the background of maternal DNA, it has been used mainly for noninvasive prenatal diagnosis of paternally inherited disorders including fetal gender [24, 25], Rh status [12], myotonic dystrophy [26], achondroplasia [13], cystic fibrosis [27], congenital adrenal hyperplasia [28] and β-thalassemia major [29]. In 2006, Tungwiwat et al. [30] have developed a real-time quantitative semi-nested polymerase chain reaction (PCR) method for identifying the fetal α^0-thalassemia in maternal plasma by using a two-step PCR. Plasma DNA was amplified conventionally using α^0-thalassemia-specific primers and a portion of the first PCR product was subjected to a semi-nested real-time q-PCR using the SYBR green I chemistry for fluorescence detection. Increase fetal DNA concentrations have been reported for several pregnancy-related complication. The value of cell-free fetal DNA in maternal plasma as an indicator for pre-eclampsia has first been reported by Lo et al. [31]. It was increased approximately 5-fold in women with pre-eclampsia. The same effect was observed by Zhong et al [32]. They used a *Taq*Man assay for the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), which is present in all genomes, for determination of the total amount of circulating DNA present in the maternal plasma samples. The result showed that increases deoxyribonucleic acid levels corresponded with the severity in pregnancies complicated by pre-eclampsia. It is therefore interesting to determine the plasma DNA levels in pregnancies with Hb Bart's hydrops fetalis, a condition commonly associated with pre-eclampsia. This should further evaluate the uses of maternal plasma DNA level in pregnancy with severe α^0-thalassemia in Thailand.

In addition, the hemoglobinopathies refer to a diverse group of inherited disorders characterized by a reduced synthesis of one or more globin chains (thalassemia) or the synthesis of a structurally abnormal hemoglobin. In Thailand, more than 30 hemoglobin variants have been reported. Most mutations do not result in any abnormalities in properties or clinical manifestation [17]. The key to identifying the globin gene mutation in carriers and affected patients is an understanding of the genotype-phenotype relationships of various globin gene mutations and the effects of interaction when several mutations are co-inherited. An appreciation of these phenotypes is needed to facilitate the definitive diagnosis of the causative mutations to inform management and counseling. Hematological and family studies provide essential clues to the different interactions and are fundamental to DNA diagnostics of the Hb disorder. Since an introduction of the prevention and control of thalassemia in Thailand, there are accumulations of unknown abnormal Hbs at the Thalassemia Service Unit, Faculty of Associated Medical Sciences. Further characterization of these abnormal Hbs by both hematological and molecular methods should lead to ways to development of appropriate diagnostic methods for use in daily practice. This should provide further understanding on the genotype-phenotype correlations associated with these unknown variants as well as facilitate an effective prevention and control of hemoglobinopathies in Thailand.

3. Objectives of the study

3.1 To determine the amount of maternal plasma DNA in pregnancies with and without Hb Bart's hydrops fetalis.

3.2 To further characterize molecularly and hematologically unknown abnormal Hbs encountered at the CMDL, Faculty of Associated Medical Sciences, Khon Kaen University.

4. Anticipated outcomes

4.1 Basic knowledge on the amount of maternal plasma DNA as measured by GAPDH assay in pregnancies with and without Hb Bart's hydrops fetalis which might be used as a predictive marker of the development of this syndrome.

4.2 Genotype-Phenotype interaction and diagnostic assays of Hb variants commonly found in Thailand.