CHAPTER IV

DISCUSSION AND CONCLUSION

α-thalassemia heterozygotes causing from the ($--^{SEA}$) deletion is the commonest mutation found in Southeast Asia, including Thailand. This mutation is 20.5 kb in length deletion of both α-globin genes in *cis*. Homozygosity of this deletion ($--^{SEA}/--^{SEA}$) fatally affects the infants causing death just before birth or shortly after birth. This condition is conventionally known as Hb Bart's hydrops fetalis syndrome. Couples of these thalassemia carriers have 25 % risk in each pregnancy conceiving a fetus with Hb Bart's hydrops fetalis syndrome. The heterozygous carrier rate of the ($--^{SEA}$) deletion α-thalassemia 1 is ranging from 3.5% in Northern Taiwan (Chui and Waye, 1998) 4.5% in Hong Kong (Lau *et al.*, 1997) 4.14% in China (Xu *et al.*, 2004) to 14% in Northern Thailand (Chui and Waye, 1998).

In Thailand, thalassemia is one of the important chronic health problems and has to be continuously solved, especially in Northern part of Thailand (Tangvarasittichai *et al.*, 2005; Lemmens-Zygulska *et al.*, 1996). The prevalence reported of α-thalassemia carriers was as high as 20-30 % (Winichagoon *et al.*, 1992). At the Mahraj Nakorn Chiang Mai Hospital, the main hospital of Northern Thailand, were reported to have α-thalassemia 1 (SEA type) 6.6% (Wanapirak *et al.*, 2004). As this information, identification of α-thalassemia 1 (SEA) type is essential for the thalassemia prevention and control program for preventing new case of Bart's

hydrops fetalis. Determination of the α-thalassemia carrier phenotype requires the CBC analysis and Hb typing, especially the measurement of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and finding microcytosis (MCV < 80 fL) or hypochromia (MCH < 27 pg) (Panyasai *et al*., 2002; Wang *et al*., 2000). α-thalassemia 1 (SEA type) usually have a MCH below 27 pg, Hb A_2 is below 3.5 and Hb F level is normal (Old, 2003). Chui and Waye (Chui and Waye, 1998) found that the mean and SD of MCV and MCH for ($--^{SEA}/\alpha\alpha$) carriers are 67.8 ± 3.3 fL and 21.8 ± 1.2 pg, respectively. They also found that the hemoglobin levels for these men and women are 135 ± 10 g/L, and 121± 10 g/L, respectively. In most of α-thalassemia 1 (SEA type) have normal Hb levels, normal Hb A_2 and usually do not have significant anemia feature but show only mild anemia pattern or microcytosis.

The problems of screening for α-thalassemia 1 (SEA type) are the requirement of the combination of many methods and each method having its own limitation in detecting of α-thalassemia heterozygotes. The specific screening method is to detect Hb H inclusion bodies. Excess β-globin chains can form homotetrameric Hb H (β_4). After supravital staining of RBC with the redox agent such as brilliant cresyl blue dye, Hb H being unstable, precipitates and aggregates to form tetra erythrocytic inclusions, which is present in approximately 1 of 10,000 RBCs in individuals with α-thalassemia minor (Pan *et al*., 2005). However, this method is often unsatisfactory in sensitivity. Wang *et al*. (Wang *et al.* 2000) reported that no person with -α/αα or -α/-α genotypes had positive result for the test and only 79% of persons with the --/αα genotype had positive for Hb inclusions. Therefore, genotyping by PCR-based method should be confirmed. The multiplex PCR was established for thalassemia

diagnosis. The PCR method is more effective and more sensitive for the diagnosis of α-thalassemia 1 than Hb H inclusion bodies (Bergstrome and Poon, 2002). Although genotyping is essential for detection carrier status, the complexity of this technique limits its applicability as a screening method and not always available in areas where molecular analysis is not affordable. In addition, PCR is high sensitivity that it tends to amplify trace contaminant and consequently generates false-positive results (Chern and Chen, 2000). Moreover, DNA analysis is still relative expensive and available in only a few specialized laboratories.

Recently, an ELISA was developed for detection of embryonic ζ-globin chains. This technique was later used to diagnose of α-thalassemia 1 (SEA type) in adulthood with high sensitivity and specificity (Ma *et al*., 2002). However, Lafferty *et al*. (2000) found that ζ-globin ELISA kit were also positive in persons with ($-\alpha^{3.7}/-\alpha^{3.7}$), ($-\alpha^{3.7}/\alpha\alpha$), ($-\alpha^{4.2}/\alpha\alpha$) or ($\alpha\alpha/\alpha\alpha$) genotypes.

In this study, an alternative screening method for detecting of α-thalassemia heterozygote, especially α-thalassemia 1 (SEA) type using sandwich ELISA was developed. The ELISA employed 2 mAbs, anti-Hb Bart's mAb clone Thal GJA and anti-hemoglobins mAb clone Thal N/B. The ascitic fluid containing mAb Thal GJA and mAb Thal N/B were produced in BALB/C mice. Then, they were purified by affinity chromatography using the HiTrap Protein G column. The specificity of the purified mAb was confirmed by indirect ELISA. The results demonstrated that mAb Thal GJA only reacted with Bart's hydrops fetalis hemolysate. MAb Thal N/B showed positive results in Bart's hydrops fetalis hemolysate, cord blood hemolysate and normal hemolysate. The specificity of mAb Thal GJA was further evaluated using purified form of Hb Bart's, Hb F, Hb A, Hb A_2 and Hb E by ELISA. It was found

that mAb Thal GJA reacted to only purified form of Hb Bart's and did not cross-react with other hemoglobins. The results indicated that mAb Thal GJA was an Hb Bart's specific mAb and can be used in the development of sandwich ELISA for detection of Hb Bart's for α-thalassemia 1 carrier screening purpose.

For sandwich ELISA, horseradish peroxidase (HRP) conjugate is needed. mAb Thal N/B which react all hemoglobins was selected for use as secondary antibody. In order to produce HRP conjugated Thal N/B, one-milligram protein of mAb Thal N/B was labeled with HRP (Porstmann *et al*., 1985; Imagawa *et al*., 1982). The HRP labeled Thal N/B mAb was then tested for using as conjugate in the sandwich ELISA. In this study, the optimization of primary antibody and conjugate used in the ELISA were first determined. It was found that 100 μg/ml of mAb Thal GJA for coating plate and 1.25 μg/ml of HRP conjugated Thal N/B used as conjugate were the optimal concentrations. The optimized ELISA has very high sensitivity which could detect less than 10 ng/ml of Hb Bart's. The developed ELISA was then used to determine the Hb Bart's in various type of thalassemia and normal hemolysates.

A total of 91 EDTA-blood samples of adult Thai subjects who attended at Maharaj Nakorn Chiang Mai hospital were deliberately selected from blood samples by screening for blood having microcytosis using routine complete blood count. By hematological data using semi-automated hematology analyzer the samples were divided into two groups: In group 1, which consists of 60 samples, hematological data was diagnosed as α-thalassemia heterozygote or β-thalassemia heterozygote. Of the remaining 31 samples, in group 2, were hematological parameters normal. All samples of the both groups were repeated CBC and followed by one-tube osmotic

fragility test (OF test). It was found that a total of 56 samples in group 1 were positive for the OF test and 4 samples in this group were negative. Although 31 sample normal controls (group 2) showed negative results, 4 samples of them were later found to be α-thalassemia 2 heterozygote with 3.7 kb deletion by DNA analysis. From the results, the used OF test has low specificity for detection of α-thalassemia heterozygote because anemias with reduced MCV and MCH can also be positive. This might increase the workload of PCR later.

To identify the α-thalassemia heterozygote, 60 samples in group 1 were subjected to microscopic examination of the Hb H inclusion bodies. PCR analysis was used to confirm the diagnosis of the α-thalassemia heterozygote. 34 of 40 samples that had Hb H inclusion bodies were heterozygous of α-thalassemia. In addition, it was found that there are as high as one-third of β-thalassemia heterozygote or Hb E heterozygote without α-thalassemia 1 (SEA deletion) and α-thalassemia 2 (both 3.7 and 4.2 kb) deletions had Hb H inclusion bodies positive. These data demonstrate that Hb H inclusion bodies test has poor sensitivity and subjective. Additionally, 9 samples with negative results by Hb H inclusion bodies were found to be α-thalassemia heterozygote. This is another limitation in the use of Hb H inclusion bodies as a screening test. However, the determination for Hb H inclusion bodies by concentration method may increase its sensitivity. It was reported that, by the concentration method, Hb H inclusion bodies could be detected in α-thalassemia heterozygote up to 92% (Intapan, 1981).

In order to verify the developed sandwich ELISA for screening thalassemia carriers, all 91 hemolysate were subjected for the determination of Hb Bart's. It was

found that 24 out of 26 samples who were α-thalassemia 1 (SEA type) had Hb Bart's concentrations between 6.21 μg/ml to 125.57 μg/ml, while double heterozygotes of β-thalassemia and α-thalassemia 1 carrying ($--^{SEA}$) deletion gene had Hb Bart's concentrations between 4.50 μg/ml to 156.2 μg/ml. Double heterozygotes of Hb E and α-thalassemia 1 with SEA deletion had Hb Bart's concentrations ranged between 8.81 μg/ml to 36.92 μg/ml. Range of the Hb Bart's concentrations was 4.55 μg/ml to 84.44 μg/ml in double heterozygotes of β-thalassemia and α-thalassemia 2 (3.7 kb deletion). There was one sample each with double heterozygotes of Hb E and α-thalassemia 2 (4.2 kb deletion) and heterozygous of α-thalassemia 2 in which Hb Bart's concentrations was 83.21 μg/ml and 6.41 μg/ml, respectively. In normal population, 25 of 27 samples had Hb Bart's concentrations was less than 4.21 μg/ml. Surprisingly, 2 out of 27 normal samples had high level of Hb Bart's (5.92, and 10.01 μg/ml).

In general thalassemia screening, elevation of Hb A_2 is the main diagnostic β-thalassemia heterozygote (Weatherall and Clegg, 2001). Double heterozygotes of α-thalassemia 1 with β-thalassemia can reduced level of Hb A_2, however, its levels are still higher than normal (Li *et al*., 2006). Li *et al*. (2006) showed that the presence of a α-thalassemia allele does not suppress the increase in Hb A_2 in β- thalassemia heterozygote. Accordingly, they are mistakenly identified as pure β-thalassemia heterozygotes, therefore screening for α-thalassemia 1 ($--^{SEA}$) in β- thalassemia heterozygotes is highly recommended in order to achieve a prevention of severe α-thalassemia. In fact Siriratmanawong *et al*. (2001) established rapid PCR method for the simultaneous detection of β-thalassemia and α-thalassemia 1 gene for diagnosis of

complex αβ-thalassemia syndrome. In this study, 4 of β-thalassemia heterozygotes and 2 of Hb E heterozygote samples contained Hb Bart's level was greater than cutoff value 4.50 μg/ml. Although the published prevalence of (--^{FIL}), (--^{MED}), (--$^{20.5}$), and (--^{THAI}) deletions is low (Bernini *et al*., 2001), in this study, blood samples were randomly collected in which these deletion may occur. In addition, these samples had not detectable SEA deletion, 3.7 and 4.2 kb deletions. All mention samples had either coinheritance of β-thalassemia heterozygotes or Hb E heterozygote with others α-thalassemia type deletion or single-point mutation or oligonucleotide insertions and deletions involving the canonical sequence that control gene expression, the so called 'non deletion variants, in which the affected gene is denoted α^T (Weatherall and Clegg, 2001) such as $\alpha\alpha^{CS}$ which have been reported in South East Asian (Boonsa *et al*., 2004). Therefore, these samples should be investigated further. The other 4 of β-thalassemia heterozygotes and 5 of Hb E heterozygote samples had low level of Hb Bart's concentrations was less than 4.30 μg/ml. Twenty-five out of twenty-seven normal samples contained low level of Hb Bart's concentrations was less than 4.2 μg/ml while two samples contained high level of Hb Bart's. It was probable that these two samples have rare deletion or acquired form of α-thalassemia. Gene mapping and sequencing are requested for its confirmation.

Taken together, in this study, a sandwich ELISA was developed for measurement of Hb Bart's in hemolysates. Our results indicate that the developed ELISA had highly sensitivity and specificity. By using a cutoff value (4.5 μg/ml), the sensitivity and specificity of this test for the detection of the α-thalassemia 1 with (--^{SEA}) deletion heterozygote were 95% and 71 %, respectively. For α-thalassemia 1

with ($--^{SEA}$) deletion and α-thalassemia 2 heterozygotes with 3.7 and 4.2 kb deletions, the ELISA had sensitivity and specificity of 86% and 77%, respectively.

For Hb H disease (--/-α), the most frequently results from the interaction of α-thalassemia 1 (--/αα) and α-thalassemia 2 (αα/α-). Other less frequent genotypes an also result from the co-inheritance of α-thalassemia 1 (--/αα) and a nondeletional (--/$\alpha^T\alpha$) or homozygosity for nondeletion defects ($\alpha^T\alpha$/ $\alpha^T\alpha$). However, the loss of the α2 gene by nondeletion mutations (--/$\alpha^T\alpha$) results in a more severe phenotype and have level of Hb Bart's higher than the loss by deletion (--/-α) (Liebhaber, 1989). In Thailand 40-50% of individuals with Hb H disease carry the α^{CS} mutation (--/-α^{CS}) (Fucharoen *et al*., 1988). Therefore, quantitation of Hb Bart's in Hb H disease may classify between common of deletional form of Hb H disease (--/-α) and nondeletional form. From this information, it is of interest to apply the developed sandwich ELISA for determination the Hb Bart's level in Hb H disease.

Conclusions

In the current time, it is known that approximately 5-6% elevated level of Hb Bart's are associated with α-thalassemia heterozygotes, however, there is seldom appropriated method to identify α-thalassemia carriers. Results of the present study demonstrate that developed sandwich enzyme-linked immunosorbent assay is a useful, simple and inexpensive screening test to identify adult heterozygote of the (--^{SEA}) α-thalassemia 1 deletion and α-thalassemia 2 heterozygotes. Furthermore, developed sandwich ELISA has highly sensitivity (95%) so that it would be suited to use in high-volume screening program to identify adolescence and reproductive age with carrying (--^{SEA}) deletion that at risk of conceiving fetuses with Hb Bart's hydrops fetalis.