IV. DISCUSSION

α-Thalassemia is the most prevalent hemoglobinopathy in Thailand. The clinical disorders associated with severe α-thalassemia range from the moderately severe to transfusion dependent anemia characteristic of Hb H disease, to the lethal Hb Bart's hydrops fetalis syndrome. Hb H disease is the most severe phenotype compatible with life. The most common phenotype (--/−α) is usually not severe enough to interfere with the activities of daily living. However, the nondeletion form of Hb H disease (--/$\alpha^T\alpha$) has found in a more severe symptom with some patients being transfusion dependent (Paglietti *et al.*, 1986, Moi *et al.*, 1987; Kattamis *et al.*, 1988). In this nondeletion form, Hb Constant Spring is the most prevalent mutation that dysfunctions the α_2-globin gene; therefore, the reliable and convenient detection of this gene is useful for the diagnosis, treatment, and genetic counseling.

Hemoglobin Constant Spring was first observed by conventional hemoglobin electrophoresis in hemolysate obtained from Hb H disease patients (Weatherall and Clegg, 1965). However, this method usually fails to detect Hb CS in the heterozygotes; due to the very small amount of this hemoglobin (<1%), the instability of the protein, and the obscurity from Hb A_2 on the electrophoresis. This led to the development of several DNA-based diagnostic approaches.

Traditionally, the diagnosis of known point mutation or small deletion depended on allele specific oligonucleotide hybridization with its genomic DNA. The procedures of this method are tedious, time consuming, and technically demanding. With the advent of PCR technology, the manipulation on any specific gene is much easier and more reliable. If the mutation creates or abolishes a restriction site, it is easy to detect the mutations after digestion its specific PCR product with the correspondent restriction enzyme. Unfortunately, most of the mutations do not create or abolish restriction site. Therefore, oligonucleotide specific hybridization with PCR product are usually manipulated. Although PCR reduces many steps in the allele specific hybridization method, such as restriction enzyme digestion, gel electrophoresis and sometimes Southern blotting; detection with oligonucleotide specific hybridization is still difficult and usually involved in hazardous and cumbersome radioactive detection system. Even though many nonradioactive detection systems have been invented, they were not well documented.

The detection of Hb CS gene followed the development of DNA technology. It was first detected by allele specific oligonucleotide hybridization on restriction fragments of genomic DNA (Kosasih *et al.*, 1989; Laig *et al.*, 1990); followed by oligonucleotide specific hybridization on α_2-specific PCR product, with radioactive detection system (Kropp *et al.*, 1989; Hsie *et al.*, 1989), or with nonradioactive detection system (Fucharoen *et al.*, 1989). These methods met all the difficulties involved in hybridization and detection system.

In the year 1991, Kropp and his coworkers described a method of Hb CS gene detection by asymmetrically primed selective amplification/ temperature shift fluorescence polymerase chain reaction. The one step PCR that used asymmetric priming and a temperature shift to accomplish dual effects of α_2-specific amplification and discrimination of normal and Hb CS gene by allele-specific fluorescence primers. However, the method need the fluorescent primers and fluorescent detection system, which is not common in many molecular biology laboratories. Moreover, the need of extracting PCR product from their unincorporated primer was obviously demanded since it interfered the interpretation of the result. The interpretation could be more difficult if the temperature or the PCR reaction mixture was a little deviated from the protocol, which result in the priming of the wrong primer.

As described above, the most convenient and easiest method of detection one point mutation is the study of RFLP pattern from its specific PCR product, when the mutation creates or abolishes a restriction site. With the same principle as in site directed mutagenesis, the designed mismatched primer can also create a restriction site to a particular mutation. This principle was employed to detect Hb CS gene by Chang and his coworkers (1993). The 2-base-mismatched primer created a restriction site for restriction enzyme Tth 111I when the template was Hb CS gene. Unfortunately, their primers could also amplified $\psi\alpha_1$-globin gene. Thus, it could not discriminate homozygous and heterozygous Hb CS gene and the misinterpretation may occur if the mutation was in $\psi\alpha_1$-gene.

With the ongoing search for new restriction enzymes, more restriction sites have been found and are matched to the loci of more mutations. At the termination codon of α_2-globin gene, it locates a restriction site for restriction enzyme Mse I. The mutations at this locus abolish the restriction site and can make a different RFLP pattern from the normal. Hb CS is one of the mutation that occurs in this locus and can be excluded from the normal termination codon. With this principle, Makonkawkeyoon (1993) had developed a rapid method of detecting abnormal termination codon on α_2-globin gene. The method is very useful for population screening because all the four mutations that occur on this locus dysfunction the gene and lead to the thalassemic phenotype. However, this method cannot discriminate these four mutations apart .

In the present study, the method of Makonkawkeyoon was employed to detect the abnormal termination codon on α_2-globin gene. The sample that found to possess the mutation on this locus was subjected to semi-nested PCR with a restriction site created primer introduced by Chang, as described above. Thus, that sample was able to characterized whether it possessed Hb CS gene or not. The semi-nested PCR from the α_2-specific PCR product overcame the nonspecificity found in Chang's method. The whole results were concluded in Fig. 14; the first PCR product and its RFLP pattern after cutting with Mse I were shown in lane "a" and "b", the semi-nested PCR product and its RFLP pattern after cutting with Tth 111I were shown in lane "c" and "d", respectively.

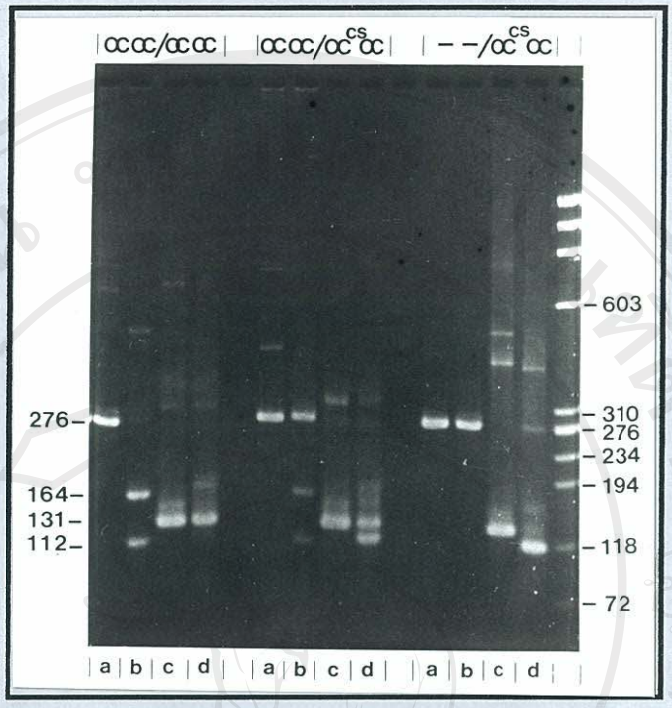

Figure 14 : The RFLP Pattern of Different Genotypes

The first round PCR product before cutting with Mse I are shown in lane "a" and after cutting are shown in lane "b". The semi-nested PCR before cutting with Tth 111I are shown in lane "c" and after cutting are shown in lane "d". DNA molecular weight marker (ϕX174 DNA/Hae III) is shown in the last lane. The genotype of each sample is shown above the picture. They were run on 3% agarose gel in 0.5x TBE buffer.

The method is simple and reliable. The first step of screening for abnormal termination codon are then confirmed by the detection of Hb CS gene. However, there are some precautions involving in the PCR and in the restriction enzyme cutting procedures.

The cross contamination are the frequent phenomenon happened in the PCR procedures. A likely mode of cross contamination is via aerosols normally generated during sample processing, as in mixing by vortex action, opening of microcentrifuge tubes, pipetting, ejecting pipette tips from micropipettors, and centrifugation. Aerosol particles can be large as 20 μm in diameter or about $4x10^{-6}$ μl in volume; therefore, one microdroplet of this size produced from a typical PCR will carry about 24,000 copies of an amplified product (Innis *et al*, 1990). This is more significant when doing with the nested PCR. The precautions should be aware at the very first steps of genomic DNA extraction. In the population screening, the step of DNA preparation should be finished before starting PCR procedures. The areas of DNA extracting, PCR preparation and PCR product processing should be separated. It should be highly aware with semi-nested PCR procedures, when the volume of target DNA was small and the cross contamination is likely.

In the step of restriction enzyme cutting, it should be aware that the amount of enzyme, the incubation time, buffer, and the substrate of restriction enzyme should be optimal for the complete digestion.

The method was used to detect the abnormal termination codon and Hb CS gene in northern Thailand populations. Of the 191 cord blood samples; all except 5 were found to possess normal termination codon on both α_2-globin gene. The other 5 samples were found to be Hb CS heterozygote. From this result, the prevalence of Hb CS in northern Thailand populations is 2.62% and the frequency of α^{CS} is 0.013.

When compared to the data provided by Laig and his coworkers (1990), the gene frequency of α^{CS} in northern Thailand presented there was more than double the figure presented here(0.033 vs. 0.013). The 151 samples used in that study were from the annual army induction examinations. At the same year, Chamrasratanakorn was reported the prevalence of α^{CS} gene in northern Thailand in her thesis for Master's degree, the 112 samples used in her study was also from the annual army induction examinations. The prevalence of Hb CS gene in her study was 3.57% (4 out of 121) and the frequency of α^{CS} gene was 0.0178. Since these two reports were from the healthy adults which were a selected group of people at the specific area, the value may not represent the whole populations. Study in non selected consecutive cord blood of newborns should give a more accurate figure. Nevertheless, neither of these studies could represent the northern Thailand populations, since the number of samples investigated in all studies were small. The accurate gene frequency is yet to come after the routine diagnosis of this particular gene has been established.

The method was also used to detect Hb CS gene in 18 samples that were characterized by Southern hybridization to possess the genotype of (--/$\alpha^T\alpha$) (Muangmoonchai, 1994). From both RFLP patterns, 12 samples were found to have Hb CS gene on their only α_2-globin gene. Therefore, these patients have the genotype of (--/$\alpha^{CS}\alpha$). The only one sample that had normal termination codon may possess a mutation in other region. The direct nucleotide sequencing may be needed to solve this mutation.

Of the twelve samples that found to possess Hb CS gene; the semi-nested PCR from some samples were not completely cut with restriction enzyme Tth 111I. They left a faint band at the position of semi-nested PCR product. This may be resulted from the following reasons.

1. The partially purified PCR product that used as DNA template for semi-nested PCR still contained small amount of genomic DNA from the first step PCR. The amplification product could also come from the α_1-globin gene and be the minor product produced.

2. The enzyme digestion in some particular tubes were partially inhibited by some reagents in the semi-nested PCR product.

3. There were some nonspecific products occurred from the step of semi-nested PCR. Some of these products could be cut with Tth 111I and the digested product may have the size near the semi-nested PCR product.

4. It may come from cross contamination via aerosol from the normal α_2-globin PCR product.

For the other 5 samples (H14-H18), the RFLP patterns showed that there were two species of DNA template, one for the normal termination codon and one for the α^{CS} gene. These samples had also been characterized by Southern hybridization to possess the genotype of (--/$\alpha^{T}\alpha$); therefore, they should not possess two α_2-globin template as seen from both RFLP patterns. These samples were repeated three times to ensure the correction of the procedures during PCR reaction. Thus, the results reflected to the DNA template itself. Because the RFLP patterns were similar to Hb CS heterozygote, these samples were subjected to test for the ($--^{SEA}$) deletion by PCR, as described by Bowden *et al.*, (1992). Every samples were found to possessed the ($--^{SEA}$) deletion as shown in the 0.66 kb PCR product in Fig. 13, confirmed with the result from Southern hybridization. Therefore, they were not the samples from Hb CS heterozygote. Because these DNA samples were collected since 1990 and have been used for many purposes, it is likely that these samples were contaminated.

The gene frequency of α-thalassemia 2 in northern Thailand is 0.0991 (Hundrieser *et al.*, 1991), while the frequency of α^{CS} gene from this study is 0.013; thus, the compound heterozygote of α-thalassemia 1 and α-thalassemia 2 (--/-α) should be 7.6 times more than the compound heterozygote of α-thalassemia 1 and α^{CS} gene (--/$\alpha^{CS}\alpha$). However, from the study of Muangmoonchai (1994), 21 Hb H patients who came to thalassemia clinic at Maharaj Nakorn Chiang Mai during the year 1992 were characterized by Southern hybridization to possess the genotypes as follow; 13 samples were (--/$\alpha^{T}\alpha$), 6 samples were (--/$\alpha^{-3.7}$), and 2 samples

were (--/$\alpha^{-4.2}$). The nondeletional Hb H disease samples were further characterized in this study, all but one of these samples possessed α^{CS} gene. Thus, 12 patients had genotype of (--/$\alpha^{CS}\alpha$) compare to 8 patients with the genotype (--/-α). The proportion of Hb CS-Hb H disease patients were even greater than the deletional Hb H disease patients.This can reflect the more severity of α^{CS} gene than the loss of one α-globin gene as found in the gene expression studies. Therefore, Hb Constant Spring should have more consideration and routine diagnosis should be established.