II. RESEARCH DESIGNS AND METHODS

II.1 Research Design for Detection of Hb Constant Spring Gene

According that Hb CS gene is characterized by a mutation (TAA--> CAA) at the termination codon of α_2-globin gene, the method of Polymerase Chain Reaction (PCR) was applied to amplify specifically on this area. The PCR products were then the subject for restriction enzyme Mse I, which recognize the sequence TTAA, in order to screen out normal termination codon samples. The samples that showed partially cut or no cut were expected to have Hb CS gene. In order to proof this expectation, the PCR products were subsequently taken further step of semi-nested PCR with a special primer that can produce restriction site of restriction enzyme Tth 111I when the site has Hb CS gene. The products that could be cut with Tth 111I were considered to have Hb CS gene.

The method of each procedure was given in detail later in this chapter. The diagram below shows the whole procedure orderly.

BLOOD SAMPLES

↓ Genomic DNA preparation

GENOMIC DNA

↓ Polymerase Chain Reaction (PCR)

PCR PRODUCT

↓ Restriction enzyme Mse I cut

PRODUCT OF Mse I CUT

↓ Gel electrophoresis
Ethidium bromide staining

---------- **RESULT** ----------

↓ **completely cut samples** (normal termination codon)

↓ **uncut or partially cut samples** (homozygous or heterozygous abnormal termination codon)

PCR PRODUCT

↓ Semi-nested PCR

SEMI-NESTED PCR PRODUCT

↓ Restriction enzyme Tth 111I cut

PRODUCT OF Tth 111I CUT

↓ Gel electrophoresis
Ethidium bromide staining

---------- **RESULT** ----------

↓ **completely cut or partially cut** (homozygous or heterozygous Hb CS)

↓ **Uncut** (other type of abnormal termination codon)

II.2 Method

II.2.1 Samples

II.2.1.1 Cord blood

The samples were collected from a total 191 random newborn subjects from the delivery room of Department of Obstetrics and Gynecology, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand, during March to May, 1994. All the subjects had parents who lived in northern Thailand. There were 167 samples from Chiang Mai , 16 from Lampoon , 2 from Lampang, 2 from Phetchaboon and one each from Payao , Maehongson , Sukhothai and Naan.

The blood samples were kept in 15 ml-polypropylene tubes which contained 1 ml. of 0.5 M EDTA.

II.2.1.2 Hb H Disease Patients

The blood from twenty eight Thai patients with hemoglobin H disease who came for treatment at the Thalassemic Clinic, Maharaj Nakorn Chiang Mai, Chiang Mai, Thailand, during the year 1990 and 1992, were kept. Hb H disease patients were characterized by the presence of Hb H on conventional hemoglobin electrophoresis and by the inclusion body found after staining with brilliant cresyl blue. The DNA from these blood were

extracted and were subsequently analyzed for the α-thalassemic genotype by the method of Southern hybridization (Muangmoonchai R., 1994). The 18 samples which were characterized as nondeletion form(s) of these patients were the subject of this study.

II.2.2 Genomic DNA Preparation

Genomic DNA preparation was modified from the method described by Miller *et al.* (1988). The first step was to separate nucleated cells from 5-10 ml. of the whole blood. White blood cell, the major nucleated cells in blood, were kept from the buffy coat between plasma and packed red cells after centrifugation the whole blood at 3000 rpm (SAFEGUARD CENTRIFUGE, CLAY ADAMS) for 5 minutes. Then they were washed with 45 ml of Red Cell Lysis Buffer (RCLB) in order to get rid of the intact red blood cells. The mixture was subsequently centrifuged at 1000 rpm (IEC INTERNATIONAL CENTRIFUGE, MODEL PR-2) for 10 minutes and the supernatant was discarded. The step was repeated once or twice to ensure the purity of the cells.

The pellet, which contained white blood cells, was then resuspending in 8 ml Nuclei Lysis Buffer. After mixing vigorously with 0.5 ml 10 % Sodium Dodecyl Sulfate (SDS), the cells were lysed and the mixture became viscous. It was added with 0.5 ml Proteinase K solution and incubated in shaking water bath at 55°C overnight . The mixture was then taken another step of protein separation. The digested protein was

separated by salting out with 5 M. NaCl at the ratio of 1 to 4 volumes of the mixture. To complete protein precipitation, the mixture was kept at 4 ^{o}C for 20 minutes. Then, it was centrifuged at 4 ^{o}C, 3000 rpm (IEC INTERNATIONAL CENTRIFUGE, MODEL PR-2) for 25 minutes. The clear supernatant was subsequently transfer to a new tube and was added two volumes of ice-cold absolute ethanol. After inverting the tube gently, the DNA was precipitated and suspended in the solution. The white fluffy DNA was then hooked out, rinsed with 70 % ethanol to wash the excess salt, and kept dry at room temperature for 20 minutes. Finally, the DNA was dissolved in 0.5 ml Tris- EDTA (TE) buffer and was ready to use for the experiment.

The concentration of DNA was estimated by spectrophotometric determination. An OD of 1 at wavelength of 260 nm corresponds to approximately 50 μg/ml for double-stranded DNA. The obtained DNA was diluted to 1 % solution in TE buffer before measuring.

II.2.3 Polymerase Chain Reaction

II.2.3.1 Components of the Polymerase Chain Reaction

Primers

The sequences of α_2 globin specific primers were obtained from the method described by Makonkawkeyoon and her coworkers (1993). They were synthesized by Bio Service Unit, Department of Biochemistry, Mahidol University, Bangkok, Thailand.

Leftward primer (L1) : TGC GGG CCT GGG CCG CAC TGA
MW : 6456.20
Concentration : 585.9 μg/ml or 0.09 μmol/ml.

Rightward primer (R1) : GCC GCC CAC TCA GAC TTT ATT
MW : 6315.20
Concentration : 754.5 μg/ml or 0.12 μmol/ml

Primers were used at the concentration of 0.5 μM in 100 μl reaction mixture. Therefore, each reaction contained 0.42 μl of Rightward primer and 0.55 μl of Leftward primer.

In order to ensure the unity of primers concentration in each reaction, the 1 ml dilution of these two primers had been prepared, in which 10 μl of this dilution contained 0.42 μl and 0.55 μl of the Rightward and Leftward primer, respectively.

Deoxynucleotide Triphosphates (dNTPs)

dNTPs were used at the concentration of 200 μM for each dNTP in the reaction mixture. The stock of 2 mM dNTPs had been prepared and 10 μl was used in each reaction.

Taq DNA Polymerase

Taq DNA Polymerase (Promega) was provided at the concentration of 5 units/μl. The enzyme was diluted to concentration of 2.5 units/10 μl with sterile deionized water before use.

Target DNA

The genomic DNA was adjusted to the concentration of approximately 0.5 μg/μl. The 2 μl of each sample (contained approximately 1 μg of DNA) was dispensed in each reaction .

Buffers and $MgCl_2$

The 10 x Taq reaction buffer and 25 mM $MgCl_2$ were provided along with Taq DNA Polymerase (Promega). The concentration of $MgCl_2$ used in this experiment is 1.25 mM. Therefore, each reaction contained 5 μl of 25 mM $MgCl_2$.

Each amplification reaction was carried out in a 0.5 ml. microfuge tube, each tube contained the PCR components as follow,

10 x buffer	10.0	µl
25 mM $MgCl_2$	5.0	µl
2 mM dNTPs	10.0	µl
diluted primer solution (50 pmol each)	10.0	µl
genomic DNA solution	2.0	µl
Taq DNA polymerase	2.5	units
sterile deionized water to	100.0	µl

After mixing the mixture, it was then covered with 80 µl of mineral oil (Sigma M 5904). This prevented evaporation of the sample during repeated cycles of heating and cooling.

The reaction mixture above was taken to perform in a Perkin-Elmer Cetus Instrument DNA thermal cycler. The conditions for denaturation, annealing and polymerization were as follows.

Cycle	Denaturation	Annealing	Polymerization
First cycle	95°C, 6 min	62°C, 1:30 min	74°C, 0:30 min
Subsequent cycles (2^{nd}-35^{th})	95°C, 1 min	62°C, 1:30 min	74°C, 0:30 min
Last cycle	-	-	74°C, 5:00 min

The reaction mixture was soaked at 4 °C until it was kept. The PCR products were then analyzed by 2 % agarose gel electrophoresis and ethidium bromide staining . The pUC 18/Hpa II cut was used as molecular weight markers.

II.2.4 The purification of PCR product

The PCR product was partially purified before any step of restriction enzyme cut. First, the mineral oil was removed. Then, 200 µl of absolute ethanol was dispensed in each tube, mixed thoroughly and kept at -20 °C for at least 2 hours to ensure the precipitation of PCR product. The precipitated DNA was recovered by centrifugation at 12,000 rpm (Sigma 202 MC) for 15 minutes. The supernatant was removed and the pellet was left at room temperature until it was completely dry. Then 50 µl of sterile deionized water was added to dissolve the pellet.

II.2.5 Restriction Enzyme Mse I Cut.

The total 15 µl Mse I reaction mixture was contained 2 µl of partially purified PCR product, 2 units of restriction enzyme Mse I (Biolabs), 1.5 µl of 10 x buffer, 0.15 µl of 100 x BSA, and sufficient amount of sterile deionized water.

The reaction mixture was incubated at 37 °C for at least 2 hours before analyzing with 2.5 % agarose gel electrophoresis.

II.2.6 Semi-nested PCR

The components for semi-nested PCR were the same as II.2.3.1, except for the leftward primer and target DNA, changed as follow.

Leftward primer (L2) (for semi-nested PCR) : G CTG ACC TCC AGA CAC CGT

The target DNA were PCR products that were not cut or partially cut with Mse I. The partially purified PCR product were dispensed at an amount of 0.5 µl for each reaction.

Amplification reaction mixture was carried out in 0.5 ml microfuge tube, each tube contained the PCR components as follow,

10 x buffer	10.0	µl
25 mM $MgCl_2$	5.0	µl
2 mM dNTPs	10.0	µl
diluted primer solution (50 pmol each)	10.0	µl
diluted PCR product	0.5	µl
Taq DNA polymerase	2.5	units
sterile deionized water to	100.0	µl

After mixing the mixture, it was then covered with 80 µl of mineral oil (Sigma M 5904). This prevented evaporation of the sample during repeated cycles of heating and cooling.

Semi-Nested PCR Conditions

Cycle	Denaturation	Annealing	Polymerization
First cycle	95°C, 5 min	50°C, 2 min	74°C, 20 sec
2nd-11th cycle	95°C, 30 sec	50°C, 2 min	74°C, 20 sec
12th-31th cycle	95°C, 30 sec	55°C, 1 min	74°C, 15 sec
Last cycle	-	-	74°C, 5 min

The reaction mixture was soaked at 4 °C until it was kept. The products were then analyzed by 2.5 % Agarose gel electrophoresis and ethidium bromide staining. The ϕX174 DNA/Hae III Cut was used as molecular weight marker.

II.2.7 Restriction Enzyme Tth 111I Cut

After semi-nested PCR was performed, the product was then partially purified by the method above (II.2.4), and was subjected for restriction enzyme Tth 111I cut.

The total 20 μl Tth 111I reaction mixture was contained 5 μl of partially purified semi-nested PCR product, 3 units of restriction enzyme

Tth 111I (Promega), 2.0 µl of 10 x buffer, 0.2 µl of 100 x BSA, and sufficient amount of sterile deionized water.

The reaction mixture was incubated at 65 °C for at least 2 hours before analyzing with 3.0 % agarose gel electrophoresis.

II.2.8 Agarose Gel Electrophoresis

Agarose gel electrophoresis was applied in many steps of this study. The percentage of agarose varied from 2 % to 3% , depending on the resolution needed in each step. It was used at 2 % for analyzing the PCR product, at 2.5 % for analyzing the product of Mse I-cut, and at 3 % for analyzing the semi-nested PCR cut with Tth 111I.

The agarose gel was prepared by dissolution sufficient amount of powdered agarose in 0.5 x TBE buffer to the designed concentration and volume. After melting the mixture on the hot plate with a stirring rod, the gel was cooled to 60-70 °C before molding. A comb with 5 mm-wide and 2 mm-thick was put in the melted gel to prepare the slots. After the gel was completely set (30-45 minutes at room temperature), carefully removed the comb and the sealing edge of the mold. Then 0.5 x TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the gel to a depth of about 1 mm.

The samples were mixed with gel loading buffer and then were slowly loaded into the slots using an automatic micropipettor. The voltage of 4 V/cm (the distance between the electrodes) was usually used. In analyzing PCR products and the cut of PCR product with Mse I, the gel was run for 1.5 hours. In analyzing semi-nested PCR products and the cut of them with Tth 111I, the gel was run for 2.5 hours.

II.2.9 Ethidium Bromide Staining

After electrophoresis, the gel was submerged in 0.5 μg/ml ethidium bromide solution for 30-45 minutes and then was destained in water for one hour.

II.2.10 Photography

Photographs of gels were made using Foto/UV 260 Transilluminators (FOTODYNE@) and MP-ST^{TM} Photographic System (FOTODYNE@). The film was Polaroid type 667. The lens was opened at f/5.6 or f/8 and the exposure time was set at 1 sec. The films were processed for 30 seconds as recommended by the company.