CHAPTER II

MATERIALS AND METHODS

2.1 Subjects

The subjects in the present study include 100 out-patients with other problems attending the Out-patient Laboratory Unit (OPD 11) at Maharaj Nakorn Chiang Mai Hospital were recruited for the determination of HFE polymorphism, 22 non-thalassemic individuals recruited from the Blood Bank Unit, Maharaj Nakorn Chiang Mai Hospital, 23 homozygous β-thalassemia and 34 β-thalassemia/HbE recruited from The Thalassemia Clinic, Department of Medicine and The Pediatric Thalassemia Clinic, Department of Pediatrics, Faculty of Medicine, Chiang Mai University and Maharaj Nakorn Chiang Mai Hospital. Venous blood samples were collected and stored in ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. Clotted blood samples were also collected from all subjects.

2.2 Laboratory investigations

Laboratory investigations carried out in the present thesis were categorised into 2 groups: hematological investigations and DNA analysis. In the first, the laboratories to be carried out consisted of complete blood count (CBC), determination of zinc protoporphyrin (ZPP) levels, Hb identification, determination of serum iron (SI), total iron binding capacity (TIBC) and transferrin saturation (TS), determination of blood lead level.

2.2.1 Hematological investigation

2.2.1.1 Complete Blood Count (CBC)

The CBC was performed using automated blood cell analyzer (Sysmex). The hematological parameter scollected were hemoglobin (Hb) concentration, hematocrit (Hct) level, mean corpuscular

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

2.2.1.2 Determination of zinc protoporphyrin (ZPP) levels

Intra-erythrocytic ZPP was measured under the principle of hematofluorometry by using the portable ProtoFluor Z Hematofluorometer machine (Helena Laboratories, Beaumont, Texas) and commercially available ProtoFluor Reagent (Helena Laboratories, Beaumont, Texas). By adding ProtoFluor Reagent to whole blood, the hemoglobin is derivertized to a product having the spectral characteristics of oxyhemoglobin in the region where the hematofluorometer operates. Thus, the need for oxygenation is required which allows the determination of ZPP with greater accuracy and precision, even in moderately aged and deoxygenated blood.

Practically, there are 2 steps for measuring the ZPP level: ProtoFluor Z Hematofluorometer calibration and ZPP determination. The calibration of the ProtoFluor Z Hematofluorometer was accomplished by using ProtoFluor Calibrators (High and Low levels) following the operating procedure as described in the Operator's Reference Manual. Briefly, a drop of ProtoFluor Low Calibrator was firstly placed directly on a sample coverslip , spread using the tip of the vial so that the calibrator completely covered the sample area shown. The level of the calibrator was then read from the hematofluorometer. If the read level was not the same as shown for that low calibrator, the hematofluorometer was adjusted until the desired value of ZPP was obtained. Finally, the whole process was repeated using High Protofluor Calibrator. Once the correct ZPP values from both calibrators were obtained, the machine was then ready to use.

The determination of intra-erythrocytic ZPP levels in whole blood samples was undertaken using the ProtoFluor Reagent. Using a pasteur pipette, one drop of whole blood sample was placed in a small test tube (12 x 75 mm), two drops of ProtoFluor Reagent added and mixed by brief shaking. Then, a drop of specimen was poured onto a glass coverslip which had been placed into the sample holder, spread the drop using the tip of the test tube so that the specimen covers the appropriate area

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

of the coverslip. The measurement was finally processed as instructed in the Operator's Manual. As determined by this principle, the normal intra-erytrocytic ZPP has been found to be 30-80 μmol ZPP/mol heme (59).

2.2.1.3 Determination of influence of bilirubin on ZPP levels

Bilirubin is yellow pigment in the serum and can interfere with the measurement of ZPP under the priciple of hematofluorometry used in the portable ProtoFluor Z Hematofluorometer machine. The association of total bilirubin and the ZPP levels (previously determined) were evaluated. In this investigation, the samples were classified according to the total bilirubin levels. Each blood sample was divied into 2 parts; unwashed sample in which it was in an original form when measured, while another was washed with NSS prior to addition of NSS to the original Hct level. The ZPP levels were then compared between unwashed whole blood and washed blood samples.

2.2.1.4 Hb identification by high performance liquid chromatography (HPLC)

Hemoglobin typing in blood samples could be determined by passing the hemoglobin solution through a weak cation-exchange high performance liquid chromatography or HPLC. With the help of sophisticated analytical software, hemoglobin fractions were automatically computed. This task was accomplished using the PVS99 System (Primus Corperation, Kansus City, MO, USA). Hemolyzed samples were automatically injected into the column during the flow of appropriately blended buffers (mobile phases 1 and 2). Hemoglobin species migrated through the column at rates determined by their individual physical properties. Upon elution from the column, sample components passed through the spectrophotometric detector where detection occurs at a wavelength of 413 nm ± 2 nm. Following elution of all hemoglobin species, original conditions were re-established prior to the injection of the next sample. All critical events including sample injection, reagent flow rates, and composition were carefully timed to provide maximum reproducibility. All

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

processes were electronically controlled through the computer. The computer processed the signal from the detector and calculates the retention time and percent concentration of each peak. As the sample was chromatographed, it was displayed on the monitor in real time. The computer produces printed reports with the samples identification information, date and time, followed by the chromatogram with retention times indicated at the apex of each peak.

2.2.1.5 Iron studies

The iron studies means the evaluation of the iron status in peripheral blood including the determination of serum iron (SI), total iron binding capacity (TIBC) and transferrin saturation (TS). The levels of these iron parameters reflect the iron metabolism in the body.

A. Serum Iron (SI)

The method for determination of SI is based on that recommended by the International Committee for Standardization in Hematology (ICSH) (60). In principle, a mixed acid reagent was used to release the transferrin-bound iron, to reduce the ferric to ferrous ions and to precipitate the serum proteins. The ferrous iron in the remaining supernatant is colour-developed using buffered Bathophenanthroline sulphonate and the absorbance of the resulting pink solution and a "blank" (iron-free-water) are measured.

Practically, 1.0 ml test sample and 1.0 ml working iron standard and 1.0 ml iron-free-water (blank) were placed in the first iron-free tube (Appendix C for a practical procedure to prepare iron-free tubes), 1.0 ml Mixed Acid Reagent (Appendix C) added to precipitate serum protein and liberate iron from transferrin, vortexed and stood for 15 minutes at RT to ensure complete precipitation reaction. After that protein precipitate was sedimented by centrifugation at 5,000 rpm for 10 minutes. Then, 1.0 ml of the optically clear supernatant was collected into the second iron-free test tube and 1.0 ml Chromogen (Appendix C) added, mixed and left for at least 15 minutes at RT to ensure complete color developing reaction. Then, the final pink-colored solution was measureed for an

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

absorbances at 535 nm againt DW in a spectrophotometer. (The colour is stable for several hours at RT)

Calculation

$$\text{Serum Iron } (\mu g/dl) = \frac{A_T - A_B}{A_S - A_B} \times 100$$

A_T = Absorbance of test sample

A_S = Absorbance of standard (100 μg/dl)

A_B = Absorbance of blank

Normal range (60): Male = 70-170 μg/dl

Female = 60-150 μg/dl

B. Total Iron Binding Capacity (TIBC)

Sufficient amount of iron was added to saturate the unbound transferrin in serum, any excess of iron was subsequently removed using magnesium carbonate ($MgCO_3$) powder (Appendix C) to absorpt the excess iron left in the suspension followed by heavy centrifugation before supernatant collection for iron assay, the procedure of which was the same as that for SI determination.

In practice, a set up 3 test tubes was prepared for each sample. 1.0 ml of test sample was placed in the first tube followed by addition of 1.0 ml Iron Saturating Solution (Appendix C). The mixture was then left at RT for 15 min to ensure complete iron saturation on unbound transferrin. After that, approximately 0.15 g $MgCO_3$ was added to the mixture followed by mixing intermittantly. The absorption by $MgCO_3$ lasts 15 min followed by centrifugation at 5,000 rpm for 10 minutes. The 1.0 ml of clear supernatant was the transferred to the second iron-free test tube prior to the assay for iron content in the third iron-free tube as described earlier under the section of SI.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Calculation

$$TIBC\ (\mu g/dl) = \frac{A_T - A_B}{A_S - A_B} \times 100 \times 2$$

A_T = Absorbance of test sample

A_S = Absorbance of standard (100 μg/dl)

A_B = Absorbance of blank

2 = Dilution factor due to addition of equal volume of iron saturating solution

Normal range (60) = 280-400 μg/dl

C. Transferrin Saturation (TS)

Transferrin saturation (TS) is defined by the proportion of the actual proportion of serum transferrin that has already been bound by the ferric ion. It can simply be calculated by the following equation:

$$TS\ (\%) = \frac{SI\ (\mu g/dl)}{TIBC\ (\mu g/dl)} \times 100$$

Normal range (60) = 20-50 %

2.2.1.6 Determination of blood lead levels

The determination of blood lead levels was accomplished under an atomic absorption spectrometry method using the Varian SpectrAA-400Z^{TM} atomic absorption spectrometer. In principle, lead metal in the whole blood was atomized by strong heat generated by graphite furnace before the detection of signal from this atomized lead ion by the detector. Practically, 100 μl whole blood sample (fresh or frozen) or control materials were mixed with 900 μl Working Modifier

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

provided in the kit in a 2.0-ml graphite-furnace-sample dispenser cup, carefully mixed with a transfer pipette to insure homogeneity. This treated blood samples were then injected into the machine under the instruction provided by manufacturer. The standard materials were also treated and processed in the same manner as the samples to creat the standard curve used to enumerate the correct values of lead levels in unknown samples. The blood lead levels were then expressed in μg/ml. Generally, the reference values of this metal in blood of children is < 10 μg/dl and in blood of adult is < 25 μg/dl.

2.2.2 DNA analysis

2.2.2.1 DNA preparation from buffy coat (61)

Genomic DNA was prepared from buffy coat by using the standard phenol/chloroform extraction. The step-by-step procedure was as follow:

Day 1

The 1-ml frozen buffy coat was thawed, 3.0 ml of 0.5% NP-40 (non-ionic detergent) added, spun at 3000 rpm for 5 min and supernatant discarded. Then, 750 μl lysis buffer in combination with 40 μl SDS (10%, v/v) and 100 μl Proteinase K (5 mg/ml in 50 mM EDTA) were added followed by overnight incubation in 37°C waterbath.

Day 2

Cell lysis was checked by mixing the solution up and down using pasteur pipette for a couple times to ensure that it was not clumpy and then 200 μl of 5 X ANE buffer was added followed by 500 μl phenol saturated with tris pH 8.0 and 500 μl chloroform, mixed well by shaking end-over-end and spun at 3000 rpm for 10 min. After that, aqueous (upper) layer was aspirated with sterile plastic pipette and transferred to a fresh and clean tube. The phenol/chloroform extraction was repeated until the interface is clear (usually once or twice). Finally, 1 ml chloroform was added to remove residual phenol, mixed well by shaking and spun for 5 min at 3000 rpm. The clear upper phase was then aspirated and put into a fresh clean tube before the addition of 100 μl CH_3COONa (3M) pH 5.6 and 2.5 ml deeply cold absolute ethanol to precipitate the genomic DNA. The precipitated DNA was then

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

seen as small white fiber. If no DNA is seen at this stage, the solution must be stored at –20°C overnight to enhance the precipitation.

Day 3

The tube was spun at 3000 rpm for 10-20 min at 4°C, then supernatant aspirated, washed with 70% ethanol, spun at 3000 rpm for 10 min at 4°C and air dry. Dried DNA pellet was then dissolved in sterile DW. This takes a few hours or maybe overnight depending on an amount of DNA obtained. The original stock DNA should not be frozen and thawed several times, thus it must be kept in –20°C or –70°C if possible. The working solution should be kept in –20°C and let it thaw slowly.

2.2.2.2 DNA preparation from Chelex™

DNA was extracted from whole blood using the Chelex method (62) with some modifications (63). Chelex resin is a chelating agent that removes divalent cation including Mg^{++} which could act as cofactor for DNase enzyme. In practice, 40 µl of EDTA blood was mixed with 1 ml of 0.5% Triton X-100 in 1.5 ml tubes, vortexed and centrifuged at 14,000-rpm for 1 minute. The supernatant was removed, 1 ml of water added, centrifugated as above and supernatant removed again. A Chelex-100 suspension was then added to the pellet until a 1-2-mm-thick layer was obtained, followed by 110 µl distilled water. Then the mixture was incubated at 56°C at least 2 hours or overnight prior to heating at 100°C boiling water for 7 minutes. Incubation at 56°C was to activate the Chelex-100 resin while heating aimed to destroy the cell and cellular components, such as proteins, to liberate the genomic DNA. The extracts were then stored at 4°C until use.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.2.2.3 Survey for the HFE gene polymorphism

HFE genotyping

Two regions of HFE gene containing the proposed mutations (C282Y and H63D) were amplified by PCR prior to restriction fragment length polymorphism (RFLP) analysis. The reaction mix as well as the thermal cycles was similar in the amplification of DNA section covering the C282Y and H63D mutations. In practice, the PCR was carried out in a 25-μl reaction mix containing 5 μl undiluted genomic DNA obtained from phenol/chloroform extraction, 100 μM each dNTP, 2 units of Taq DNA polymerase (Fermentus), 100 ng of each primer (H63D; H63DF: 5'-ACATGGTTAAGGCCTGTTGC-3', H63DR: 5'-CTTGCTGTG GTTGTGATTTTC C-3' and C282Y; C282YF: (5'-CAAGTGCCTCCTTTGGTGAAGGTGACACAT-3', C282YR: 5'-ATCTCACTGCCATAATTACCTCCTCAG-3') (64) in 10 mM Tris, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 5 min and the final extension was prolonged to 5 min. The MJ Research PTC-200 Peltier Thermal Cycler was used. The 294-bp and 343-bp amplified products were generated for the H63D and C282Y, respectively. 5 μl were then removed, mixed with 5 μl of 0.05% methylene blue, and loaded on a gel of 2% agarose. After electrophoresis at 100 V for 30 min, the gel was stained with ethidiumbromide and visualized under medium-wavelength ultraviolet illumination.

The 20 μl amplified products of H63D primers were subsequent incubated, in 25 μl total volume, at 37°C for at least 9 hours with 1.0 unit *Mbo* I(Fermentas) in 10 mM Tris HCL (pH 8.5), 10 mM MgCl2, 100 mM KCl and 0.1 mg/ml BSA (1 x Buffer R). Following the *Mbo* I digestion, the digested products were separated in 2.0% agarose gel electrophoresis. Those products lacking the mutation (Wild type) yielded restriction fragments of 138, 99, and 57 bp. However, the mutation at this position (Mutant type) removes the cutting site of this restriction enzyme and only 237 and 57-bp digested fragments were generated (Figures 2.1 A and B)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

The 20 μl amplified products of C282Y primers were subsequent incubated at $37^{0}C$ for at least 9 hours with 1.0 unit *Rsa* I (Fermentas) in 10 mM Tris HCL (pH 8.5), 10 mM MgCl2, 100 mM KCl and 0.1 mg/ml BSA (1 x Buffer R). Following the *Rsa* I digestion, the digested products were separated in 2.0% agarose gel electrophoresis. Those lacking (Wild type) the mutation yielded products of 203 and 140 bp whereas those bearing the mutation at this point (Mutant type) had additional *Rsa* I cutting site resulting in digested products of 203, 111, and 29 bp (Figures 2.2 A and B)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

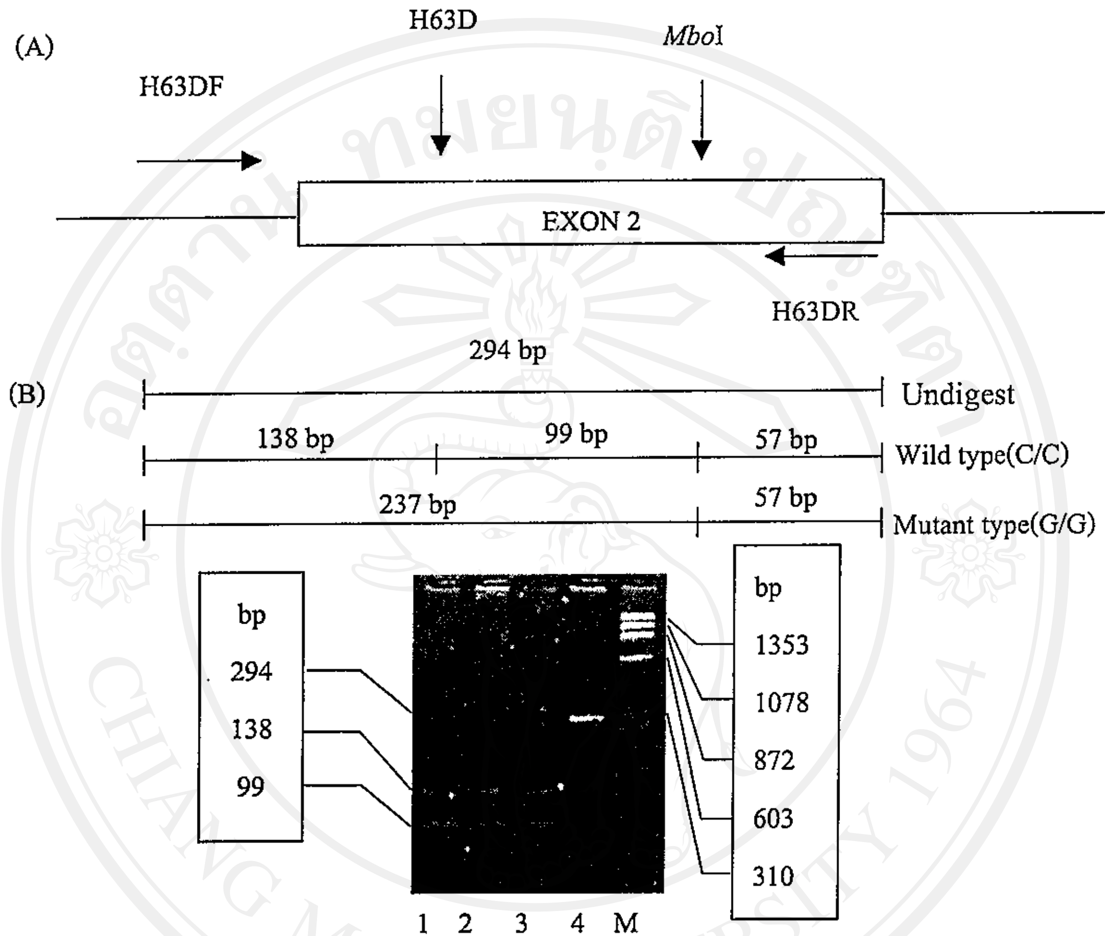

Figure 2.1 (A) Schematic representation of the DNA segment of the HFE gene. The primers H63DF and H63DR were used to specifically amplify this segment for the detection of the C→G transversion in codon 63 in exon 2. Natural cutting site for *Mbo* I is marked by arrows in which the 5' cutting site is removed in the mutant type for H63D.

(B) *Mbo* I digested PCR products run on 3.0% agarose gel. Lane 1-3 represent individuals absent for the C→G transversion. Lane 4 was undigested PCR product. ϕ x174 DNA (*Hae*III digest) was used as a size marker (Lane M) with sizes (in base pair, bp) of the digested fragments shown.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

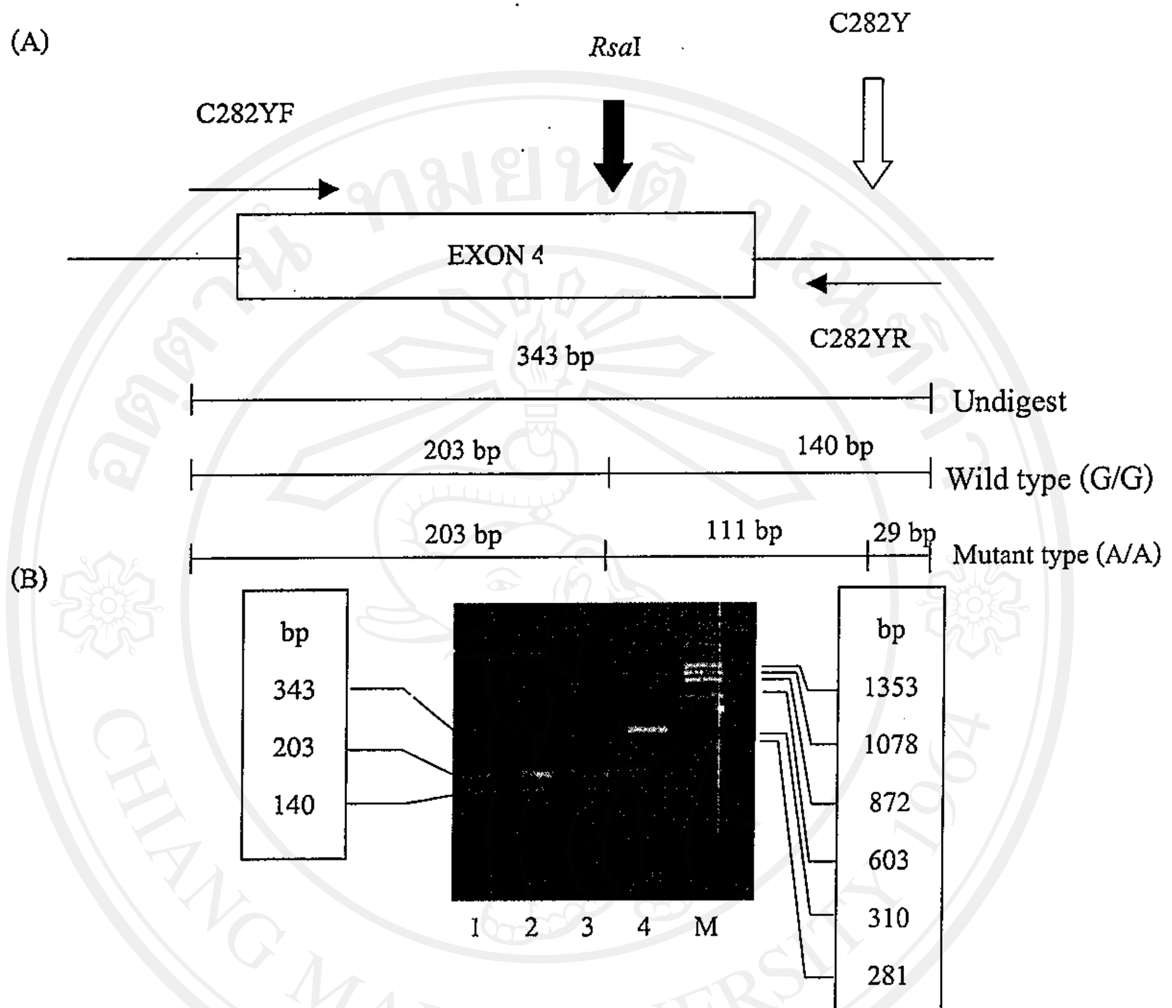

Figure 2.2 (A) Schematic represertation of the DNA segment of the HFE gene. The primers C282YF and C282YR were used to specifically amplify this segment for the detection of the 845G→A transsition in codon 282 in exon 4. . Filled arrow is natural cutting site for *Rsa* I. The C282Y creats new cutting site for *Rsa* I which is indicated by blank arrow.

(B) *Rsa* I digested PCR products run on 3.0% agarose gel. Lane 1-3 represent individuals absent for the G→A transsition. Lane 4 was undigested PCR product. ϕ x174 DNA (*Hae*III digest) was used as a size marker (Lane M) with sizes (in base pair, bp) of the digested fragments shown. *29 bp fragmen is not shown on this figure.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.2.2.4 Detection of β-thalassemia mutations

The multiplex ARMS-PCR was performed for the detection of 2 β-thalassemia mutations commonly found in Thailand (A-T substitution at codon 17 and 4bp deletion (-TTCT) at codons 41/42). The DNA was prepared directly from buffycoat using the Chelex^{TM} -DNA preparation technique mentioned earlier. Two reactions (Normal and Mutant) were performed for each sample. In the normal or wild-type reaction, a total volume of 25 μl, the reaction was performed containing 7 μl Chelex-extracted genomic DNA, 200 μM of each dNTP, 0.2 μM of α-globin gene specific primers used as internal control (P1: 5'-GCG ATC TGG GCT CTG TGT TCT-3', P_2: 5'-GTT CCC TGA GCC CCG ACA CG-3') ,and 0.4 μM of S-primer serving as a common primer (5'-ACC TCA CCC TGT GGA GCC AC- 3'), 0.3 μM of N-17 (5'-CTC ACC ACC AAC TTC ATC CAC GTT CAC ATT- 3'), 0.2 μM of N-41/42 (5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA-3'), 0.3 units Taq DNA polymerase in 10 mM Tris, pH 8.8, 50 mM KCl and 1.8 mM $MgCl_2$. In contrast, in the mutant reaction, a total volume of 25 μl, the reaction was performed containing 7 μl Chelex-extracted genomic DNA, 200 μM of each dNTP, 0.2 μM of α-globin gene specific primers used as internal control (P_1, P_2), and 0.4 μM of S-primer serving as a common primer, 0.2 μM of M-17 (5'-CTC ACC ACC AAC TTC AGC CAC GTT CAG CTA-3'), 0.2 μM of M-41/42 (5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3'), 0.3 units Taq DNA polymearse in 10 mM Tris, pH 8.8, 50 mM KCl and 1.8 mM $MgCl_2$. A total of 35 thermal cycles was performed with each cycle comprising the DNA denaturation at 95°C for 2 min, primer annealing at 65°C for 1 min and primer extension at 72°C for 2 min. The initial denaturation lasts 5 min and the final extension was prolonged to 5 min. The MJ Research PTC-200 Peltier Thermal Cycler was used. The cycling reaction was performed in a programmable control block. 5 μl were then removed, mixed with 5 μl of 0.05% methylene blue, and loaded on a gel of 2% agarose. After electrophoresis at 170 V for 25 minutes, the gel was stained with ethidiumbromide and visualized under medium-wavelength ultraviolet illumination (Figures 2.3).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

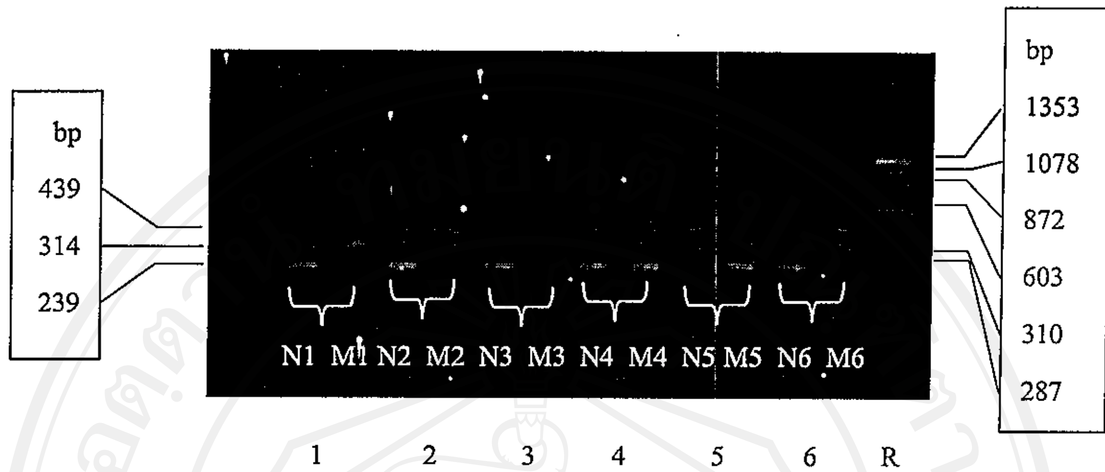

Figure 2.3 PCR products of multiplex ARMS-PCR to detect cd 41/42(-TTCT) and cd17 (A-T) in β-globin gene and run on 2% agarose gel. No.1 is individuals negative for these two mutations, No.2 is heterozygote for cd 41/42 (-TTCT), No.3 represents heterozygote for cd 17 (A-T), No.4 is individual compound heterozygous for cd 17 (A-T) and cd 41/42 (-TTCT), No.5 represents the homozygote for cd 17(A-T), No.6 is individual homozygous for cd 41/42(-TTCT). R represents ϕ x174 (*Hae* III digest) DNA size marker. N and M represent normal and mutant reactions, respectively.

2.3 Statistical analysis

The descriptive and inferential statistics including mean, standard deviation (SD) and correlation were calculated. Using Student's *t*-test, the differences of the analysed parameters between each type of β-thalassemia disease were computed.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved