CHAPTER 3

MATERIALS AND METHODS

Chemicals and Reagents

Agarose, ethylenediamine tetraacetic acid, ethidium bromide, proteinase K were obtained from Sigma Chemical Co., St. Louis, MO, USA. Absolute ethanol was purchased from Merck, Darmstadt, Germany. Restriction enzyme (*Sma*I and *BamH*I) were obtained from New England Biolabs Inc. Bevery, MA, USA. Taq polymerase and 100bp DNA marker were obtained from Gibco BRL® Life Technologies NY, USA. U.S.A. QIA amp® DNA Minikit were obtained from QIAGEN Inc. USA.

Equipments

The PCR Autorized Thermal Cycler (Mastercycler Model) No 00352 was obtained from Mondotech Germany The Ultraviolet Benchtop Transilluminator (bio Doc-It ™ System) Modle M-20, Unit was obtained from Upland. CA 9176 U.S.A. and Mini-Gel Electrophoresis Unit was obtained from Cosmobioco., LTD. Tokyo, JAPAN.

Methods

1. Subjects

One hundred and sixty-two unrelated Thais residing in the Southtern region of Thailand (90 males, 72 females, aged 17-70 years were enrolled in this study. All subjects were classified as native Southern Thais according to family history of their parents and grandparents. Subjects were healthy as assessed by medical history and physical examination. All subjects gave written informed consent before participation in the study. The subjects were asked about amount and frequency of smoking, alcoholic beverages,

beverages, diseases, and recent medication. The study protocol was approved by the Ethical Research Committee of the Faculty of Medicine, Prince of Songkla University.

2. Blood Sample Collection

Five ml of venous blood samples were collected in the tubes containing ethylenediaminetetraacetic acid. Whole blood sample was centrifuged at 3000 g for 15 min and the buffy coat was collected and stored at -20°C for preparation of genomic DNA.

3. Preparation of genomic DNA

Genomic DNA (gDNA) was purified from 200 µl of buffy coat using QIAamp® DNA Mini kits as follow: 20 µl of QIAGEN Protease was pipeted into the bottom of a microcentrifuge tube. 200 µl of AL buffer was added to the sample and mixing by pulse-vortexing for 15 seconds. The tube was incubated at 56°C for 10 min and then centrifuged gently to removed the drops from the inner side of the lid. 200 µl of 95% ethanol was added to the sample, and mixed again by pulse-vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge was centrifuged to remove the drops from the inside of the lid. Then, carefully applied the mixture to the QIAamp spin column and centrifuged at 6000 X g for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube, and discarded the tube containing the filtrate. The QIAamp spin column was opened carefully and added 500 µl of AW1 buffer and centrifuged at 6000 X g for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube, and discarded the tube containing the filtrate. The QIAamp spin column was then opened carefully and 500 µl of AW2 buffer was added and centrifuged at full speed (10,000 X g) for 5 min. The QIAamp spin column was placed in a clean 1.5ml collection tube, and discarded the tube containing the filtrate. Finally the QIAamp spin column was opened carefully and added 200 µl of AE buffer. The tube was incubated at room temperature for 1 min, and then centrifuged at 6000 g for 1 min to elute gDNA. The gDNA was stored at 4°C until used.

4. Genotyping of CYP2C19

Two major mutant alleles of CYP2C19, *CYP2C19*2* and *CYP2C19*3*, were analyzed by PCR-RFLP techniques. Genotyping procedures for detection of the *CYP2C19*2* defect were modified slightly by use of more specific primers, forward primer (5-CAGAGCTTGGCATATTGTATC-3) and reverse primer (5-GTAAACACACAACTAGTCAATG-3) (Goldstein et al., 1996). Genotyping procedures for the identification of *CYP2C19*3* defect were performed with forward primer (5-AAATTGTTTCCAATCATTTAGCT-3) and reverse primer (5-ACTTCAGGGCTTGGTCAATA-3) (de Morais et al., 1995). A 25 μl PCR reaction for *CYP2C19*2* contained 1 μl gDNA, sterile water 15.3 μl, 10x PCR buffer 2.5 μl, 50 mM $MgCl_2$ 1 μl, dNTP mixture (2.5 mM each) 2 μl, forward primer 1 μl, reverse primer 1μl, and Taq polymerase 0.2 μl. A 25 μl PCR reaction for *CYP2C19*3* contained 1 μl gDNA, sterile water 15.3 μl, 10x PCR buffer 2.5 μl, 50 mM $MgCl_2$ 1 μl, dNTP mixture (2.5 mM each) 2 μl, forward primer 1 μl, reverse primer 1 μl, and Taq polymerase 0.2 μl. PCR amplification was performed using a PCR Autorized Thermal Cyler (Master cycler). An initial denaturation step at 94°C for 5 min was followed by 37 cycles comprising a denaturation step 94 ^{0} C for 45 seconds, annealing at 53 ^{0}C for 45 seconds and extention at 72°C for 45 seconds. The final extention was subsequently performed at 72°C for 7 min. The PCR product was stored at 4°C.

The PCR product of exon 5 was digested with *Sma*I as follow: a 10 μl of the mixture containing 7.5 μl of sterile water, 2 μl of 10 x *Sma*I buffer NEB 4 and 0.5 μl of *Sma*I (10 u) was aliquoted into a tube then added 10 μl of the PCR products. The tube was incubated at 25°C overnight.

The PCR product of exon 4 were digested with *Bam*HI as follow: a 10 μl of mixture containing with 7.3 μl of sterile water, 2 μl of 10x *Bam*HI buffer, 0.5 μl of *Bam*HI (12.5 u), and 0.2 μl of 100x BSA was aliquoted into a tube then added 10 μl of the PCR products. The tube was incubated at 37°C overnight.

About 10 μl of the digestion product was mixed with 3 μl of loading dye and loaded onto agarose gel containing ethidium bromide. Electrophoresis was run at 100 volts for 30 min. The DNA fragments were visualized under UV light.

Statistical analysis

Allele frequencies of CYP2C19 were estimated by counting the number of alleles observed in all individuals. For example, the frequency of *CYP2C19*1* allele in N individuals was estimated by (2n*1/*1 + n*1/*2 + n*1/*3) /2N where n*1/*1 is the number of individuals homozygous for *CYP2C19*1*, n*1/*2 and n*1/*3 is the number of individuals heterozygous for *CYP2C19*1/*2* and *CYP2C19*1/*3* alleles, respectively (Weir, 1990). χ^2 test was used for testing whether or not genotype frequencies were follow Hardy-Weinberg equilibrium. 95% confidence limits for allele frequency estimates were computed as $p \pm 1.96\sqrt{p(1-p)/2N}$, where p is the estimated allele frequency. Allele frequencies in different racial populations were compared using the Z test.