CHAPTER 4

RESEARCH DESIGN AND METHODS

4.1 An overview

The present study was to determine the polymorphisms of the *CYP2A13* gene in Northern Thai population. The population of subjects for this study was recruited from healthy volunteers who visited the blood bank sector of Maharaj Nakorn Chiang Mai hospital, Chiang Mai Province for blood donation. These individuals were Thai nationality and born in the Northern region of Thailand. All subjects answered the questionnaire in face-to-face interviewing which included questions about demographic characteristics, medical history and smoking history. Blood samples were collected from a peripheral vein of each subject into EDTA tubes and subsequently prepared for DNA isolated. Then, the isolated genomic DNA was subjected for determination of the *CYP2A13* genetic variations by a PCR-based strategy. A total of six SNPs in exon 1, 2, 3, 5, and 3'-UTR of *CYP2A13* gene were analyzed. In order to avoid amplification of homologous gene sequences i.e., *CYP2A6* and *CYP2A7* pseudogene, the two-step PCR method was applied to each genomic DNA samples. In the first-step, a long-distance PCR method was used to amplify the full-length *CYP2A13* gene. Subsequently, the PCR products were used as the template for the second PCR-step. In the second step, the four variants of 74G>A, 1662G>C, 3375C>T and 7520C>G were distinguished by the PCR amplification coupled with RFLP analysis, the 578C>T variation was determined by mismatched

allele-specific amplification (mASA) method whereas the 7571G>C variation was determined by tetra-primer PCR method. Finally, the frequency distributions of each SNPs were analyzed statistically and some of samples were selected for haplotype structure analysis. The summary of the research design is shown in Figure 5

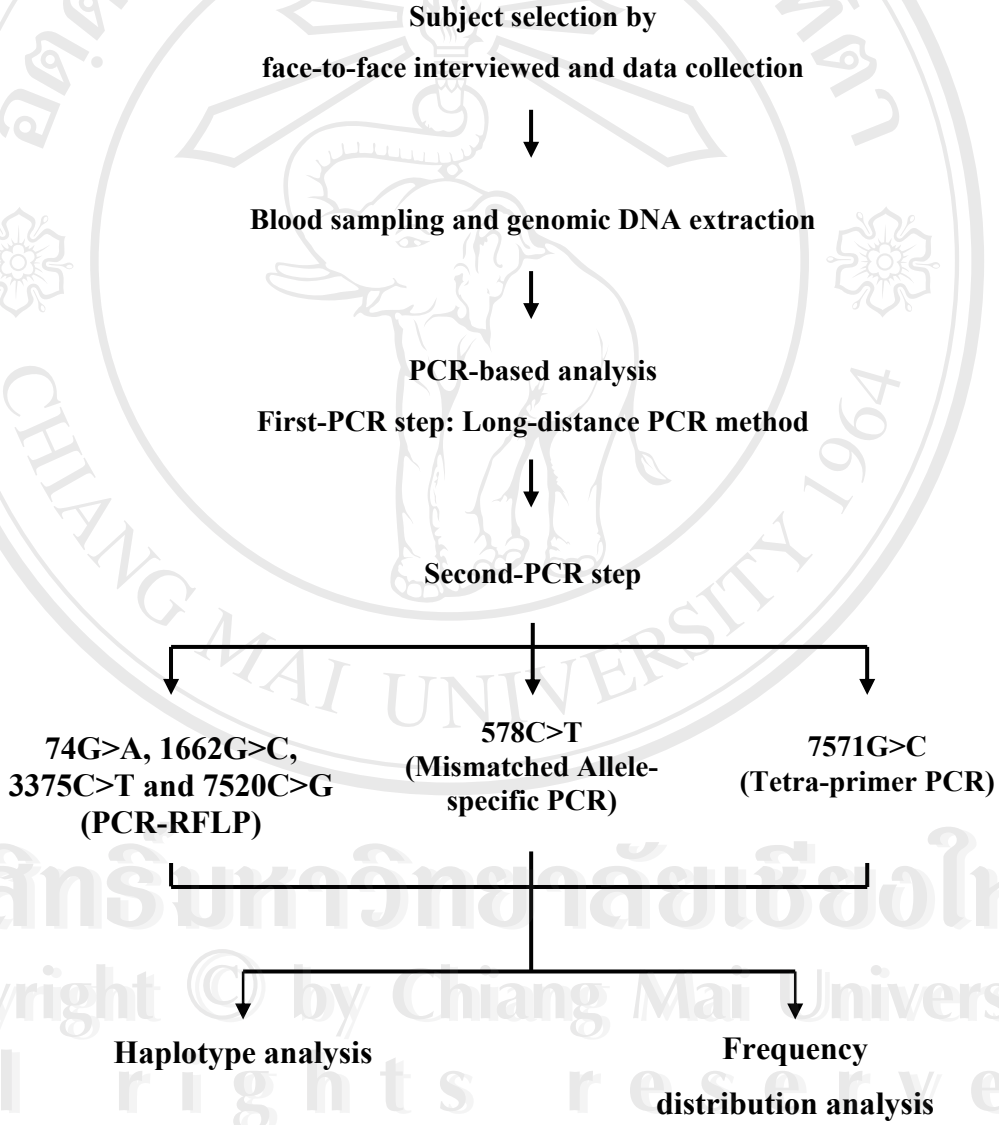

Figure 5 An overview of research design

4.2. Methods

4.2.1 Study population

The population of eligible subjects for inclusion in this study was recruited from the blood bank sector of Maharaj Nakorn Chiang Mai hospital, Chiang Mai Province between September 2004 and May 2005. This group composed of 175 male and 161 female blood donors (total 336 subjects). All subjects met the following criteria: healthy volunteer either males or females, age more than 20 years old, Thai nationals who were born in the upper Northern region of Thailand. These individuals had no history of cancer and were non-smokers. A description of the study and its components were provided and enrollments were solicited. By using a uniform questionnaire (Appendix D), a face-to-face interview, some information for every subject was collected, including demographic characteristics, personal medical history, education, job history, family cancer history and history of smoking. All subjects were asked to donate peripheral venous blood after informed consent was obtained from each subject. To maintain participant confidentiality throughout the study, all participant records and sample information were coded. All study protocols, questionnaires and consent forms of this study were approved by the Institutional Ethical Committee on Human Experimentation, Faculty of Medicine, Chiang Mai University, Chiang Mai Province, Thailand.

4.2.2 Preparation of blood samples

At the time of enrollment, approximately 5 ml of whole blood samples was collected from a cubital vein into the standard 5-ml EDTA vacutainertube for prevention of blood clotting and mixed well by inverting the tube four to five times then kept in refrigerator (4°C) until used. Subsequently, the peripheral whole blood sample was centrifuged at 1,300 x *g* (2,500 rpm) for 15 min at room temperature. After centrifugation, three different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing packed leukocytes and the bottom layer of concentrated red blood cells. The plasma was removed by pipetting then the buffy coat was carefully transferred to a new 1.5 ml microcentrifuge tube and a repeated centrifugation was performed at 1,300 x *g* (2,500 rpm) for 15 min at room temperature. Finally, the pellet of red blood cells was discarded and the residual plasma was removed from the buffy coat by pipetting. The buffy coat was stored at -20°C until genomic DNA extraction.

4.2.3 Preparation of genomic DNA

DNA sample used for the genotyping of the *CYP2A13* variants were isolated from peripheral white blood cells (buffy coat) using QIAamp DNA blood mini kit® according to the manufacturer's instructions. As following: 20 µl of QIAGEN Protease were pipetted into the bottom of 1.5 ml microcentrifuge tube then 200 µl of buffy coat and Buffer AL were added and mixed well. The sample was incubated at 56°C for 10 min and followed by brief centrifugation at 6,000 x *g* (8,000 rpm) for 10 sec to remove drops form the inside of the lid. The sample was precipitated with 200

μl of 100% ethanol and repeated centrifugation at 6,000 x *g* (8,000 rpm) for 10 sec to remove drops from the inside of the lid. The mixture was carefully applied into the QIAamp spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at 6,000 x *g* (8,000 rpm) for 1 min. The QIAamp spin column was placed in a new clean 2 ml collection tube and 500 μl of Buffer AW1 was added into the spin column without carefully wetting the rim then centrifuged at 6,000 x *g* (8,000 rpm) for 1 min. The QIAamp spin column was placed in a new clean 2 ml collection tube and 500 μl of Buffer AW2 was carefully added into the spin column without wetting the rim then centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Then the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube. The final volume of 200 μl of Buffer AE was added and incubated at room temperature for 1 min then centrifuged at 6,000 x *g* (8,000 rpm) for 1 min. Finally, DNA eluted in Buffer AE was stored at 4°C until analysis.

4.2.4 Design of oligonucleotide primers

Oligonucleotide primers for PCR analysis in this study were synthesized by OPERON™. The sequences and locations of the primers is shown in Table 3 Primers CYPF, CYPR and Ex5R were selected by referring to published papers (Zhang et al., 2002; 2003). The rest of the primers used in this study were designed according to published *CYP2A13* gene sequence from the completed human genome data base (GenBank accession no. AC008962) (Appendix G) using Primer 3 software. The primer properties were characterized using Oligonucleotide Properties Calculator

(http://www.basic.northwestern.edu/biotools/oligocalc.html) and aligned using the BLAST program at NCBI to check for their specificity.

4.2.5 Amplification of *CYP2A13* Gene by Long-Distance PCR

In order to avoid the amplification of other homologous CYP2A subfamilies such as *CYP2A6* and *CYP2A7* pseudogene, the two-step PCR method was applied to each genomic DNA samples. In the first PCR step, a full-length *CYP2A13* fragment (figure 6) spans 9,456 bp (starting at 1,117 bp before the ATG start codon and ending at 869 bp after the TGA stop codon) was generated with forward primer CYPF and reverse primer CYPR. This first PCR amplification was carried out in a Perkin Elmer thermal cycler 2400 instrument (Applied Biosystems, Foster City, CA). The reaction mixtures, in a total volume of 10 μl, contained about 50 to 200 ng of genomic DNA as a template, 1X LA PCR buffer [10 mM Tris-hydrochloric acid (HCl) (pH 8.0), 60 mM Potassium chloride (KCl)], 2.5 mM each dNTP, 0.2 μM each primer, and 0.5 unit of *TaKaRa LA Taq*™ (TAKARA BIO Inc., Japan). After an initial denaturation at 94°C for 2 min, 35 cycles of amplification, each consisting of a denaturation at 94°C for 45 s, annealing at 64.5°C for 45 s, and an extension at 68°C for 10 min, were carried out, followed by a final extension at 68°C for 10 min. An H_2O blank (no template) control was routinely used for detecting potential contamination of reagents. The PCR products were electrophoresed on 0.7% ethidium bromide-stained agarose gel in 1X TBE buffer at 110 V for 45 min. The marker 1000 bp DNA ladder was run adjacent to the test samples to confirm the correct size of the amplified bands. The negative control is amplification from a sample without DNA for checking

Table 3 Sequences and locations of the *CYP2A13* oligonucleotide primers used

Primer name and sequence (5'-3')[a]	Location (base change)[b]	Amino acid change	Amplification size (bp)	Method
CYPF: GTG GAA CTA AAG ACG CAG AGC AAG AG[c] CYPR: TGC CTG CAC ATG ATC ACA AAC ATG CG[c]	Full length CYP2A13 gene	-	9,456	Long-distance PCR
Ex1F: CTG TGT CCT AAG CTG TGT GGG Ex1RFLP: CTT CCC CCT GCT CTT CCT CTG **G**	Exon 1: 74G>A	Arg25Gln	247	Mismatched PCR-RFLP, *Hha* I
Ex1F: CTG TGT CCT AAG CTG TGT GGG Ex2R: GGG AGA GAA GAC CAG ACA GGG Ex2WtF: TGA GGA GTT CAG CG***G*** G***C*** Ex2MtF: TGA GGA GTT CAG CG***G*** G***T***	Exon 2: 578C>T	Arg101Stop	898 186	Allele-specific PCR
Ex3F: GCA CTC GCG CTG AAT CCA TCT C Ex3R: TGT TGA GCC GAA TCC CAG CGC	Exon 3: 1662G>T	Gly144Arg	306	PCR-RFLP, *Hae* II
Ex5F: CCT GGC ACC TAA TCC ACG TGA Ex5R: TGG CTT TGC ACC TGC CTG CAC[c]	Exon 5: 3375C>T	Arg257Cys	300	PCR-RFLP, *Hha* I
UtrF: TCC CTA GAG AGT GCA GCC GG UtrR: GTG AGG TGA GCG TGC AAC GC	3'UTR: 7520C>G	-	576	PCR-RFLP, *Xma* III
UtrF: TCC CTA GAG AGT GCA GCC GG UtrR: GTG AGG TGA GCG TGC AAC GC UtrWtF: GGC TAA GAA TGG GGG CAG T***G*** UtrMtR: TCT CCC CTT CCT TCC CC***G***	3'UTR: 7571G>C	-	576 389 223	Tetra-primer PCR

[a]Primers were designed according to the GenBank sequence accession no. AC008962

[b]Location of primers referred the ATG translation start codon (nucleotide 66807 in AC008962)

[c]From Zhang et al. (2002, 2003).

contamination of reaction. After completed electrophoresis the amplified fragments were visualized by Ultraviolet Transilluminator, equipped with computer program analysis (Gel Doc, Biorad). The PCR products of the long-distance PCR were diluted ten times and subsequently used as template for detection of six variants of *CYP2A13*.

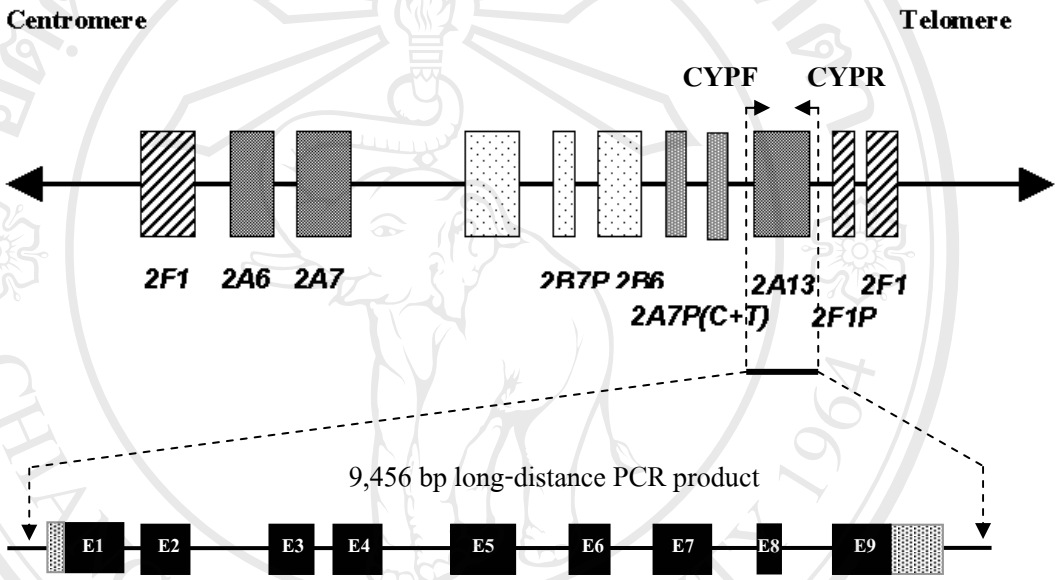

Figure 6 Schematic representation of long-distance PCR for amplification whole *CYP2A13* gene. Black boxes and dotted boxes represent exons of *CYP2A13* and 3'-UTR, respectively. Lines represent introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows (CYPF and CYPR).

4.2.6 The second PCR-step

In the second step, the first-step PCR products were used as a template to genotype the six variant alleles with three difference PCR-based methods. The PCR amplification coupled with RFLP analysis was applied to determine the four variant alleles of 74G>A, 1662G>C, 3375C>T and 7520C>G. The mismatched allele-specific amplification (mASA) method was used to detect 578C>G variation and tetra-primer method was employed to determine 7571G>C variation.

4.2.6.1 Detection of the 74G>A (Arg25Gln) of *CYP2A13* in exon 1

For specific detection of the 74G>A variant allele in exon 1, a method employing a deliberate mismatch primer to create a diagnostic RFLP (mismatch PCR-RFLP) was applied. PCR amplification was performed in the final volume of 25 µl containing 1X PCR buffer [10 mM Tris-hydrochloric acid (HCl) (pH 8.0), 60 mM Potassium chloride (KCl)]; 2.0 mM Magnesium chloride ($MgCl_2$); 200 µM each dATP, dGTP, dTTP, dCTP; 0.25 µM each primers; 0.5 unit LA Taq DNA polymerase and 1 µl of one-tenth diluted long-distance PCR product as a template using primer Ex1F (5'-CTG TGT CCT AAG CTG TGT GGG-3') and mismatched primer Ex1RFLP (5'-CTT CCC CCT GCT CTT CCT CTG **G**-3'). A 247 bp segment (figure 7) was amplified in a PCR conditions composed of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 60 sec, followed by final extension at 72° C for 3 min in the automatic thermal cycler.

Since the polymorphism of 74G>A is not located in a site of a restriction enzyme, a *Hha*I restriction site was created by introducing a G-C change at the

3'penultimate position (bold underlined letter) of the reverse primer Ex1RFLP. The primer Ex1RFLP had one mistmatch compared with the normal the CYP2A13 sequence, introducing an artificial *Hha*I restriction site (5'...GCGˆC...3) in the PCR product of the normal allele but not in that of mutant allele. Then PCR amplification products were digested with the restriction enzyme *Hha*I which cuts the 74G normal allele into two bands (24 and 223 bp), but dose not cut the 74A mutated allele.

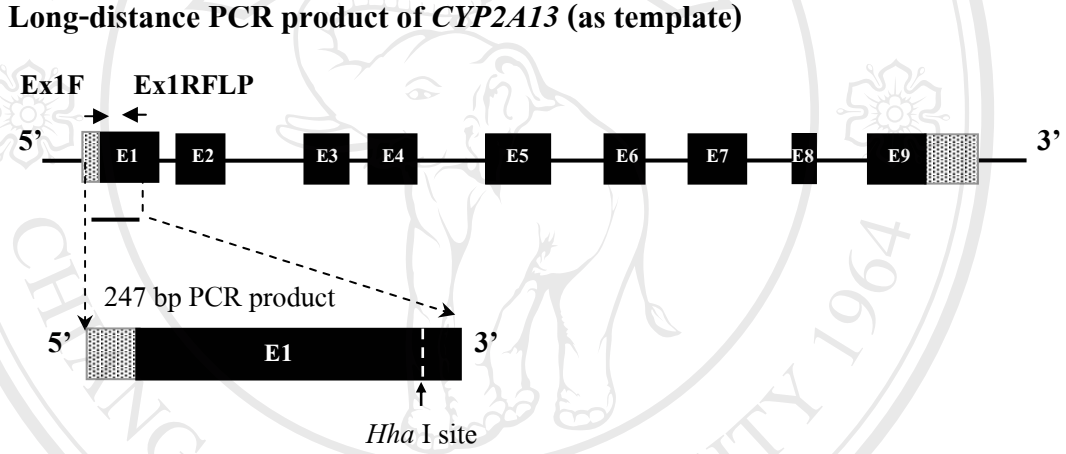

Figure 7 Schematic representation of Mismatch PCR-RFLP for the detection of 74G>A in exon 1 of *CYP2A13* gene. Black boxes and dotted boxes represent exons of *CYP2A13* and 3'-UTR, respectively. Lines represent introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows. The amplified DNA was digested by *Hha*I. The restriction sites of *Hha*I are indicated by vertical arrows. Mismatch PCR-RFLP assay for analyzing the 74 G>A polymorphisms. A mismatch PCR generates a 247 bp amplicon which was digested by *Hha*I to fragments of 24 and 223 bp for the 74G normal allele amplicon, but the 74A mutated allele is uncut.

Each reaction consisted of 5 µl of PCR product; 1X Tango™ buffer [33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] and 5 units of *Hha*I in total volume of 10 µl. The reactions were incubated at 37^{o}C overnight. The digestion pattern was determined by agarose gel electrophoresis.

4.2.6.2 Detection of the 578C>T (Arg101Stop) in exon 2 of *CYP2A13* gene.

The mismatch allele-specific amplification (mASA) was developed to detect the 578C>T allele in exon 2 of *CYP2A13* gene (figure 8). The method operates on the basis of the specific amplification of a target allele by the polymerase chain reaction with extension primers designed such that their 3' end is placed at the mutation site. When this base is complementary to that of the specific allele, the DNA segment is amplified; when it is not complementary, primer extension would be effectively blocked by a single mismatch at the ultimate or penultimate base pair of the primer/template duplex this is not generally observed under conditions commonly employed in PCR. However, in some instances a single 3'-mismatched base does allow amplification to proceed, the placement of additional mismatch base at the -3 position form 3' end of primer ameliorates this problem and allows increased PCR specificity.

The mismatched allele-specific amplification (mASA) for the 578C>T was performed in a reaction mixture (25 µl) containing of 1 µl of the one-tenth diluted long-distance PCR product; 1X PCR buffer; 2.0 mM $MgCl_2$; 200 µM each dNTP; 0.5 µM Ex2WtF or Ex2MtF forward primer; 0.5 µM Ex2R reverse primer and 0.5 unit Taq DNA polymerase. The PCR conditions were as follows: initial denaturation at 94°C for 1.30 min followed by 35 cycles of reactions composed of denaturation at 95

°C for 30 sec, annealing at 70°C for 30 sec and extension at 72°C for 45 sec, then the final extension at 72°C for 3 min in an automatic thermal cycler. The PCR products were determined by agarose gel electrophoresis.

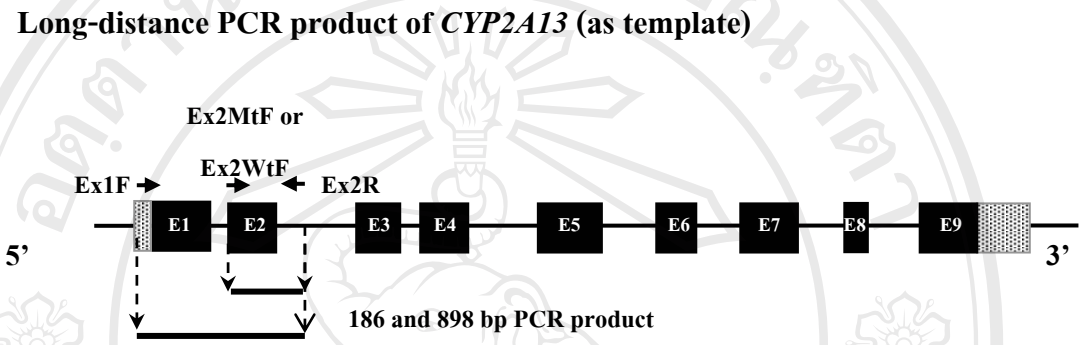

Figure 8 Schematic representation of allele-specific PCR for the detection of 578C>T in exon 2 of *CYP2A13* gene. Black boxes and dotted boxes represent exons of *CYP2A13* and 3'-UTR, respectively. Lines represent introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows.

4.2.6.3 Detection of the 1662 G>C (Gly144Arg) in exon 3 of *CYP2A13* gene.

For detection of the polymorphic 1662 G>C in exon 3of *CYP2A13* gene, PCR-RFLP was applied using a forward primer Ex3F (5'-GCA CTC GCG CTG AAT CCA TCT C-3') paired with a reverse primer Ex3R (5'-TGT TGA GCC GAA TCC CAG CGC-3') (figure 9). PCR amplification was performed in the automatic thermal cycler in a total volume of 25 µl containing 1 µl of the one-tenth dilued long-distance PCR product, 2.5 µl 10X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.25 µM of

each primer, 0.5 U of Taq DNA polymerase. The PCR conditions involved an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturationat 95°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 5 min.

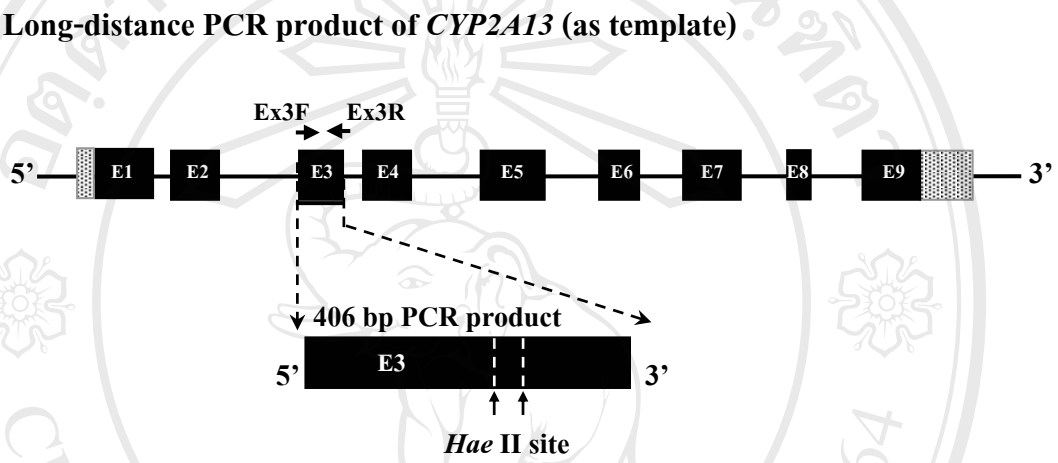

Figure 9 Schematic representation of PCR-RFLP for the detection of 1662G>C in exon 3 of *CYP2A13* gene. Black boxes and dotted boxes represent exons of *CYP2A13* and 3'-UTR, respectively. Lines represent introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows (Ex3F and Ex3R). The amplified DNA was digested by *Hae*II. The restriction sites of *Hae*II are indicated by vertical arrows. PCR generates a 406 bp amplicon which was digested by *Hae*II restriction enzyme. PCR products were digested with *Hae*II, which cuts the wild-type allele to give two bands (177 and 229 bp) and the 1662G>C mutant allele to give three bands (42, 177 and 187 bp).

The PCR products (406 bp) were subjected to restriction enzyme digestion with *Hae*II. The reaction consisted of 5 µl of PCR product; 1X Tango™ buffer [33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] and 5 units of *Hae*II in total volume of 10 µl. The reactions were incubated at 37°C overnight. The samples were then analyzed by electrophoresis on agarose gel stained with ethidium bromide. *Hae*II cuts the wild-type allele to give two fragment (177 and 229 bp), but the 1662G>C mutant allele to give three bands (42, 177 and 187 bp).

4.2.6.4 Detection of the 3375 C>T (Arg257Cys) in exon 5 of *CYP2A13* gene.

PCR-RFLP was applied to detect the 3375C>T variant allele in exon 5 of the *CYP2A13*, using a forward primer Ex5F paired with a reverse primer Ex5R (figure 10). PCR amplification was performed in the automatic thermal cycler in a total volume of 25 µl containing 1 µl of the one-tenth diluted long-distance PCR product as a template; 2.5 µl 10X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.25 µM of each primer, and 0.5 U of Taq DNA polymerase. The PCR conditions involved an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturationat 95°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 5 min.

Following PCR, restriction enzyme digestion reactions were set up as follows: 5 µl PCR product, 5 units of restriction enzyme (RE), and 1X RE buffer [33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] in total volume of 10 µl. *Hha*I (10 units/µl) or *Apal*I (10 units/µl) were used to digest the 7520C>G mutation-containing fragments. The reactions were incubated at

37°C for 2 hr. for *Hha*I and 4 hr. for *Apal*I. Digestion products were determined by agarose gel electrophoresis.

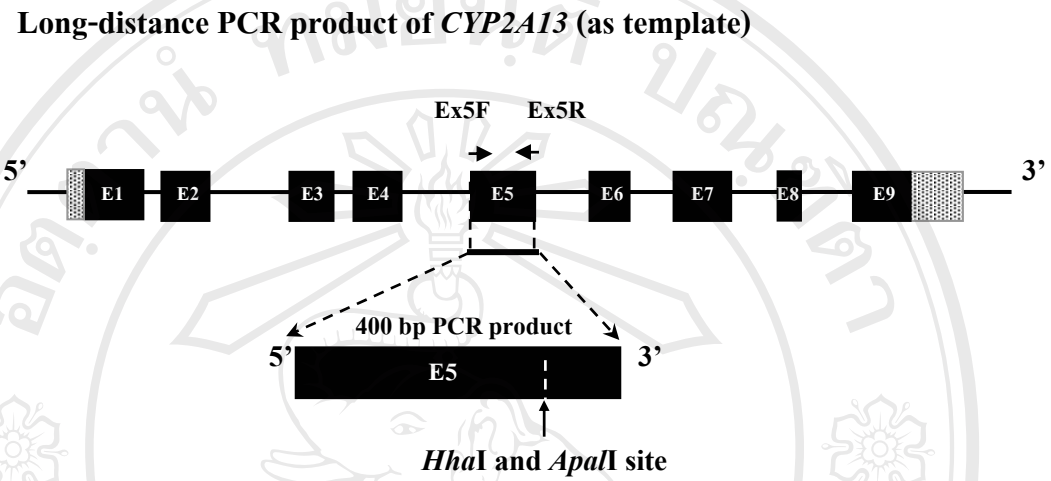

Figure 10 Schematic representation of PCR-RFLP for the detection of 3375C>T in exon 5 of *CYP2A13* gene. Black boxes and dotted boxes represent exons of *CYP2A13* and 3'-UTR, respectively. Lines represent introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows (Ex5F and Ex5R). The restriction sites of *Hha*I and *Apal*I are indicated by vertical arrows. PCR generates a 406 bp amplicon which was digested by *Hha*I and *Apal*I restriction enzyme. PCR products were digested with *Hha*I, which cuts the wild-type allele to give two bands (99 and 301 bp), but does not cut the 3375C>T allele, thus producing only one band (400 bp). In addition, all samples were also digested with *Apal*I, which does not cut the wild-type allele but cuts the 3375C>T allele to give two bands (100 and 300 bp).

4.2.6.5 Detection of the 7520C>G in 3'UTR of *CYP2A13* gene.

The detection of polymorphic 7520C>G in the 3'UTR of *CYP2A13* gene was performed using PCR-RFLP (figure 11). PCR Amplification was performed in total volume of 25 µl. Each PCR reaction contained volume of 25 µl containing 1 µl of the one-tenth diluted long-distance PCR product as a template, 2.5 µl 10X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.25 µM of each primer, and 0.5 U of Taq DNA polymerase. The forward primers UtrF (5'-TCC CTA GAG AGT GCA GCC GG-3') and reverse primer UtrR (5'-GTG AGG TGA GCG TGC AAC GC-3') were used to generate a 576 bp genomic fragment that contains the 7520C>G mutation site in the *CYP2A13* gene. The PCR conditions involved an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturationat 95°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 60 sec, with a final extension at 72°C for 5 min in an automatic thermal cycler.

Following PCR, restriction enzyme digestion reactions were set up as follows: 5 µl PCR product, 5 units of restriction enzyme (RE), and 1X RE buffer [33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] in total volume of 10 µl. *Xma*III (10 units/µl) were used to digest the 7520C>G mutation-containing fragments. The reactions were incubated at 37°C for 6 hr. and subjected to gel eletrophoresis.

Long-distance PCR product of *CYP2A13* (as template)

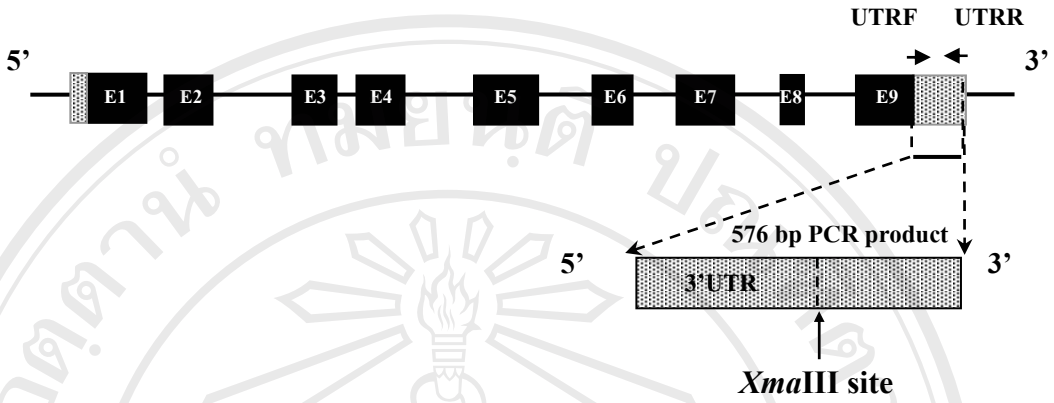

Figure 11 Schematic representation of PCR-RFLP for the detection of 7520C>G in 3'untranslated region of *CYP2A13* gene. Black boxes and dotted boxes represent exons of *CYP2A13* and 3'untranslated region, respectively. Lines represent introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows (UtrF and UtrR). The restriction sites of *Xma* III are indicated by vertical arrows. PCR generates a 576 bp amplicon which was digested by *Xma*III restriction enzyme, which cuts the wild-type allele to give two bands (259 and 317 bp), but does not cut the 7520C>G mutant allele, thus producing only one band (576 bp).

4.2.6.6 Detection of the 7571G>C in 3'UTR of *CYP2A13* gene.

A modified tetra-primer PCR was developed for determination of the 7571G>C of *CYP2A13* gene. Tetra-primer PCR (Ye et al., 1992)(figure 12) is used as a method to identify single nucleotide polymorphism using four primers (two allele-specific primers and two outer primers). Basically, three PCR bands are amplified; the largest band is amplified by the two outer primers, the second band is from the SNP allele, and the third band is from the normal allele. Originally, allele-specific primers that had single mismatched primer at the 3'-ends were used. For each allele, a perfectly matched primer was functional in the PCR but a mismatched primer was not functional in this system. Based on this principle, two and three PCR bands are detected for homozygotes and heterozygotes, respectively. However, the single mismatched primer at 3'-end was never functional in the PCR.

For detection the 7571G>C of *CYP2A13* in the 3'untranslated region, 20 μl tetra-primer PCR reaction were performed. The reaction mixture contained 2.0 μl 10X PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.125 μM of outer primer (UtrF and UtrR), 1.2 μM of allele-specific primers (UtrWtF and UtrMtR), 0.5 U of Taq DNA polymerase, and 1 μl of the one-tenth diluted long-distance PCR product as a template. Samples were amplified by 35 repeated cycles on an automatic thermal cycler using the following conditions: initial activation at 95°C for 3 min follow by denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 5 min in an automatic thermal cycler.

All amplification reactions were performed applying the commonly accepted precautions for avoiding carry-over contamination (Kwok and Higuchi, 1990).

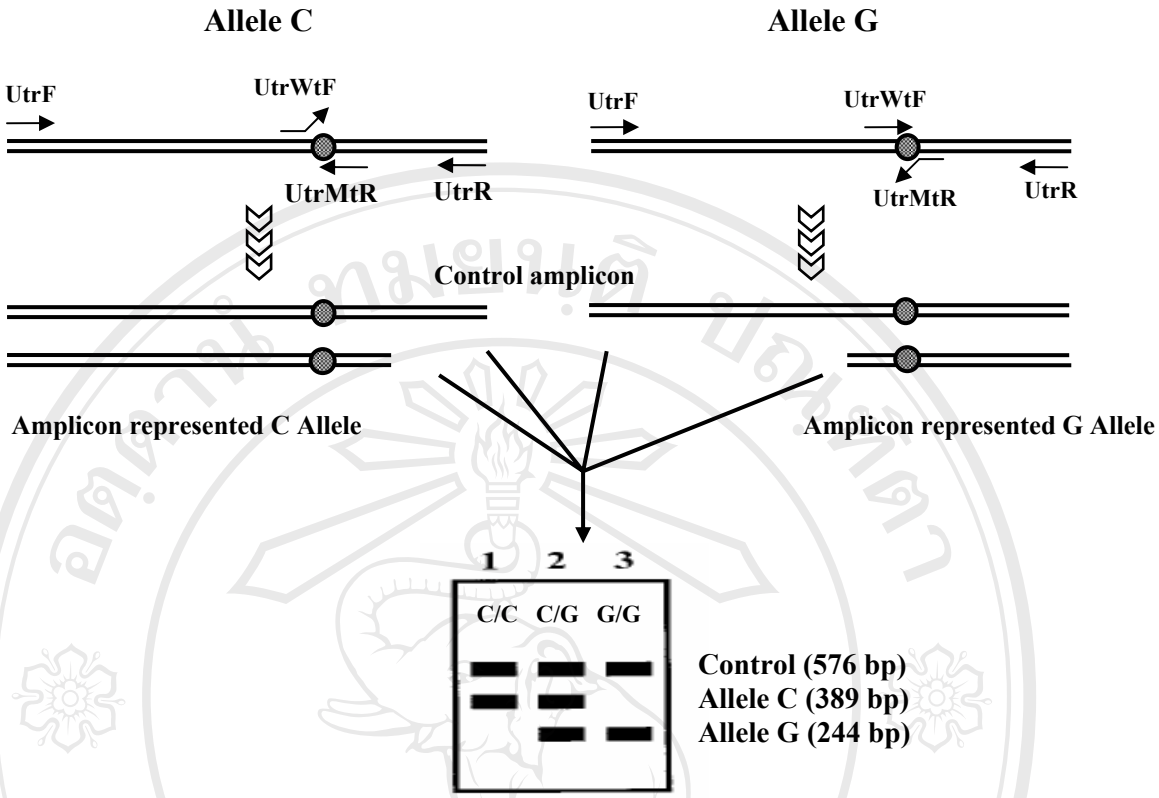

Figure 12 Schematic representation of tetra-primers PCR. Four primers are combined with DNA in a single-tube PCR reaction. The two allele-specific primers (outer primer) amplify the region of interest from both alleles serve as control band. This control fragment indicates that the PCR was successful and serves as template for the ASA in the subsequence event. The subsequent ASA produces a specific PCR product for allele G if this allele is present and primer G anneals and amplifies in combination with primer OR. A specific product of different size is generated in the presence of the allele C (right) when primer C amplifies in combination with primer OF. Separation of the different sized products by gel electrophoresis (bottom) allows genotyping of the DNA sample: lane 1, homozygous DNA for allele C (C/C); lane 2, heterozygous DNA (G/C); lane 3, homozygous DNA for allele G (G/G).

4.2.7 Electrophoresis

After PCR or RFLP reactions, an aliquot of 10 µl of PCR products or RFLP products were analyzed by electrophoresis on 1.5% or 2% ethidium bromide-stained agarose gel in 1X TBE buffer at 110 V for 45-60 min. The marker 100 bp DNA ladder was run adjacent to the test samples to confirm the correct size of the amplified bands. The negative control is PCR amplification from a sample without DNA for checking contamination of reaction. After completed electrophoresis the amplified fragments were visualized by Ultraviolet Transilluminator, equipped with computer program analysis (Gel Doc 1000, Bio-Rad, USA). All quantitative PCR reactions were performed in duplicate.

4.2.8 DNA sequencing

To validate the accuracy of genotyping by PCR-base method, samples representative of each genotype (24 samples) were selected for DNA sequencing. Amplicons encompassing the 74G>A, 578C>T, 1662G>C, 3375C>T, 7520C>G and 7571G>C mutation sites of *CYP2A13* gene were generated by PCR amplification using gDNA samples with primer pairs listed in Table 4. The PCR reaction was performed in total volume of 50 µl using the Gene Amp PCR system 9600 (Perkin-Elmer, Boston, MA, USA). The amplified products were purified using the MinElute® PCR Purification Kit according to the instructions provided by the manufacturer (QIAGEN Inc., Germany). The purified products were quantified by spectro-densitometry and were directly sequenced on one–strand. The DNA sequencing protocol was based on the dideoxy-mediated chain-termination method of Sanger and

his colleagues (Sanger et al., 1977). Thermal cycle sequencing with fluorescent labeled ddNTPs was carried out in 10 µl consisted of 4 µl ABI Prism BigDye® Terminator V 3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif.), 3.2 pmol specific primer (Table 4 in aster), and dsDNA template (2 ng per each 100 bp of the sequenced fragment). The thermocycling conditions consisted of 25 cycle of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 4 min. Sequencing reactions were desalted and the excess dye was removed by ethanol precipitation, and then re-suspended in a template suppression reagent for injection on a POP-4™ polymer capillary electrophoresis system (Applied Biosystems, Foster City, CA). The purified products were sequenced in an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer, Applied Biosystem, Foster City, USA). Sequencing data were analyzed with the assistance of ABI Prism DNA sequencing analysis software v.3.1.

Table 4 Sequences of the *CYP2A13* oligonucleotide primers used for sequencing.

Primer name	Primer name and sequence	Amplicon size
Exon 1 74G>A	Ex1F: CTG TGT CCT AAG CTG TGT GGG* Ex1R: GCC CAG CAC TGA GAT ATC ATG	556
Exon 2 578C>T	Ex1F2: GGT CTT GAT GTC AGT CTG GCG Ex2R: GGG AGA GAA GAC CAG ACA GGG*	694
Exon 3 1662G>C	Ex3F: GCA CTC GCG CTG AAT CCA TCT C* Ex3R: TGT TGA GCC GAA TCC CAG CGC	306
Exon 5 3375C>T	Ex5F: CCT GGC ACC TAA TCC ACG TGA* Ex5R: TGG CTT TGC ACC TGC CTG CAC	300
3'UTR 7520C>G or 7571G>C	UtrF: TCC CTA GAG AGT GCA GCC GG* UtrR: GTG AGG TGA GCG TGC AAC GC	578

* primers for BigDye® reaction

Each sequence obtained was verified by pairwise BLAST against the respective sequences in the GenBank reference sequence (AC008962) originally used to design the PCR primers; and standard nucleotide-nucleotide BLAST (blastn) against the nr (non-redundant) database.

4.3 Statistical analysis

Statistical analysis was conducted with the Statistical Package for Social Sciences (SPSS) program for pearson Chi-square (χ^2) test to compare allele frequency distributions between confounding factors such as gender. The lower and upper limits of the 95% confidence interval for the allele frequency were calculated, with a correction for continuity, using a program (http://faculty.vassar.edu/lowry/propl.html) that is based on methods described by Newcombe (1998). The expected genotype frequencies were calculated using the Hardy–Weinberg equation from the observed allele frequencies, $p^2+2pq+q^2=1$. The χ^2 test was used to compare the observed and calculated genotype frequencies. P values of <0.05 were regarded as significant.