ความสัมพันธ์ระหว่างอาการไม่พึงประสงค์จากยาอะมิทริปไทลีนและความหลากหลายทางพันธุศาสตร์ของยีน ซิป2ดี6 ในผู้ป่วยไทยแผนกจิตเวชโรงพยาบาลศิริราชพยาบาล

นางสาว ปราณี สื่อเจริญ

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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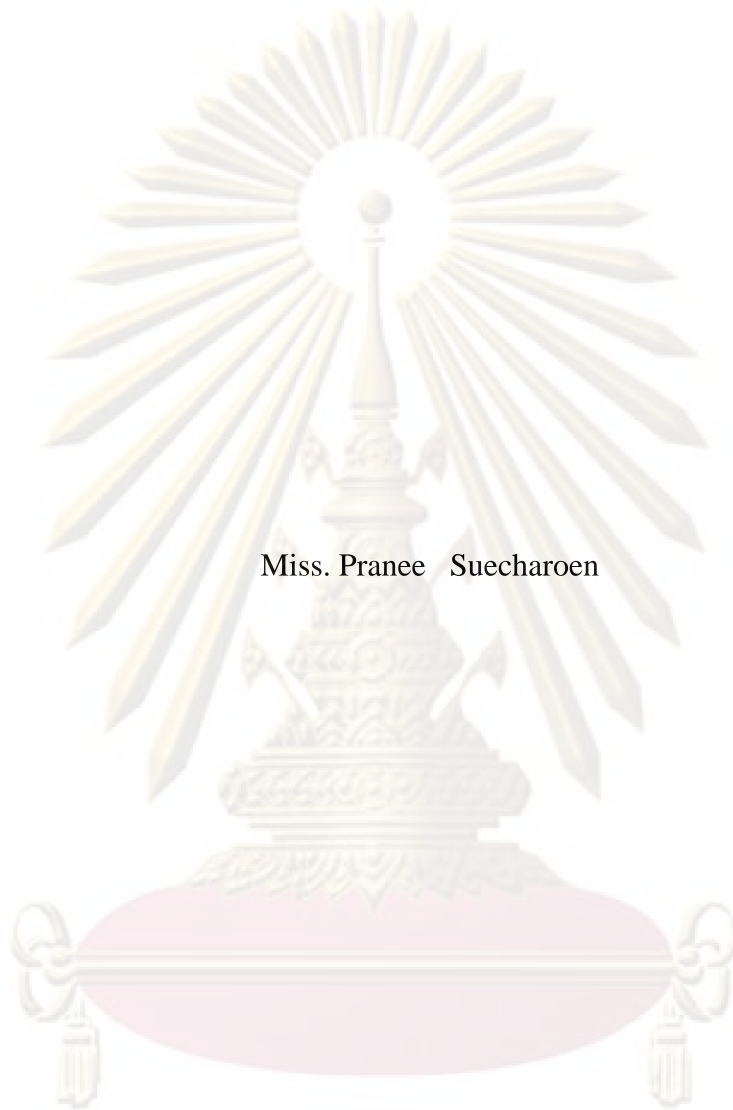
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

THE RELATIONSHIPS BETWEEN AMITRIPTYLINE ADVERSE REACTIONS AND GENETIC POLYMORPHISM OF CYP2D6 IN THAI PSYCHIATRIC PATIENTS AT SIRIRAJ HOSPITAL



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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Pharmacology
Faculty of Pharmaceutical Sciences
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Thesis Title	THE RELATIONSHIPS BETWEEN AMITRIPTYLINE ADVERSE REACTIONS AND GENETIC POLYMORPHISM OF *CYP2D6* IN THAI PSYCHIATRIC PATIENTS AT SIRIRAJ HOSPITAL
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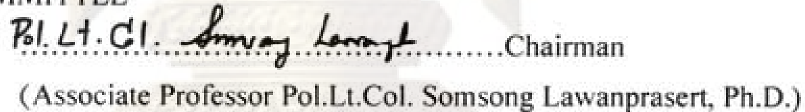
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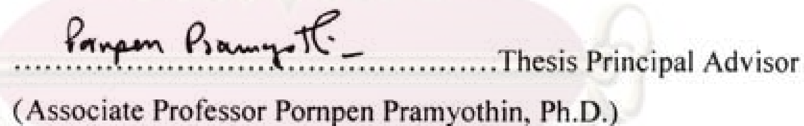
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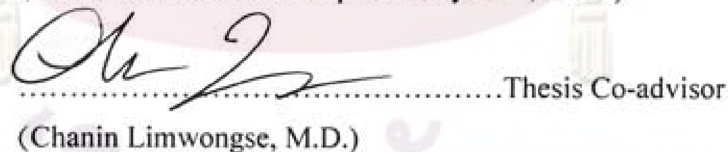
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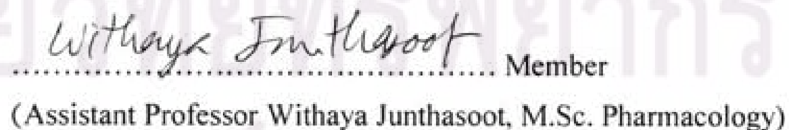
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ปราณี สื่อเจริญ:ความสัมพันธ์ระหว่างอาการไม่พึงประสงค์จากยาอะมิทริปไทลีนและความหลากหลายทางพันธุศาสตร์ของยีน ซิป2ดี6 ในผู้ป่วยไทยแผนกจิตเวชโรงพยาบาลศิริราชพยาบาล (THE RELATIONSHIPS BETWEEN AMITRIPTYLINE ADVERSE REACTIONS AND GENETIC POLYMORPHISM OF *CYP2D6* IN THAI PSYCHIATRIC PATIENTS AT SIRIRAJ HOSPITAL) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.พรเพ็ญ เปรมโยธิน อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม: นพ.ชนินทร์ ลิ่มวงศ์ อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม นพ.นันทวัช สิทธิรักษ์ จำนวนหน้า 92 หน้า.

ไซโตโครม พี450 ซิป2ดี6 เป็นเอนไซม์ที่เกี่ยวข้องกับกระบวนการเปลี่ยนแปลงยาต้านโรคจิตและยาต้านโรคซึมเศร้า โดยทั่วไปยาอะมิทริปไทลีนเป็นยาที่มีรายงานการเกิดอาการไม่พึงประสงค์แบบไม่ร้ายแรง เนื่องด้วยกลไกการออกฤทธิ์ที่ไม่เฉพาะของตัวมันเองและปัจจัยอื่นๆ ลักษณะของยีน ซิป2ดี6 มีประโยชน์ ในการใช้ทำนายความแตกต่างในการตอบสนองต่อยาและการเกิดอาการไม่พึงประสงค์ในคนแต่ละเชื้อชาติ วัตถุประสงค์ของการศึกษานี้เพื่อหาความสัมพันธ์ระหว่างการเกิดอาการไม่พึงประสงค์จากยาอะมิทริปไทลีนและความหลากหลายทางพันธุศาสตร์ของยีน ซิป2ดี6 การออกแบบการศึกษาใช้การจับคู่ระหว่างกลุ่มที่เกิดอาการไม่พึงประสงค์กับกลุ่มที่ไม่เกิดอาการไม่พึงประสงค์ซึ่งคัดแยกประเภทด้วยแบบสอบถามของ Naranjo's algorithm ได้เป็นกลุ่มละ 75 คน การประเมินการเกิดอาการไม่พึงประสงค์ใช้แบบสอบถามของการเกิดอาการไม่พึงประสงค์โดยจำแนกตามกลุ่มของอาการที่เกิด การวิเคราะห์หายีน ซิป2ดี6 ใช้เทคนิค multiplex long polymerase chain reaction (PCR) เพื่อตรวจทั้งยีนและการขาดหายไปของยีน (ซิป2ดี6*5) การทำ single base extension (SBE) ร่วมกับการวิเคราะห์โดยใช้ denaturing high-performance liquid chromatography (DHPLC) ใช้ เพื่อตรวจหา SNPs ในยีนที่ทำการศึกษา การจัดเรียงตัวของยีนใช้หาโดยวิธี 5-plex PCR จาก gDNA วิเคราะห์ร่วมกับ semi-quantitative DHPLC จากทั้งหมด 10 SNPs ที่เลือกมาวิเคราะห์ พบว่ามี 3 SNPs ที่ไม่พบ พบเพียง 7 SNPs ในการศึกษานี้ส่วนใหญ่จะเป็น ซิป2ดี6*36 -*10B (25.33%) จีโนไทป์ที่สัมพันธ์กับ ซิป2ดี6*10A หรือ *10B (23.33%) และ *1/*1 (13.33%) ตามลำดับ Haplotype บางชุดมีความแตกต่างกันทางสถิติในสองกลุ่มทดลอง อย่างไรก็ตามไม่พบความสัมพันธ์ระหว่างยีนกับคะแนนรวมของอาการไม่พึงประสงค์ ดังนั้นจึงไม่พบความสัมพันธ์ระหว่างการเกิดอาการไม่พึงประสงค์จากยาอะมิทริปไทลีนและความหลากหลายทางพันธุศาสตร์ของยีน ซิป2ดี6 ในกลุ่มตัวอย่างที่ทำการศึกษาที่ Odd ratio 2.5 แม้ว่าส่วนใหญ่จะมีการจัดเรียงตัวของยีนมีการกระจายตัวแตกต่างกันบ้างก็ตาม อย่างไรก็ตามอัลลีลที่พบบ่อยคือ ซิป2ดี6*36 -*10B และอัลลีลที่สัมพันธ์กับ *36 มียีนในกลุ่มควบคุมสูงกว่าเมื่อเทียบกับทั้งสองกลุ่ม ความสัมพันธ์น่าจะพบได้ในกลุ่มทดลองที่มีขนาดใหญ่ขึ้นร่วมกับการให้ขนาดยาที่มากขึ้นของยาอะมิทริปไทลีน การศึกษานี้เป็นการศึกษาแรกที่บอกลักษณะของยีน ซิป2ดี6 ของผู้ป่วยโรคซึมเศร้าในประเทศไทย

ภาควิชา......................เภสัชวิทยา..............ลายมือชื่อนิสิต......................ปราณี สื่อเจริญ............

สาขาวิชา...................เภสัชวิทยา.............. ลายมือชื่ออาจารย์ที่ปรึกษา..

ปีการศึกษา...............2551.......................ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..

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487 65817 33: MAJOR PHARMACOLOGY

KEY WORD: *CYP2D6* / POLYMORPHISMS / AMITRIPTYLINE / ADVERSE DRUG REACTIONS

PRANEE SUECHAREON: THE RELATIONSHIPS BETWEEN AMITRIPTYLINE ADVERSE REACTIONS AND GENETIC POLYMORPHISM OF *CYP 2D6* IN THAI PSYCHIATRIC PATIENTS AT SIRIRAJ HOSPITAL. THESIS ADVISOR: ASSOC. PROF. PORNPEN PRAMYOTHIN, Ph.D., THESIS COADVISOR: CHANIN LIMWONGSE, M.D., THESIS COADVISOR: NANTAWAT SITDHIRAKSA, M.D., 92 pp.

Cytochrome P450 2D6 is a major drug-metabolizing enzyme involved with antipsychotic and antidepressant drug metabolism. Amitriptyline, tricyclic antidepressant, was commonly reported with adverse drug reactions (ADRs) because of its non-selective action and other factors. *CYP2D6* genotyping was shown to be useful in phenotype prediction. The objective of this study was to find the relationship between amitriptyline ADRs and genetic polymorphism of *CYP2D6*. The study was designed as matched case-control recruited subjects by Naranjo's algorithm screening to obtain 75 subjects in each group. Visual analog scales of amitriptyline ADRs in questionnaires were used to identify type of ADRs and scores in both groups. *CYP2D6* genotype were detected by multiplex long polymerase chain reaction (PCR) to obtain whole gene and/or gene deletion (*CYP2D6**5). Single base extension (SBE) was subsequently performed, coupled with denaturing high-performance liquid chromatography (DHPLC) analysis in order to detect SNPs therein. Gene arrangement was determined in 5-plex PCR analyzed with semi-quantitative DHPLC. Ten SNPs were included in tested panel; three SNPs were not found in recruited subjects and seven SNPs were observed. The most common genotype found in this study are *CYP2D6**36-*10B in tandem-associated genotype (25.33%), *CYP2D6**10A or *10B-associated genotype (23.33%), and *1/*1 (13.33%), respectively. Some haplotypes are significantly different in two groups. However, the interaction between genotype and ADR scores is insignificantly associated. Hence, there are no association between *CYP2D6* genotype and amitriptyline ADRs at Odd ratio 2.5 in recruited subjects. Nevertheless, the most commonly found genotype, *CYP2D6**36-*10B and *36-associated alleles including in tandem duplication, is significant higher in control group when compared with *CYP2D6**2/*10B of both groups. The association could be found if sample size is greater together with higher challenged dose of amitriptyline. This is the first study providing *CYP2D6* genotype in depressive patients in Thai.

Department............Pharmacology............Student's signature..Pranee..Suechareon...
Field of study......... Pharmacology............Advisor's signature...Pornpen Pramyothin.....
Academic year ...... 2008.........................Co-advisor's signature.................

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ABBREVIATIONS

Abbreviations used in the text are listed bellowed:

ADRs	=	Adverse Drug Reactions
AT	=	Amitriptyline
CYPs	=	Cytochrome P450s
DHPLC	=	Denaturing high performance liquid chromatography
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxyribonucleoside triphosphates
EDTA	=	Ethylenediaminetetraacetic Acid
EM	=	Extensive Metabolizer
GM	=	Gram
HR	=	Hour
IM	=	Intermediate Metabolizer
L	=	Liter
MR	=	Metabolic Ratio
Min	=	Minute
NT	=	Nortriptyline
OD	=	Optical Density
PBS	=	Phosphate Buffer Saline
PCR	=	Polymerase Chain Reaction
OD	=	Optical Density
PBS	=	Phosphate Buffer Saline
PCR	=	Polymerase Chain Reaction
PM	=	Poor Metabolizer
RBC	=	Red Blood Cell
RFLP	=	Restriction Fragment Length Polymorphism
TCAs	=	Tricyclic antidepressants
UM	=	Ultrarapid Metabolizer
UV	=	Ultra violet
WBC	=	White Blood Cell
W.H.O.	=	World Health Organization

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CHAPTER I

INTRODUCTION

Rationale and Background

Nowadays, psychiatric disorders appear to cause more morbidity and mortality. Based on the forecasts in 2020, depression represents a major health problem second only to ischemic heart disease [1]. The diagnosis was made according to diagnostic by Diagnostic and Statistical Manual of Mental Disorder 4th Edition by Depressive disorder (DSM4) and International Classification of Disease ICD 10 criteria (ICD 10). The depressive disorder associated with suicide is frequently ranked in the first tenth order of suicidal cause in every country [2]. The suicidal incidence was commonly found at the age over 55 years with the ratio between women and men of 2:1 [3, 4]. Depressive disorder is considered to cause predominantly by an imbalance of neurotransmitter level. Other conditions are also accounted for the causative factors such as post-traumatic mental problem, chronic physical disorders, drug addiction and genetic factors. Depressive disorder can be treated effectively depending on the factors that mainly cause depression which is different in each individual. Common treatment of depression can be divided into 3 approaches. First, psychological therapy can be performed using such as these following strategies; Interpersonal Psychotherapy (IPT), Cognitive-Behavioral Therapies (CBT) and Rational-emotive Therapy (RET). Second, Electroconvulsive therapy (ECT) is a technique that uses electrical impulse to stimulate brain cells in order to improve neurotransmitter balance. This strategy can alleviate depressive symptoms especially in serious depressive patient who are unable to treat with antidepressant [5]. Third, Pharmacological therapy is achieved by using medicine such as antidepressants. Currently, depressive treatment with antidepressant is widely used in order to inhibit the reuptake of neurotransmitters in the brain which resulting in an increase of neurotransmitter level. This type of treatment requires the use of drug for individual patients since they exhibit a wide range of drug response although the same drug and dosage are given. The variation of individual drug response could be affected from several factors e.g. age, dietary intakes, concomitant used drugs, lifestyle and genetics [6]. Drug metabolizing enzymes (DMEs) play an important role in drug metabolism and elimination and its genetic polymorphisms are found to be a factor that causes a wide range in drug response and toxicity. There are numerous studies regarding a relationship between genetic polymorphisms of DMEs and plasma drug concentration corresponding to the genotypes [7-10].

Pharmacogenomics in psychiatry research is conducted in an attempt to identify genetic variations [11] that has an effect on the accumulation or depletion of drug concentrations, to associate genetic polymorphisms with potential risk to cause psychiatric disorders or adverse event from drug uses. The cytochrome P450 2D6 (CYP2D6) is phase I drug metabolizing enzyme that known to be responsible for metabolism of almost 20-25 percent of all commonly prescribed drugs such as antidepressant, antiarrythmics, neuroleptic drugs, opiates, and antihypertensive drugs [12]. It is currently one of the most well studied enzymes providing tremendous information available for its genotype and clinical implication. *CYP2D6* genetic polymorphisms can be classified based on phenotype as extensive metabolizers (EM), intermediate metabolizer (IM), poor metabolizers (PM), and ultrarapid metabolizer (UM) [13, 14]. Individual who carries 2 null-alleles is characterized as PM which exhibits nonfunctional enzyme activity due to the extremely low *CYP2D6* expression [13]. PM phenotype is prone to exhibit adverse reaction [15-17]. when is medicated with CYP2D6 substrates especially in psychotherapy with pronounced clinical relevance. Due to psychopharmacology is still practiced empirically, the optimization of therapy for individual generally based upon trial and error [16, 18 and 19]. It was obviously found the correlation between psychiatric drug response and genetic polymorphisms of *CYP2D6* which are reported worldwide among different ethnic groups [13, 20]. The variations are resulted from a difference in blood concentration (pharmacokinetic), drug binding receptors (pharmacodynamic), and eventually clinical responses [1, 13, and 21-24]. Adverse drug reaction (ADR) is a common problem arising from drug uses in patients. World Health Organization (WHO) defines adverse reaction as “a reaction which is noxious and unintended which occurs at dose normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function” [25]. Formerly, majority of depressive patients often receive tricyclic antidepressants (TCAs) as an initial treatment. For instance, amitriptyline has been used to treat depression for more than three decades since 1961 and it is still widely prescribed although the newer antidepressants were introduced to the market. Amitriptyline and some antidepressant drugs are well known for its relatively narrow therapeutic range and high toxicity manifested in pronounced anticholinergic symptoms, gastrointestinal symptoms, cardiovascular symptoms and others [26-31]. CYP2C19 and CYP2D6 play an important role in the metabolism of amitriptyline. It is first metabolized by CYP2C19 giving nortriptyline as an active metabolite. Then CYP2D6 metabolizes nortriptyline to an inactive form by hydroxylation [1, 24 and 32].

Therefore, the defective CYP2D6 enzyme function possibly gives an accumulation of active metabolite which consequently cause ADRs related high plasma drug concentration.

The several studies reported the relationship between adverse reactions and *CYP2D6* polymorphism that the prevalence of PMs and IMs in the adverse reactions group was significantly higher than those in non adverse reaction group [1, 24, 29, 30 and 33]. These studies did show a nonsignificant of dysfunctional *CYP2D6* alleles in heterozygously treated depressive patients with adverse drug reactions, failed to genotype with response drugs correlations. Recently, two newer antidepressants (paroxetine and mirtazepine) can not be predicted to adverse events in depressive patients [1].

In contrary, UMs have been correlated with therapeutic response and have never been reported with adverse reactions [34, 35]. The ultimate goal of pharmacogenomics monitoring is to personalize drug therapy which focused on the impact of genetic variation on the expression and function of drug-metabolising enzymes in order to determine the most effective and safest dosage of the best drug for a particular patient before the first dose is given. Therefore, pharmacogenetic information has the potential to improve patient compliance with drug therapy. There are no information regarding the relationship between *CYP2D6* genetic polymorphisms and adverse drug reactions have been conducted in Thai population. This study aims to examine the influence of *CYP2D6* variability on drug response in depressive patients by evaluating adverse reactions of amitriptyline. *CYP2D6* polymorphisms in Thai population were preliminarily investigated (unpublished data). However, the aspect of determining ADRs of commonly prescribed dose with genetic polymorphisms has never been done before. This study will be the first to prove whether *CYP2D6* polymorphisms have any association with ADR when administering amitriptyline in Thais. This data could lead to the application of genotype-adjusted dosing of amitriptyline in Thai depressive patients.

Hypothesis

Adverse reactions of amitriptyline and genetic polymorphism of *CYP2D6* have an association in Thai depressive patients at Siriraj Hospital.

Objectives

To find the relationship between adverse reactions of amitriptyline and genetic polymorphism of *CYP2D6* in Thai depressive patients at Siriraj Hospital.

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Expected Outcomes

The study could be used as a preliminary data for dose adjustment of amitriptyline in order to tailor each patient with the best dosage regimen corresponding to their genotype. Consequently, adverse drug reaction could be prevented and patient compliance could be increased providing high efficacy of drug treatment.

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CHAPTER II

LITERATURE REVIEWS

1. Major Depressive Disorder (MDD)

Among psychiatric disorders, depression is one of the most prevalent problems that represent as a major health issue in the 21st century [1]. Several studies reported the prevalence of depressive disorder which women are two to three times more likely to have than men [1, 2 and 3]. In the past, psychiatrists believed that depression was a result of both troubling thoughts and emotions in a person. Current studies revealed the three most important factors include defective biological processes, genetic factors and environmental factors. Biological causes are due to the chemical changes in the brain. Research data indicated that people suffering from depression have imbalances of two neurotransmitters which are serotonin and norepinephrine in the brain. The deficiency in serotonin may cause sleep problems, irritability, and anxiety associated with depression. Likewise, a decreased amount of norepinephrine which regulates alertness and arousal may contribute to the fatigue and depressed mood of the illness. Genetic factors are also contributed with the support from genetic research that depressive disorders have family linkage. For instance, if one identical twin suffers from depression or manic-depressive disorder, the other twin will have a 70 percent chance to suffer from the same illness. Finally, environmental factors resulted from stressful and emotional situations such as the death of parent during childhood are considered causative as well.

Depression is a common mental disorder which can be characterized by depressed mood. It is an illness that involves the body, mood, and thoughts affecting the way a person eat and sleep, the way one feels about oneself, and the way one thinks about things. The signs and symptoms of depression include loss of interest in activities that once were interesting or enjoyable, feeling of guilt or self-worth, disturbed sleep or appetite, low energy, and poor concentration. The diagnosis of mental disorders that is widely used to classify are the criteria from International Classification of Disease (ICD10) and the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSMD4).

International Classification of Diseases (ICD10)

International Classification of Diseases (ICD10) was endorsed by the 43rd World Health Assembly in May 1990 and came into use in WHO Member States since 1994. This classification is the latest series which has its origins in the 1850s. The ICD10 has become the international standard diagnostic classification for all general epidemiological and many health management purposes. These include the analysis of the general health situation of population groups and monitoring of the incidence and prevalence of diseases and other health problems in relation to other variables. Since then, ICD10 was used to classify diseases.

1.1 Mood disorder (F32-34)

F32 Depressive episode

F32.0 Mild depressive episode

(1) Without somatic symptoms

(2) With somatic symptoms

F32.1 Moderate depressive episode

(1) Without somatic symptoms

(2) With somatic symptoms

F32.2 Severe depressive episode without psychotic symptoms

F32.3 Severe depressive episode with psychotic symptoms

F32.8 Other depressive episodes

F32.9 Depressive episode, unspecified

F33 Recurrent depressive disorder

F33.0 Recurrent depressive disorder, current episode mild

(1) Without somatic symptoms

(2) With somatic symptoms

F33.1 Recurrent depressive disorder, current episode moderate

(1) Without somatic symptoms

(2) With somatic symptoms

F33.2 Recurrent depressive disorder, current episode severe without psychotic symptoms

F33.3 Recurrent depressive disorder, current episode severe with psychotic symptoms

F33.4 Recurrent depressive disorder, currently in remission

F33.8 Other recurrent depressive disorder

F33.9 Recurrent depressive disorder, unspecified

F34 Persistent mood (affective) disorders

F34.0 Cyclothymia

F34.1 Dysthymia

F34.8 Other persistent mood (affective) disorders

F34.9 Persistent mood (affective) disorder, unspecified

At present, treatments of depressive disorders use several therapies but pharmacotherapy is widely accepted as a main treatment. Classification of pharmacotherapy in antidepressive treatment comprise of Tricyclic Antidepressants (TCAs), Selective Serotonin Reuptake Inhibitors (SSRIs), Monoamine Oxidase Inhibitors (MAOIs) and other antidepressant drugs as shown in Table 1.

Table 1 Drugs used for treatment of depressive disorders [6, 13-15 and 18]

Tricyclic and related antidepressant drugs (TCAs)	
Amitriptyline hydrochloride	Nortriptyline
Amoxapine	Protriptyline hydrochloride
Clomipramine hydrochloride	Trimipramine
Desipramine	Maprotiline hydrocholride
Doxepin	Mianserin hydrochloride
Imipramine	Trazodone hydrochloride
Lofepramine	Viloxazine hydrocholride
Monoamine oxidase inhibitors (MAOIs)	
Phenelzine	Tranylcypromine
Isocarboxazid	Moclobemine
Selective Serotonin-Reuptake Inhibitors (SSRIs)	
Fluoxetine	Sertraline

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Fluvoxaime maleate	Venlafazine
Paroxetine	
Other antidepressant drugs	
Alprazolam	Mirtazepine
Bupropion	Nefazodone hydrochloride
Flupenthixol	Reboxetine
Lithium	Tryptophan

2. Amitriptyline hydrochloride (a mee trip′ ti leen)

Amitriptyline (AT) is a tricyclic antidepressant agent (TCA) and contains a characteristic three-ringed nucleus structure that is indicated to treat various forms of depression. It is often used in conjunction with other psychotherapies and analgesic (for certain chronic and neuropathic pain) or as a prophylaxis against migraine headache.

Pharmacodynamics and pharmacokinetics

Amitriptyline has onset of therapeutic effect within 7 - 21 days. It can be distributed across placenta and breast milk. Metabolism of AT is mainly mediated by hepatic enzyme giving nortriptyline (NT) as an active metabolite and other hydroxyl and conjugated derivatives. The metabolism may be impaired in the elderly. The half-life in adults is 9-25 hours (15 hours in averages) and time to peak serum concentration is within 4 hours. Renal excretion is eliminated by approximately 18 percent as unchanged drug and only small amounts is excreted in feces by bile.

Mechanism of action and metabolism of amitriptyline

The mechanism of action of tricyclic antidepressants is involved with the blockade of norepinephrine (NE) and/or serotonin (5-Hydroxytryptamine, 5-HT) reuptake at the presynaptic neuron in the central nervous system (CNS) as shown in Figures 1-2. Antidepressants have been shown to increase neurotransmitter levels rapidly by the reuptake of blockade and can occur within a few exposures of medication or at most several days after chronic administration. However, the clinical response from antidepressants requires several weeks. This time course is more consistent with postsynaptic receptor changes. Therefore the early theories have been adjusted to reflect the importance of neurotransmitter levels for the sensitivity, function, and efficiency of the receptor system.

Amitriptyline contains tertiary amine is metabolized by the cytochrome P450 2C19 (CYP2C19) into the secondary amines (e g. NT is active metabolized of AT). Amitriptyline is metabolized at N-demethylation and benzylic 10-hydroxylation whereas demethylated metabolite NT is primary metabolized by 10–hydroxylation. Both AT and NT are 10-hydroxylated by the cytochrome P450 2D6 (CYP2D6) [1, 7, 11, 24 and 32] as shown in Figure 3. The degree of demethylation of AT may be clinically important, because the metabolite form is active and may reach higher concentrations in plasma than the parent drug.

Tricyclic antidepressant agents are known as nonselective antidepressant. This implies that it not only blocks the reuptake of the monoamine neurotransmitters that is involved with α1-adrenergic, histaminergic, and cholinergic receptor system, it also produces common adverse drug reactions (ADRs). The World Health Organization (WHO) defines ADR as any response to a drug that is noxious and unintended, and that occurs at doses normally used in man for prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function [25]

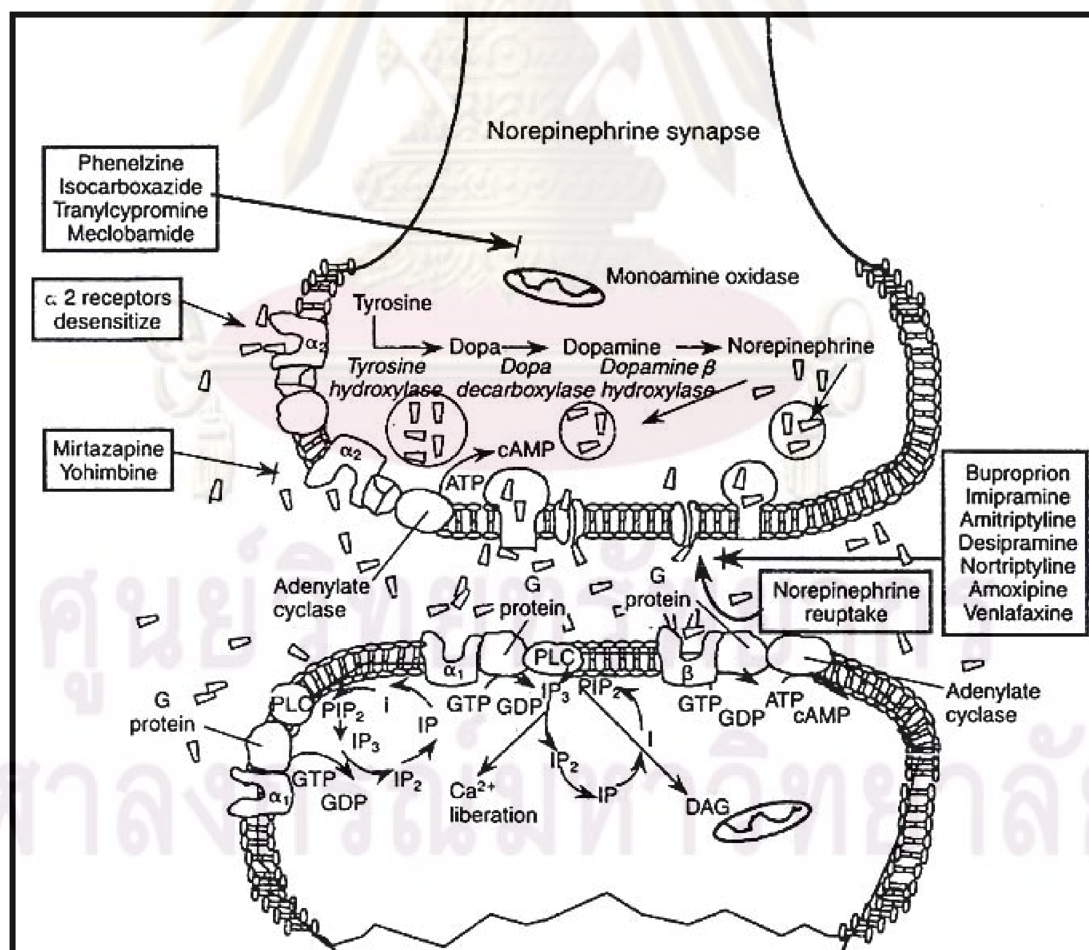

Figure 1 Mechanisms of action of antidepressant drugs: norepinephrine synapse [36].

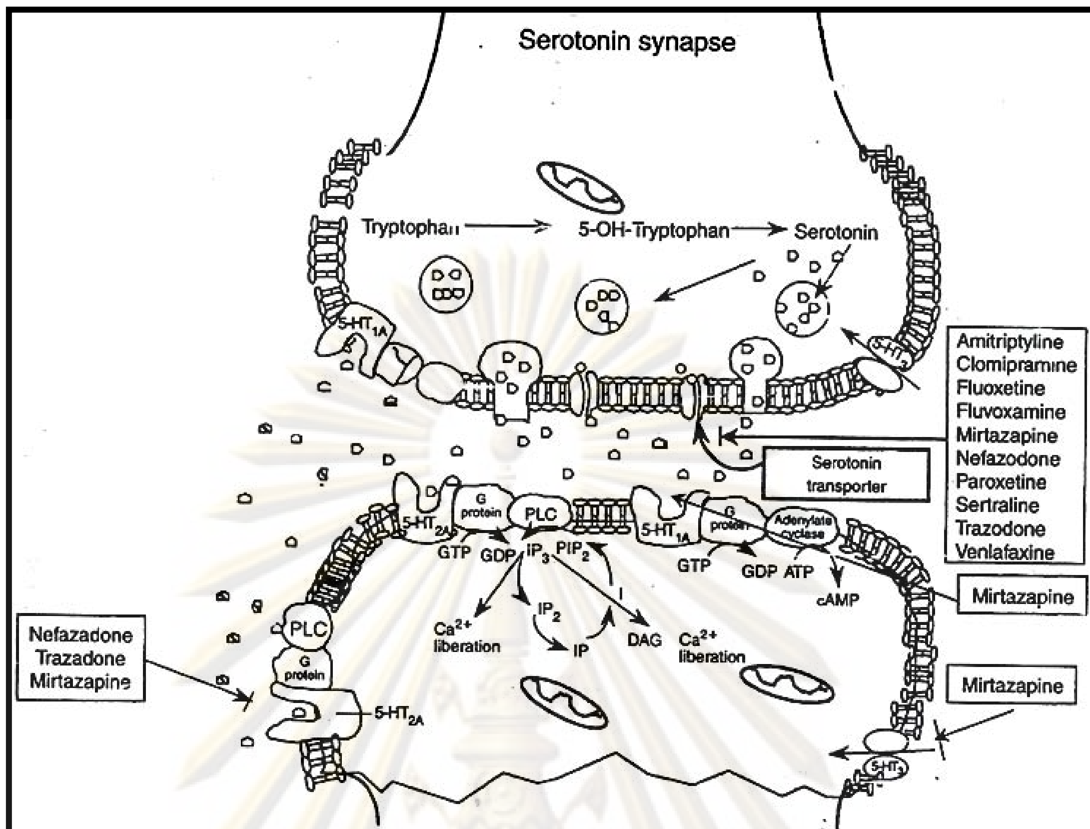

Figure 2 Mechanisms of action of antidepressant drugs: serotonin synapse [36].

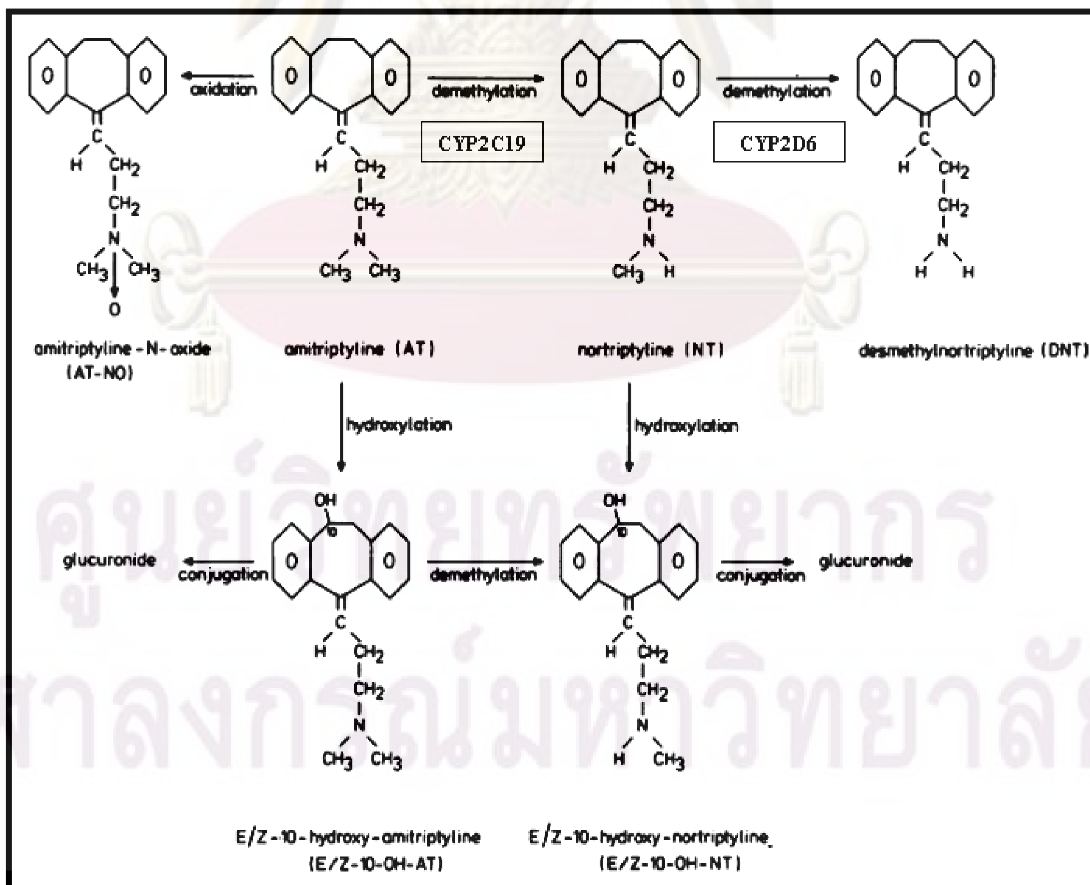

Figure 3 Chemical structures of amitriptyline and its metabolic pathway [37].

Adverse reactions of amitriptyline [1, 26-31]

The most common adverse reactions of amitriptyline can occur with great variability in the effect profile.

1. Anticholinergic action

The anticholinergic effects may be pronounced from their antagonism of muscarinic receptors, incidence of adverse reactions have 34 percent of occurring [38]. Anticholinergic effects such as drowsiness and dry mouth occur at approximately in 1 of 3 patients [31, 39]. In addition, there are urinary obstruction and gingivitis. The problem of blurred vision, urinary retention and constipation (rarely paralyticileus) occur in many elderly people.

2. Cardiovascular system

Because of significant blockade of alpha-adrenergic receptors, they produce myocardial infarction (heart attack), stroke, arrhythmias irregular heartbeat), hypotension (low blood pressure), particularly orthostatic hypotension (low blood pressure upon standing), syncope (fainting), hypertension (high blood pressure), tachycardia (rapid heartbeat), palpitation, supraventicular tachycardia, ventricular tachycardia ventricular fribrillation, prolongation of PR, QRS or QT Interval ST segment and T wave changes bundle branch block or complete heart block.

3. Central nervous system and neuromuscular system

The adverse reactions of amitriptyline in the CNS and neuromuscular are coma, seizures, hallucinations, delusions, confusional states, disorientation; incoordination, ataxia (failure of muscle coordination), tremors, numbness, tingling, paresthesias of the extremities, dysarthria (imperfect articulation of speech due to disturbances of muscular control), disturbed concentration, excitement, anxiety, insomnia, restlessness, nightmares, nervousness, drowsiness, dizziness, weakness, fatigue, headache, and tinnitus (ringing in the ear).

4. Dermatology and allergy

The dermatology and allergic reactions are skin rash, urticaria, photosensitization, edema (swelling) of face and tongue, alopecia and photosensitity.

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5. Endocrine

The increase of hormone levels can result in testicular swelling and gynecomastia in the male (development of breasts), breast enlargement in female. Infrequently, galactorrhea may also be observed with increased or decreased libido, impotence, elevation, and lowering of blood sugar levels.

6. Extrapyramidal symptoms

A number of side effects are related to dopamine blockade, and others are related to various receptor systems. The most common side effects are often termed extrapyramidal symptoms (EPS) and results from the blockade of dopamine receptors in the nigrostriatal pathway. There are three components of acute EPS these are pseudoparkinsonism, dystonia, and akathisia.

7. Gastrointestinal system

It is rare to have hepatitis (including altered liver function and jaundice) but the most occurring are nausea, epigastric distress, vomiting, anorexia, stomatitis, peculiar taste, diarrhea, increased appetite, weakness, unpleasant taste, weight loss or gain.

8. Genitourinary and Hematologic system

The most common symptoms are leucopenia and agranulocytosis.

9. Miscellaneous

It is rare with bone marrow depression, hepatic toxicity, seizures, peripheral neuropathy, photosensitivity, dysarthria, stuttering, renal failure and withdrawal symptoms.

Risk factors for adverse reactions [6, 40]

1. Age

The relationship between age and ADRs is ambiguous. Most of the older people are likely to have more ADRs because of the increased number of medications taken by the elderly. The absorption, metabolism, distribution and elimination of drugs may reduce with age and these factors may be responsible for ADRs. In newborn infants, many drug metabolizing enzyme systems are underdeveloped.

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2. Disease and drug

Drugs are usually administered to people whom a physiological or biochemical process is altered by the disease, for example hepatic drug elimination and the conversion of prodrug. Which may be substantially reduce in chronic liver disease. Caution and dosage adjustment may be required. There is a direct linear relationship between the number of drugs taken and the rate of ADRs which mostly drug allergy is associated with disease or response after mitogenic stimulation.

3. Medical history

Patients who have already had an adverse reaction to a drug are more likely to have reactions to other drug but atropy is not a factor either for ADRs in general or allergic ADRs.

4. Race

For many products, the pharmacokinetic variability seems to be no greater than the intraindividaul variability, but there is evidence of differences in both pharmacokinetics and pharmacodynamics and susceptibility to ADRs. The factors can be divided into intrinsic, which means they are inborn such as height, weight and metabolic genetic polymorphism, extrinsic, which means these are acquired such as medical practice, smoking, alcohol and diet.

5. Sex or gender

Females appear to be more susceptible to ADRs than males. The increases may be due to pharmacokinetic factors and hormonal influences. Women generally have more adipose tissue, secrete less gastric acid and have slower gastric emptying than men.

6. Genetic and pharmacogenetic factors

Genetic factors play a major role in determining drug response and handling as well as susceptibility to ADRs. Although receptors, transport process and metabolic pathways may be responsible for ADRs, metabolic process may contribute the most. Many of which are responsible for handling foreign substances (xenobiotics) especially drugs.

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3. Pharmacogenetics and pharmacogenomics [19, 29 and 41]

The pharmacologic drug response is a complex trait and is likely under polygenic control rather than simple monogenic regulation. At present, pharmacogenomic investigates drug metabolism to a broader context with an attempt to dissect the genetic control at multiple levels of the pharmacokinetic and pharmacodynamic cascade from drug absorption and transport to drug-receptor interface and beyond.

Human genetic variation and single nucleotide polymorphisms (SNPs) represent the most common DNA sequence variation in the human genome. It is thought that the complete human sequence including the coding regions, introns, and promoters will contain approximately one million SNPs. SNPs are valuable biomarker to elucidate the genetic basis of common complex disease and pharmacologic traits.

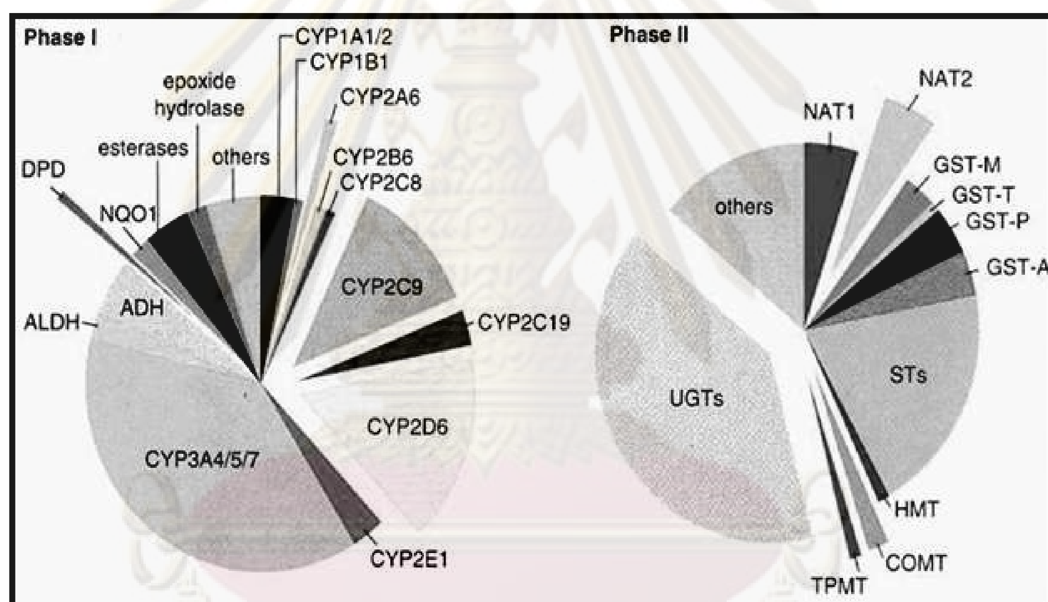
Both pharmacogenomic and pharmacogenetic research determine genetic factors that contribute to variability in drug response in individual patients which are summarized at the website http://www.pharmgkb.org/index.jsp. Pharmacogenetic strategies primarily study sequence variations in candidate genes suspected of affecting drug response, whereas pharmacogenomic strategies evaluate the entire genome. The progress made by pharmacogenetics, in many ways, is a theoretic working model describing the relationship between the genetic polymorphisms and adverse reactions.

Drug metabolism

Drug metabolism is a biochemical modification or degradation usually through specialized enzymatic systems. Drug metabolism often converts lipophillic chemical compounds into more readily excreted polar products. Its rate is an important determinant of the duration and intensity of the pharmacological action of drugs. Every organism takes up many different foreign compounds (xenobiotics) such as food components, pollutants and chemicals from the environment or drugs. Elimination of these exogenous substances out of the body often requires biotransformation through enzymes or xenobiotic metabolizing system. Drug metabolism usually mediates chemical conversion of endogenous and exogenous substances to active or inactive metabolites and intoxication or detoxification. Drug metabolism is divided into two phases: Phase I belongs to functionalization (non-synthetic) reactions where functional groups are oxidation (cytochrome P450 monooxygenase system, flavin-containing monooxygenase system, alcohol

dehydrogenase, aldehyde dehydrogenase, monoamine oxidase and co-oxidation by peroxidases), reduction (NADPH-cytochrome P450 reduces and reduced (ferrous) cytochromeP450), and hydrolysis (epoxidhydrolases, esterases and amindases). Cytochrome P450 enzymes play the major role in phase I metabolism by catalyzing the metabolism of more than half of all drugs [6]. Phase II enzymes are conjugation (synthetic) reactions, linking the functional groups created by phase I to water soluble groups provided by the cells intermediary metabolism such as UDP-glucoronosyltranaferases (UGT), N-acetyltransferases (NAT), methyltransferases (MT), sulfotransferasea (SULT) and glutathione-s-transferases (GST) as shown in Figure 4.

Figure 4 Phase I and II metabolizing enzymes and their metabolic contribution [6, 13 and 14].



The cytochrome P450 enzyme family [45, 48-52]

The cytochrome P450 enzyme is abbreviated as “CYP” and plays a major role in phase I metabolism. Their name originated from early observations describing them as a pigment with a maximum spectral absorbance at 450 nanometers in the presence of a reducing agent and carbon monoxide. The term cytochrome P450 today refers to a superfamily of heme protein present in nearly all organisms including yeast, bacteria, plants and higher animals. These proteins in eukaryotes in these proteins are embedded in the membrane of endoplasmatic reticulum by an amino-terminal membrane anchor by a heme iron which is coordinated as a conserved cysteine. Substrates of this enzyme system include endogenously synthesized compounds and

exogenous compounds such as drugs. The general stochiometric reaction catalyzed by cytochrome P450 is as follows [6]:

$$NADPH + H^+ + O_2 + SH \longrightarrow NADP^+ + H_2O + SOH$$

The reaction is referred to a monooxygenation because only one of the two oxygen atoms is incorporated into the substrate (S). The reduction equivalents provided by NADPH are transferred to the CYP enzyme by the membrane bound lipoprotein enzyme, NADPH-cytochrome P450 reductase.

The completion of the sequence of the human genome revealed the presence of 115 human CYP genes, the cytochrome P450 super family gene comprises of 57 functional genes and 58 pseudogenes which are summarized at the website http://www.drnelson.utmem.edu/cytochrome P450 html and http://cpd.ibmh.msk.su/. All members of cytochrome P450 enzymes are classified in families and subfamilies on the basis of the percentage of amino acid sequence similarity [6] as shown Figure 5. The CYP allele nomenclature can be retrieved athttp://www.imm.ki.se/CYPalleles/. Gene families that display more than 40% amino acid identity are designated by Arabic numerals (1, 2, 3, etc), examples are the numerous members of the families CYP1, CYP2 and CYP3 and so on. They are the most important phase I metabolizing enzymes and mediate from 70 to 80% of all phase I dependent metabolism of clinically used drugs. Subfamilies have 40 to 70% amino acid sequence similarity which some study found that more than 55% are identical and grouped in the same subfamily designated by capital letters (A, B, etc) such as the CYP2C, CYP2D, CYP2E as well as CYP3A subfamily. Individual genes has sequence of more than 70% identical are designated by Arabic numerals (1, 2, 3 etc) such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Finally, Specific alleles are named by Arabic numerals or followed by a capitalized Latin letter. There are no spaces between gene, asterisk and allele and the entire gene allele symbol is italicized.

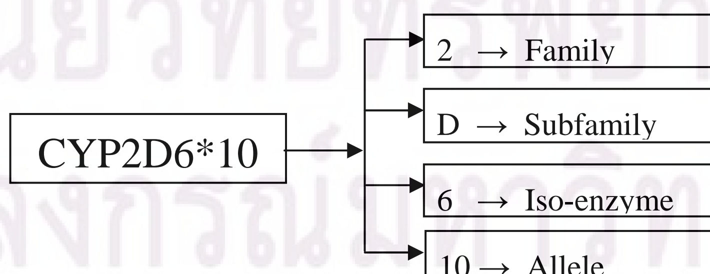


Figure 5 An example of the nomenclature of the cytochrome P450 enzymes [41].

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4. Genetic polymorphism of cytochrome P450 2D6 (CYP2D6) [52]

Genetic polymorphism or pharmacogenetic polymorphism is defined as the inheritance of a trait controlled by monogenic trait that exists in the population in at least two phenotypes. A characteristic feature of pharmacogenetic polymorphisms is that the response to drugs can have inter-individual variability as well as inter-ethnic differences. Genetic polymorphism of metabolizing enzymes influences individual drug efficacy and safety through the alteration of pharmacokinetics and disposition of drugs as shown in Figure 6.

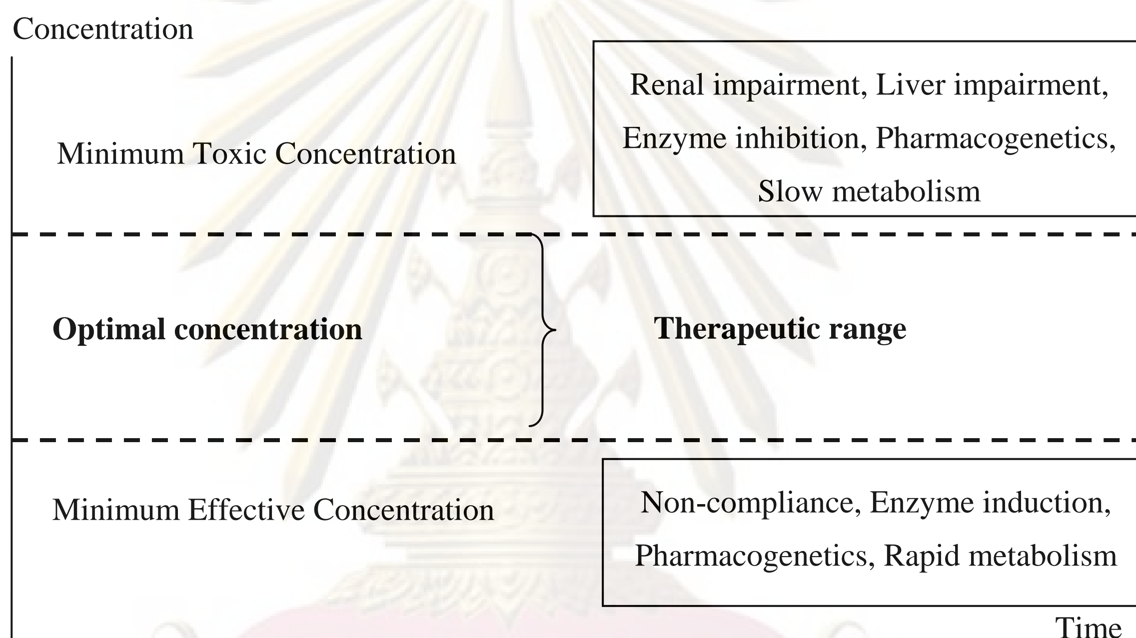

Figure 6 Classification of responses from therapeutic index and factors affecting drug concentrations [42].

Among cytochrome P450s, the largest metabolic contributions are made by CYP3A4/5 (36%), CYP2D6 (25%) is the second largest and the rest are CYP2C8/9 (16%) and CYP2C19 (8%), CYP1A1/2 (11%), CYP2E1 (4%), CYP2B6 (3%), and CYP2A6 (3%), respectively. CYP2D6 is one of the most intensively studied enzymes in pharmacogenetics since it is highly polymorphic. A recent analysis reviewed that there are over 300 drugs from diverse therapeutic classes such as analgesic, antihypertensive, antitussive, antiarrhythmic, anticancer agents, anti-infectious agents, beta-blocking agents, neuroleptic, calcium antagonists, recreational drug amphetamines and important psychotropic drugs such as antidepressant. All mentioned drug classes depend on CYPs for their metabolic clearance. An example of CYP2D6 substrates are shown in Table 2.

Table 2 Some drugs metabolized by CYP2D6 enzyme [6, 8, 13-15 and 43]

Antidepressants			
Amiflavine	Desmethylcitalopram	Paroxetine	Amitriptyline
Desipramine	Minaprine Tomoxetine	Brofaromine	Fluvoxamine
Moclobemide	Citalopram Fluoxetine	Clomipramine	Imipramine
Nortriptyline	Venlafaxine		
Antiarrhythmics			
Amiodarone	Flecainide	Procainamide	Propafenone
Mexiletine	N-propylajmaline	Sparteine	Encainide
Antihypertension			
Alprenolol	Carteolol	Indoramine	Nitrendipine
Bufuralol	Clonidine	Losartan	Oxyprenolol
Bunitrolol	Debrisoquine	Metoprolol	Propranolol
Bupranolol	Guanoxan	Nimodipine	Timolol
Antihistamine			
Azelastine	Cinnarizine	Loratadine	Promethazine
Chemotherapeutic agents			
Clotrimazole	Mefloquine	Rifampicin	Roxithromycin
Doxorubicin	Pyrimethamine	Ritonavir	Sulfasalazine
Neuroleptics			
Clozapine	Olanzapine	Risperidone	Thioridazine
Haloperidol	Perphenazine	Sertindole	Zuclopenthixol
Levomepromazine	Pimozide		
Opiates			
Codeine	Dextromethrophan	Hydrocodone Oxycodone	
Dihydrocodeine	EthylmorphineNorcodeine	Tramadol	
Miscellaneous			
Apigenine	Ethinyloestradiol	Methoxyphenamine	Budesonide
Fenoterol	Methoxypsoralen	Quercitin	Chloral hydrate
Formoterol	Metoclopramide	Serotonin	Cyclobenzaprine
4-hydroxyamphetamine	MPTP	Tacrine	Dexfenfluramine
Laudampsome	Nicergoline	Tamoxifen	Dibucaine
MDMA(ecstacy)	Ondansetron	Tolterodine	Dihydroergotamine
Methoxamine HCL	Perhexiline	Tropisetron	Dolansetron
MethoxyamphetaminePhenformin			

Substances (medicines or other compounds) that are metabolized by CYP2D6 are called substrates. Some substances that inhibit the activity of CYP2D6 enzyme are called inhibitor. For instance, amiodarone, cimetidine, fluoxetine, quinidine and ritonavir may increase the effects of substances. On the other hand, other substances called inducer that can enhance the metabolic rate of CYP2D6 may lead to subtherapeutic level of other substrates. Cytochrome P450 substrates, inducers and inhibitors are summarized at the website http://medicine.inpui.ed/flockhart.

Hepatic CYP450 expression and the metabolic activity are different among subtypes as summarized in Table 3. CYP2D6 expresses only 1 to 2% in amount but it contributes as much as 30% in metabolism. CYP2D6 genetic polymorphism is one of the most intensively studied with regards to autosomal recessive monogenetic defects in drug metabolism. One of the main biotransformation processes of psychotropic drugs is oxidation which is catalysed by enzymes of CYP2D6. Its gene is highly polymorphic with more than 70 allelic variants described so far in http://www.imm.ki.se/CYPalleles/ leading to a wide range in enzymatic activity.

Table 3 Difference between cytochrome P450 distribution and metabolic activity in the liver [41]

CYP P450	Distribution (%)	Metabolism (%)
3A	30	55
2C	20	10
1A2	13	2
2E1	7	1.5
2A6	4	1.5
2D6	2	30

Evolution of CYP2D6 polymorphism was found between 1975 and 1977 when two groups independently discovered the genetic deficiency of debrisoquine and sparteine metabolism. The discovery of genetic polymorphism in the metabolism of the two prototype drugs was an incidental observation at the same time.

Molecular genetics of cytochrome P450 CYP2D6 [44]

After cloning of the CYP2D6 gene, large numbers of the allele have been found so far and CYP2D6 gene structure and its sequence were reported since 1989. It

was found that the locus of *CYP2D6 gene* is on chromosome 22 lining in tandem with other highly homologous genes, *CYP2D8P* and *CYP2D7*, which are classified as pseudogenes. *CYP2D8P* and *CYP2D7* share 92% and 98% homology, repectively to *CYP2D6*. The active gene, *CYP2D6*, consists of nine exons and eight introns. The CYP2*D8P* is a true pseudogene contains several gene disrupting insertions, deletions, no open reading frame, and termination condons within its exons. The *CYP2D7* coding sequence contains only a single inactivating mutation, an insertion of T138 in the first exon, causing a shift of the reading frame and premature translation termination. Based on XbaI restriction fragment length polymorphisms, the *CYP2D* gene cluster is defined by different haplotypes which differ in the number of peudogenes and functional *CYP2D6*. The *CYP2D* gene cluster has localization on the long arm of chromosome 22 (q13.1) and the position of the pseudogenes and functional gene in the *CYP2D* gene cluster are shown in Figures 7-8 with exons (numbered boxes) and introns (thick lines).

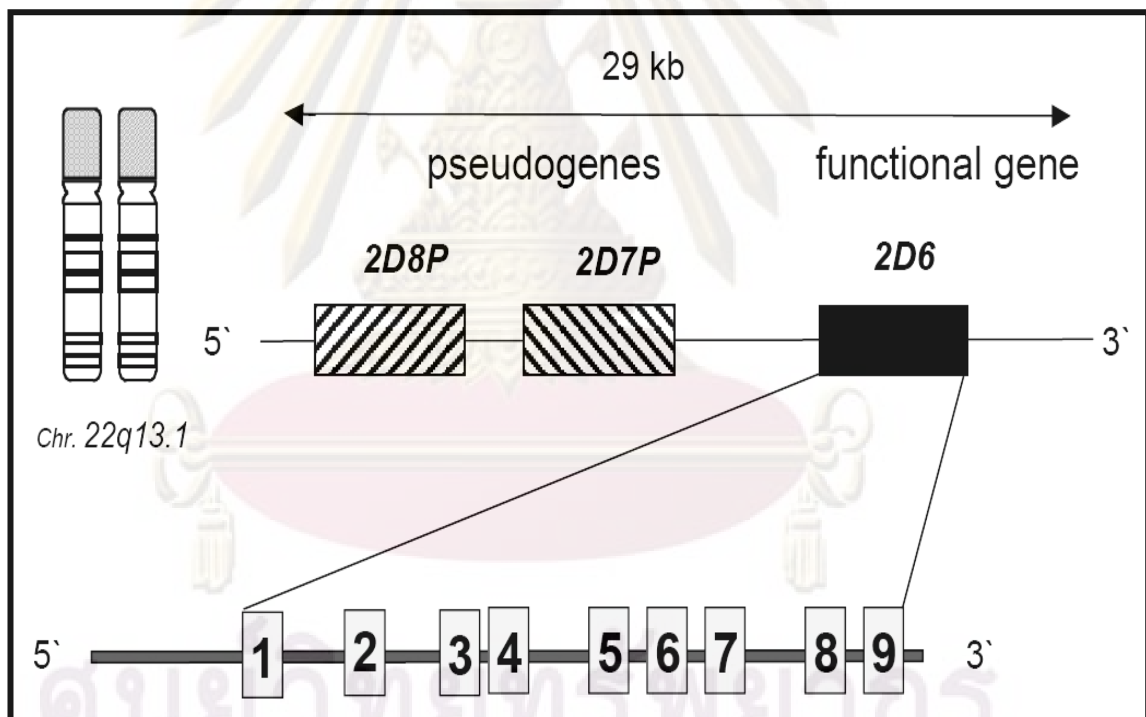

Figure 7 The structure of *CYP2D6* gene and its pseudogenes on chromosome 22 [45]

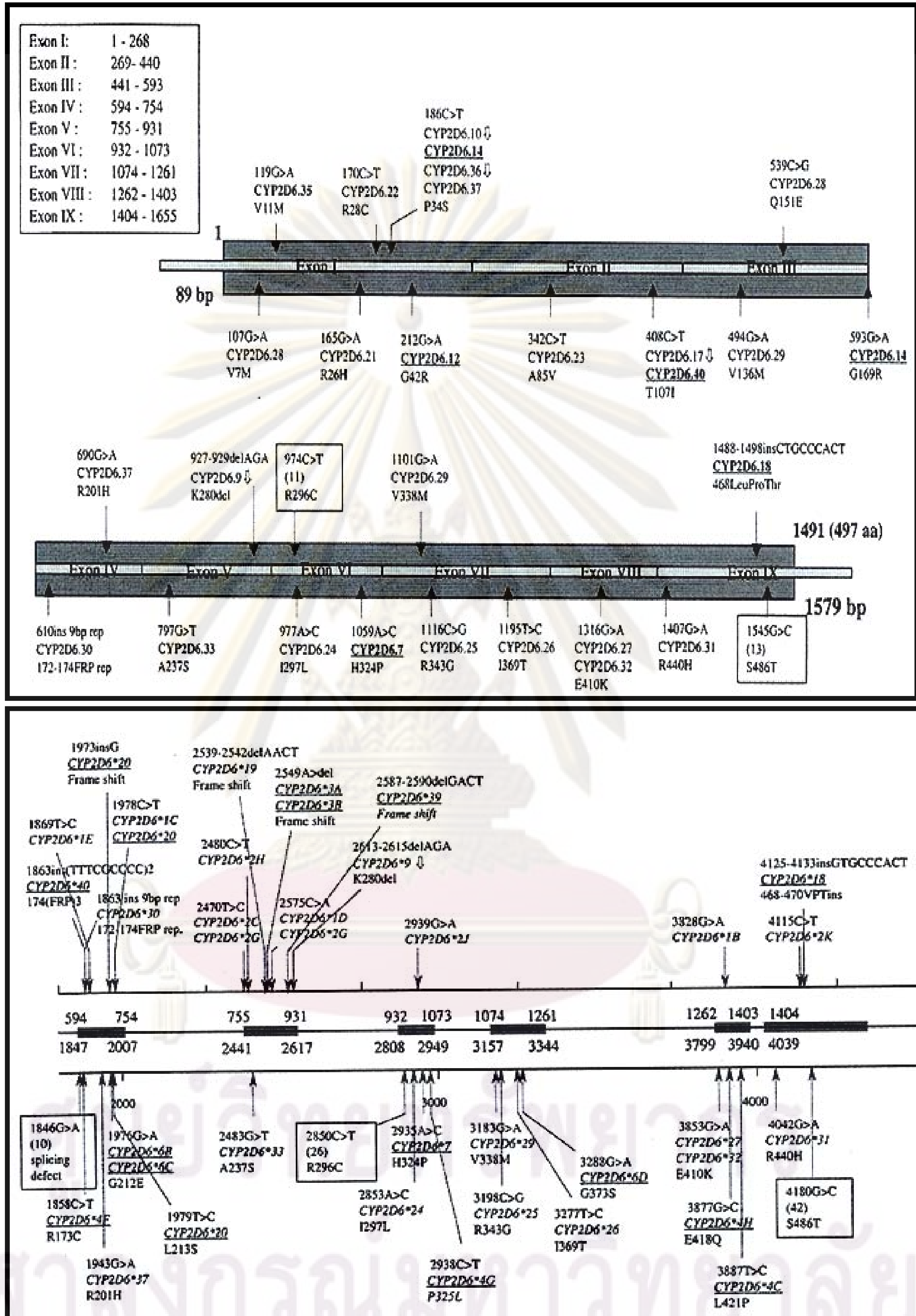

Figure 8 Some single nucleotide polymorphisms (SNPs) in *CYP2D6* gene [46].

At present, more than 60 *CYP2D6* alleles are described that encode an enzyme with inactive, decreased, increased, or normal catalytic function. The number of functional *CYP2D6* genes correlates with drug and metabolite concentrations in the plasma. In addition, genetic polymorphism of *CYP2D6* relates with adverse drug reaction from psychotropic drugs. Genotyping results must be confirmed by phenotyping that examines the rate of transformation of a drug into its metabolites and shows whether the increase or decrease in this rate is linked to a specific polymorphism. The metabolic ratio (MR) is defined as the ratio of the amounts of unchanged drug and drug metabolite.

$$MR = \frac{\text{Amount of unchanged drug}}{\text{Drug metabolite}}$$

In general, phenotype correlates with drug response which genetic polymorphism enables division of phenotype individuals within a given population into four groups, namely ultrarapid metabolizers, extensive metabolizers, intermediate metabolizers and poor metabolizers.

Alleles with increased enzyme activity or ultrarapid metabolizers (UM)

Ultrarapid metabolizers is the result of increased drug metabolism and is an autosomal dominant trait arising from gene amplification. UM genotype is found to have multiple copies of apparently functional CYP2D6*1, *2, *35 and *41 allele ×N (N = copy number of allele) as a result of unequal crossover events and other mechanisms. Their ultra metabolisms are commonly found in Ethiopian and Saudi Arabian populations. Thus, they need higher doses of psychotropic drugs to reach therapeutic blood drug level.

Alleles with normal enzyme activity or extensive metabolizers (EM)

Extensive metabolizer is a normal metabolizers found in general populations, for example the wild type allele *1 has per definition a normal enzyme activity. The *CYP2D6**2 allele carries an extended intronic part of the *CYP2D7* gene having normal metabolic activity which originates presumably from a gene conversion and two mutations, C2850T and G4180C, leading to amino acid changes (R296C and S486T). Nevertheless, *CYP2D6**1 and *CYP2D6**2 alleles have very similar activities in vivo.

Alleles with decreased enzyme activity or Intermediate metabolizers (IM)

Intermediate metabolizer is a drug metabolism which stands between EM and PM The most prevalent IM allele is of different race. Several common IM allele examples are *CYP2D6**9, *10, *17, *29 and *41, which leads to amino acid changes and metabolically deficient CYP2D6 protein. Approximately 10-15% of Caucasians, 20-30% of Africans and up to 50% Asian populations were IM of *CYP2D6* [22]. For instance *CYP2D6**10 allele, a single nucleotide alteration in exon 1 (C100T) causes a Pro34→Ser amino acid substitution, which leads to an unstable enzyme with lower metabolic activity. The *CYP2D6**10 allele seems to be more clinically important than the other *CYP2D6* mutant and has been shown to be related to the pharmacodynamics of CYP2D6 substrates [22, 47].

Allele with non functional enzyme activity or poor metabolizer, null alleles (PM)

Poor metabolizer does not encode a functional protein product. Poor metabolizer is a phenotype with no CYP2D6 being expressed. It is caused by small gene insertions/deletion or single nucleotide polymorphisms in the gene that interrupt the reading frame or interfere with correct splicing leading ultimately to premature termination. Approximately 6-10% of Caucasian and 0-2% of Asian populations are known to have PM phenotype. For example, *CYP2D6**4 is the most frequent null allele in Caucasians which occurs with an allele frequency of about 20-25% and responsible for 70 to 90 % of all PMs. It was found that the G1846A is a key mutation that causes a shift of the consensus acceptor splice site of the third intron by one base, thereby resulting in a spliced mRNA with one additional base that has an altered reading frame and premature stop codon. Drugs used in standard doses in this group may reach a marked higher level in the blood, even at toxic levels when compared with those in EM group.

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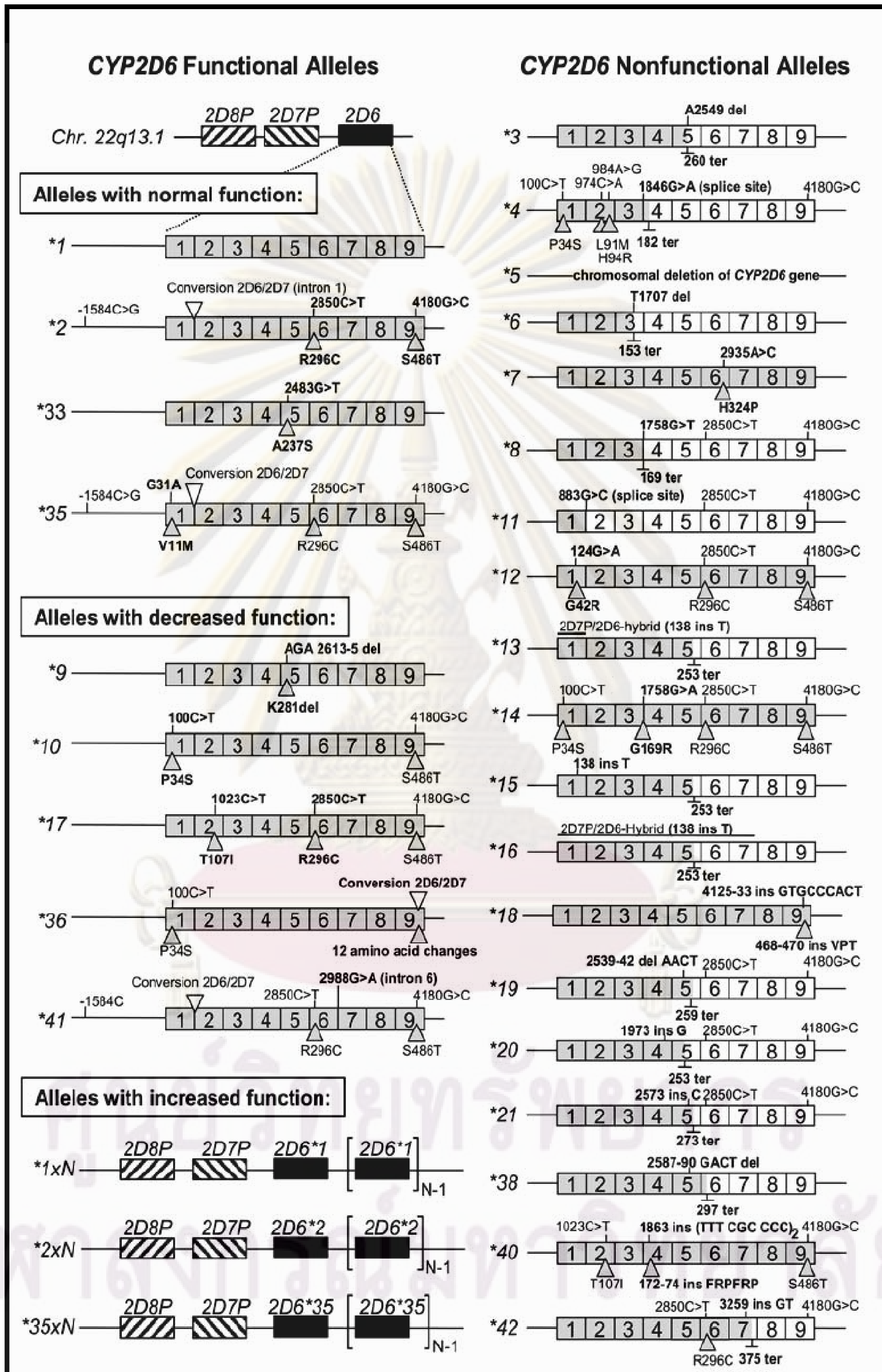

Figure 9 Division of phenotype [46]

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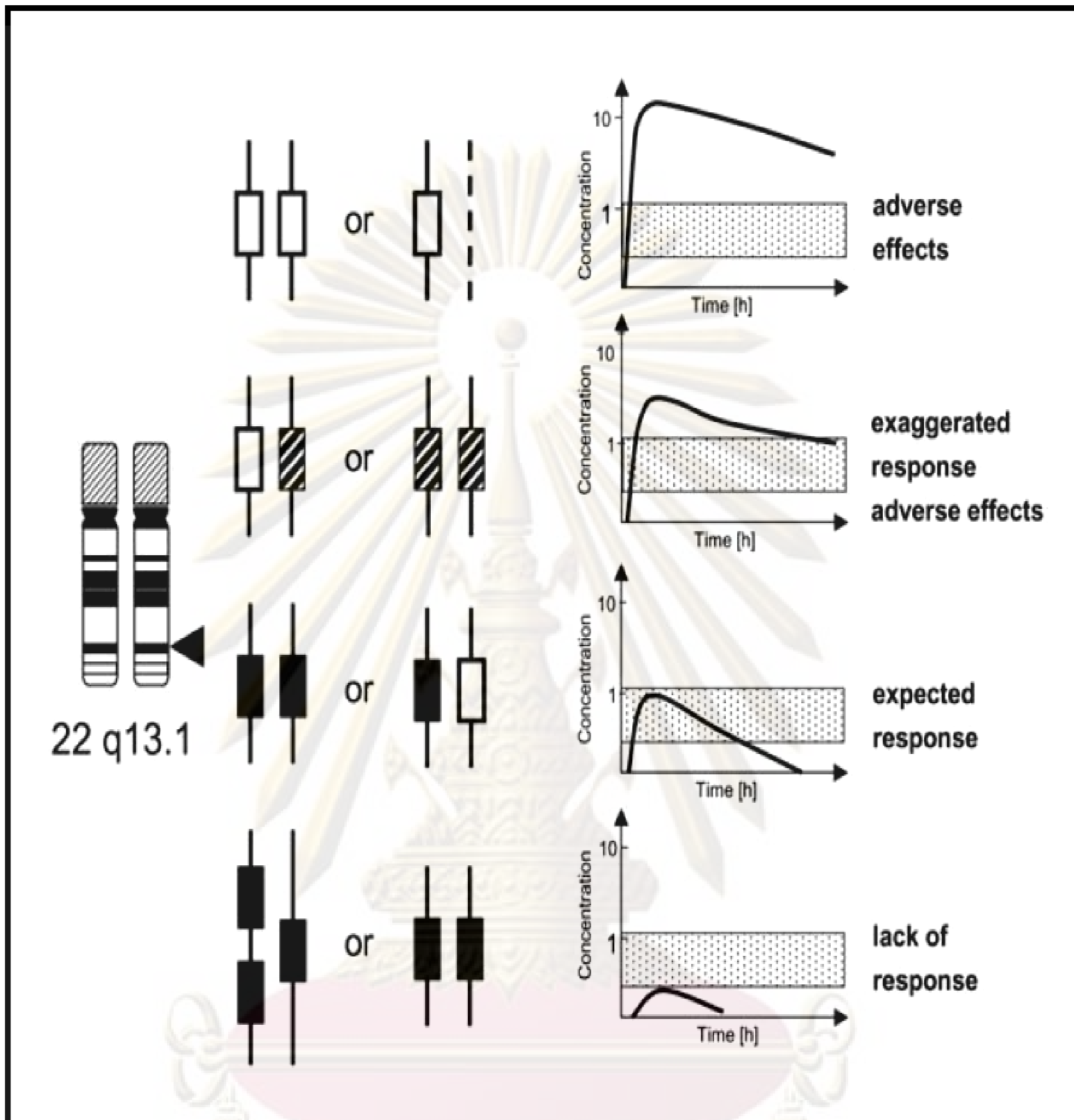

Figure 10 Scheme of *CYP2D6* genotype-phenotype relationships and their pharmacokinetic and clinical consequences. Open boxes; null allele, Black boxes; fully functional allele, Hatch boxes; decreased function allele [46]

A number of studies have explored the relationship between adverse effects of psychotropic drug and *CYP2D6* genotype. For example, a doubling of the frequency of defective *CYP2D6* alleles in depressed patients with adverse reactions compared with those without such reactions. The prevalence of different genotypes among ethnic groups is shown in Table 4. Frequencies of ultrarapid metabolizers and poor metabolizers in genotyped population as shown in Table 5.

Table 4 CYP2D6 allele frequencies in different ethnic groups [49]

CYP2D6 Alleles	Enzyme Activity	Allele frequencies (%)				
		Caucasian (n=589)	African (n=154)	Japanese (n=206)	Chinese (n=223)	Korean (n=400)
*1	Normal	36.40	34.70	43.00	37.90	33.25
*2	Normal	32.40	26.90	12.30		10.13
*3	None	2.04	0.30		0.00	0.00
*4	None	20.70	7.80	0.20	0.20	0.25
*5	None	1.95	6.20	4.50	7.20	6.13
*6	None	0.93			0.00	0.00
*7	None	0.08				0.00
*8	None	0.00			0.00	0.00
*9	Decreased	1.78				0.00
*10	Decreased	1.53	7.50	38.10	51.30	45.00
*11	None	0.00				0.00
*12	None	0.00				0.00
*13	None	0.00				0.00
*14	None	0.00		0.70	2.00	0.50
*15	None	0.08				0.00
*16	None	0.08				0.00
*17	Decreased		14.60			0.00
*18	None			0.20		0.00
*21	None					0.25
*27	Unknown					0.38
*35	Normal					0.13
*39	Unknown					0.63
*41	Decreased					1.88
*47	Unknown					0.13
Duplication		1.93	1.90	1.00	1.30	1.13
*1xN	Increased	0.51		0.50		0.13
*2xN	Increased	1.34		0.50		0.50
*4xN	None	0.08				0.00
*10xN	Decreased	0.00				0.50
Undetermined						0.25

Table 5 Frequencies of ultrarapid metabolizers and poor metabolizers in genotyped population [14, 41, 43 and 50]

Ethnic group	Total subjects	UM (%)	PM (%)
White North Americans	464	2.2	5.8
Black North Americans	246	2.4	3.3
Germans	589	2.0	7.0
South Germans	195	1.5	7.7
North Spanish	147	5.1	5.4
South Spanish	217	3.5	2.8
Swedish	270	1.0	8.0
Spanish	258	1.0	5.0
French	265	1.9	8.4
Turks	404	5.8	1.5
Koreans	152	0.3	0
Chinese	113	1.3	0
Saudi Arabians	101	21	2.0
Ethiopians	122	29	1.8
Thai	173	No data	1.2

Abbreviations; UM = Ultrarapid metabolizers (Gene duplication),
PM = Poor metabolizers

5. *CYP2D6* polymorphism detection

1. PCR amplification of the *CYP2D6* gene

To determine the *CYP2D6* genotype, the whole gene must be specifically amplified without the co-amplification of the other two pseudogenes (*CYP2D8P* and *CYP2D7*). PCR-based techniques were used to obtain the desired DNA fragments.

1.1 Principle of Polymerase Chain Reaction

The polymerase chain reaction (PCR) is one of the mainstays in molecular biology. It provides rapid means of DNA identification and

analysis. The PCR is an in vitro DNA amplification method that involves a repeated cycling process with a number of defined stages.

The PCR will amplify a precise fragment of a DNA from a complex mixture of starting material usually termed the DNA template and in many cases requires little purification of the DNA. To multiply a DNA molecule with the PCR major four materials are required: the target DNA molecule or genomic DNA (the one to be amplified); short strands of know “primer” DNA that will tag and identify the segment to be copied and provide a foundation for beginning the replication process; DNA polymerase, an enzyme that directs DNA replication; and a mixture of nucleotides, the nucleic acid (four nucleotide dNTP as A, T, C and G) building blocks from which new DNA will be formed as shown in Figure 11.

1.1.1 Conditions of PCR reaction

The PCR involves with three major steps, all performed over and over again in a highly automated PCR machine.

(a) Denaturation

In the first cycle, the double stranded (ds) template DNA is denatured by heating the reaction to 94°C to 95°C at 30 to 60 seconds to separate the ds DNA and produce two single strands.

(b) Annealing

This step allows the hybridization of the two oligonucleotide primers which are present in excess to bind to their complementary sites that flank the target DNA on the single strands in the previous stage. In anealing the usual temperature is reduced as 50 to 65°C for 30 to 60 seconds. The polymerase extends the primer segment with the available nucleotides and produces two ds DNA molecules.

(c) Extension

In the final stage of the cycle; the process is repeated, and at the end of cycle, four ds DNA molecules are present. By repeating the process in cycle yields a total of eight ds DNA molecules. The strands increase in number geometrically in succeeding cycles. The DNA synthesis step is termed

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extension and is carried out to produce complementary copies of the initial single strands from primer bound to the DNA by a thermostable DNA polymerase, most commonly known as Tag DNA polymerase. This step usually takes place at 70°C to 75°C for 30 to 120 seconds; depend on length of DNA molecules.

DNA synthesis proceeds from both of the primers until the new strands have been extended along and beyond the target DNA to be amplified. Each of these steps is repeated 30-40 times, termed cycles. The temperature is then cooled to between 40°C and 60°C.

As the system is taken through successive cycles of the three major steps; denaturation, annealing and extension, all the new strands will act as templates so there will be an exponential increase in the amount of DNA produced as shown in Figure 11.

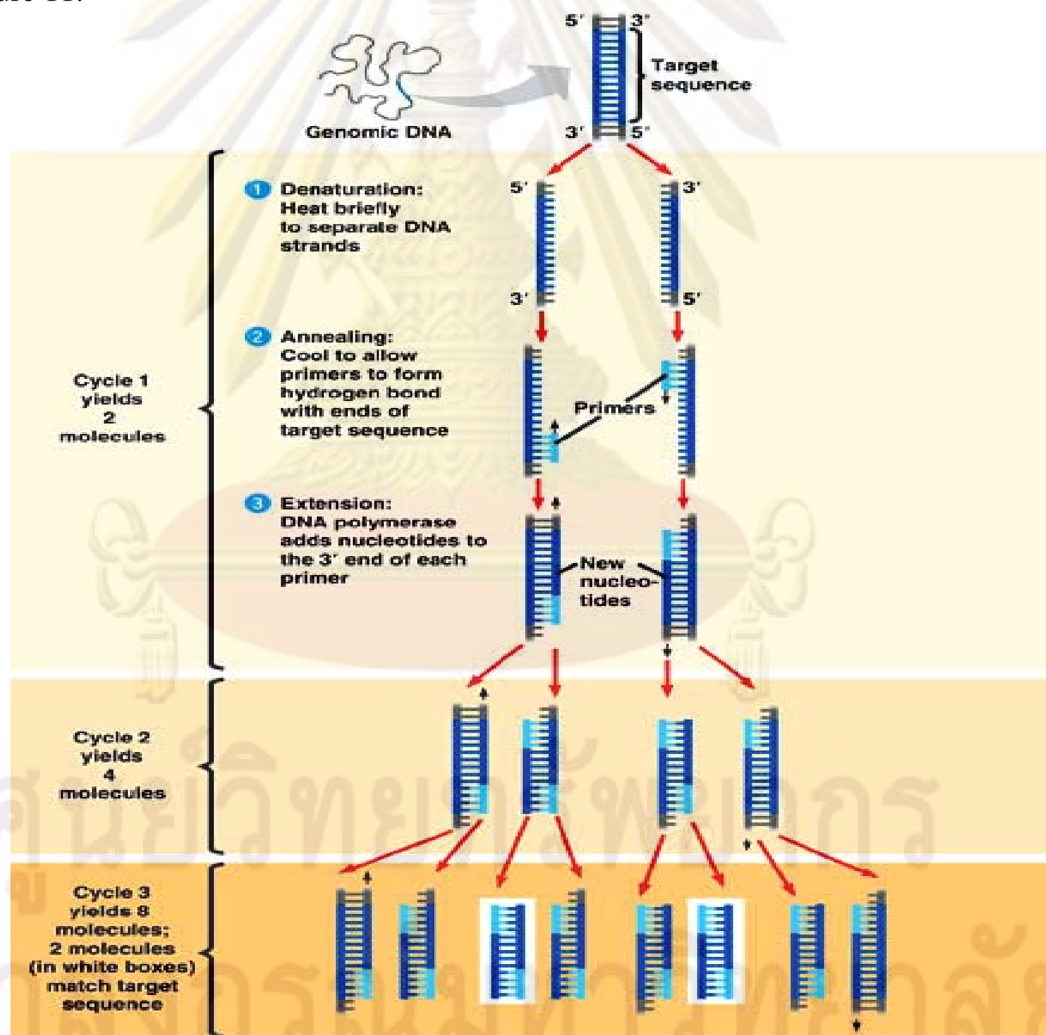

Figure 11 Principle of Polymerase Chain Reaction, all the new strands will be an exponential increase in the amount of DNA produced.

1.2 Principle of Gel electrophoresis of DNA techniques

The term electrophoresis describes the migration of a charged particle under the influence of an electric field (cations (+) and anions (−)).

Under the influence of an electric field these charged particles will migrate towards the anions to the cations of the DNA samples. Separating DNA molecules of different lengths is the standard method with gel electrophoresis.

1.2.1 Agarose gel for electrophoresis of DNA

An agarose is a linear polysaccharide, comprised of alternating units of 3,6anhydrogalactose and galactose. It is one of the components of agar a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentration of between 1% and 3%.

Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms and allowed to cool to room temperature to form a rigid gel. The gelling properties are attributed to both inter and intra molecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvertional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentration and smaller pore size are formed from the higher concentrations as shown in Table 6 and 7.

Table 6 Range of separation in gel containing different concentration (by Lifetech)

Amount of agarose in gel (%w/v)	Efficient rang of separation of linear DNA molecule (kilo base pair; kb)
0.3	5.0 – 60
0.6	1.0 – 20
0.7	0.8 – 10
0.9	0.5 – 7
1.2	0.4 – 6
1.5	0.2 – 3
2	0.1 – 2

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Table 7 DNA size migration with sample loading dyes (by Lifetech)

Agarose concentration (%)	Xylene cyanol	Bromphenol blue
0.5 - 1.5	4 - 5 kb	400 - 500 bp
2.0 - 3.0	750 bp	100 bp
4.0 - 5.0	125 bp	25 bp
Abbreviations; kb=kilobase pair; bp=base pair		

2. Single Base Extension (SBE)

2.2 Principle of Single Base Extension (SBE)

SBE methods identify a single allelic nucleotide immediately adjacent to a define primer terminus. SBE has proved to be particularly attractive for its simplicity (the minimal implementation contains only three major added components: primer, polymerase and nucleoside triphosphate substrate).

In the SBE primer extension reaction, a DNA polymerase is used specifically to extend a primer that anneals immediately to the adjacent nucleotide position to be analyzed with a single labeled nucleoside triphospate complementary to the nucleotide at the variant site. The reaction allows highly specific detection of point mutations and single nucleotide polymorphisms (SNPs). Because all SNPs can be analyzed with high specificity at the same reaction conditions, SBE is a promising reaction principle for multiplexing high-throughput genotyping arrays. It is also a useful tool for accurate quantitative PCR-based analysis.

3. Denaturing High Performance Liquid Chromatography (DHPLC)

3.1 Chemistry and chromatography of DHPLC

3.1.1 Stationary phase (non-polar)

The stationary phase (column matrix in DHPLC) is non-polar and hydrophobic. The nature of the DNA is a negatively charged phosphate group which cannot adsorb to the column matrix by itself.

3.2.2 Mobile phase (polar)

a) Ion-pairing agent (buffer)

The ion-pairing agent used in column is triethylammonium acetate (TEAA) buffer pH 7.0 which is a mediator for the binding of the DNA to the stationary phase. TEAA is a positively charged ammonium group (cation) which can be to fit to bind to the negatively charged anionic phosphase group of DNA strands. Therefore it builds a bridge between the column material and the nucleic acid as shown Figure 12.

b) Eluent (organic solvent)

The separation of the nucleic acid bond to the column is achieved at an optimized temperature by increasing the concentration of an organic solvent. Acetonitrile (ACN) is an organic solvent used in the elution buffer (forming the elution gradient) which competes with hydrophobic interactions between the column and TEAA, resulting in the elution of the nucleic acid. Therefore the fragment sizes increases as more ACN is required for elution. Retention time is also related to the size.

c) Chromatography in DHPLC

The PCR products are amplified by SBE reaction. When finished, are injected into the column which can bind to the matrix and interactions are mediated by TEAA. The elution of a particular concentration of ACN was detected with UV absorbance measured at 260 nm, depending on the size and sequence of the fragment. The peak maximum absorbance is the retention time of the DNA sample at a given CAN concentration.

3.2 Principle of DNA separation by DHPLC

The partially denaturing DHPLC used for screening dsDNA fragments for the presence of mutation (SNPs). The separation DNA is based on the principle of temperature-mediated heteroduplex analysis (TMHA), that heteroduplexed DNA can be distinguished from homoduplexed DNA by different elution profile as shown Figure 13. The optimized temperature to

begin denaturization of the dsDNA molecules is between 52 - 75°C. The number of double strand DNA PCR fragment reduces denaturation and single-stranded fragments elute earlier than dsDNA fragments due to reduced negative charge.

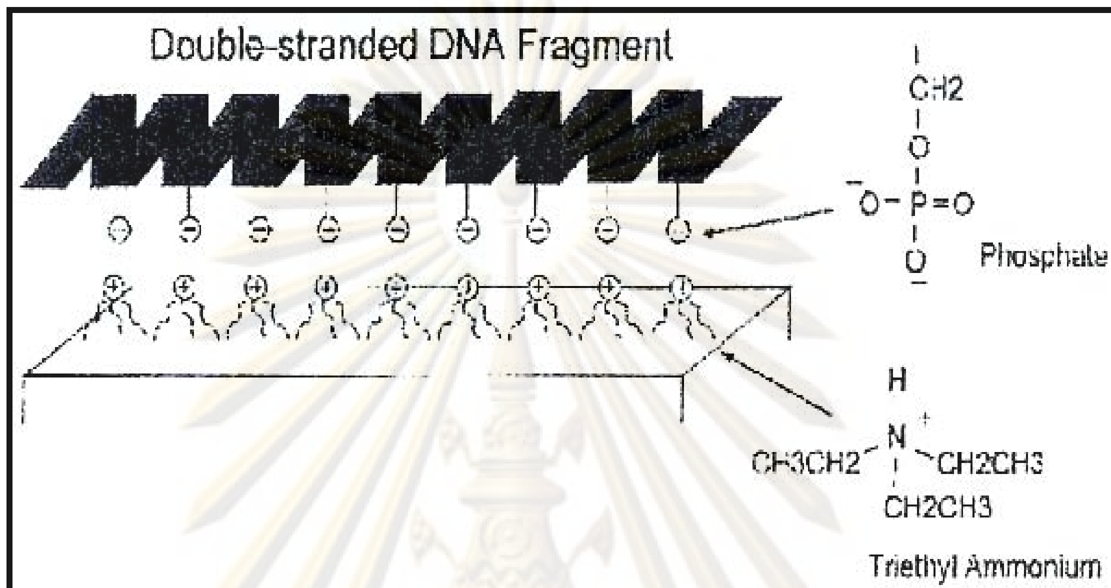

Figure 12 Binding of DNA to the stationary phase (column matrix) triethylammonium acetate (TEAA) in mobile phase.

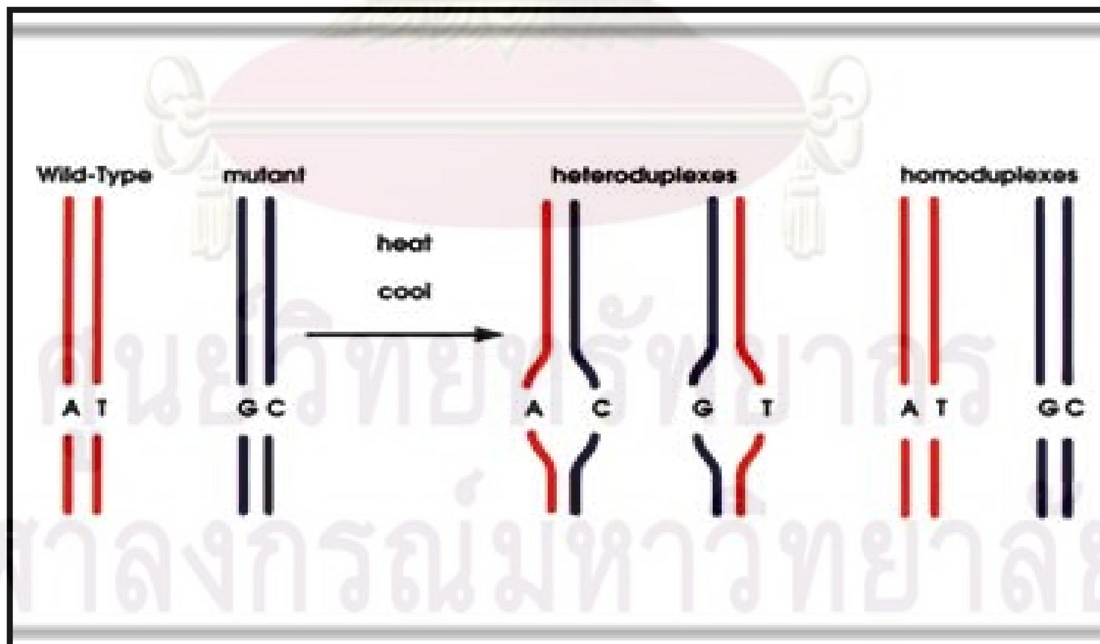

Figure 13 Principle of the partially denaturing DHPLC use for mutation detection

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3.3 Quantitative analysis of CYP2D6

CYP2D6 gene has high polymorphism effect on the difference of interindividual metabolic rate. DHPLC can be used to detect and identify the peak attributed to gene deletion and gene copy numbers, under different temperatures and condition.

4. Restriction fragment length polymorphisms (RFLP)

This step is to confirm any uncertainty in both PCR products and DHPLC profiles. Restriction endonucleases are bacterial enzyme, RFLP describes the patterns of different (polymorphisms) sizes of DNA (fragment length) that result from cutting specific sequence in the double stranded DNA with design primers as show in Figure 14. Restriction enzymes are usually named by a three letter abbreviation that identifies their bacterial original such as BstEII (Geobacillus stearothermophilus.).

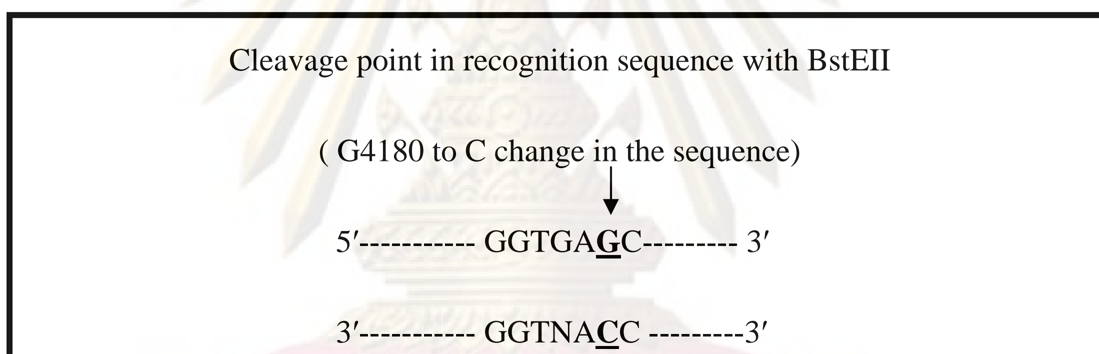

Figure 14 Detection of SNP position of *CYP2D6* polymorphism by RFLP

5. Clinical relevance of the CYP2D6 polymorphism and ADRs

Genetic polymorphisms of drug metabolizing enzyme are greatly contributed to interindividual difference in drug response of particular metabolizer groups. Approximately 25% of all clinically used drugs are metabolized by CYP2D6 especially psychiatric drug such as antidepressants. The clinical relevance of CYP2D6 polymorphisms to drug response based upon many factors such as the therapeutic index (narrow or broad) of parent compound and metabolites, drug dosage, duration of use and the contribution of other pathways of elimination. Several studied has been investigated the relationship between blood concentration with genotype. The studies determined phenotype in quantitative aspect mostly showed the genotype-phenotype correlation. The conclusions are made in agreement that the increased adverse effects

are consistently presented at high blood concentration from poor metabolizer [51, 52, 53, 54, 55, 56 and 57]. There was a case reported that the decreased activity of CYP2D6 associated with adverse effects and high blood concentration of nortriptylline while other studies reported differently [58, 59, 60, 61, 62, 63, 64 and 81]. Moreover, two case reports failed to predict adverse events in depressed patients treated with two newer antidepressants (paroxetine and mirtazepine) [1]. This may be the confounding effect from paroxetine itself that can inhibit CYP2D6 activity thus; there was no distinct association between blood concentrations and adverse events or antidepressant efficacy [47]. Antidepressant drug is classified to have moderate therapeutic index (TI) that its overdose or higher TI level will normally cause toxic or ADRs. Many studies conclude that *CYP2D6* PM phenotype have significant relationship with efficacy or adverse events.

There are some studies conducting the correlation of *CYP2D6* genotype and neuroleptic-induced movement disorder and the results are still inconsistent as shown in Table 8.

Table 8 Association of *CYP2D6* genotype and neuroleptic-induced movement disorder

Side effect	Relationship with *CYP2D6* genotype	Reference
Tardive dyskinesia	No No Yes Yes Yes	[66] [67] [68] [69] [70]
Extrapyramidal side effects	Yes No	[71] [72]
Acute dystonia Chronic movement disorder	No No (non-signtendency)	[73] [73]
Tardive dyskinesia Parkinsonism Akathisia	No (non-signtendency) No No	[74] [74] [74]

When consider adverse drug reaction in each phenotype, poor metabolizers were shown to have more serious side effects than those who are extensive metabolizer as shown in Table 9. Summary is given in table below. However, there are so few reports showed the correlation of *CYP2D6* genotype and phenotype in Asian population especially with ADR association. Since *CYP2D6* genotypes and frequencies between Caucasians and Asians are different somehow, genetic data and association results could not be applied interchangeably between two ethnic groups. This study will provide the *CYP2D6* genotypes in Thais and prove whether it has any association with amitriptyline ADRs in Thai patients. Moreover, it will give more evidence for association study conducting in Asian.

Table 9 Association of *CYP2D6* genotype with adverse reactions

Relationship with phenotype	Adverse reactions	Reference
PM	Tremor, weight loss and increased pulse rate	[75]
PM	Parkinsonian like side effects	[76]

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CHAPTER III

MATERIALS AND METHODS

1. Subjects

The study was conducted from June 2007 to October 2007 at Siriraj Hospital, Bangkok, Thailand. The subjects of this study were depressive disorder patients from out-patients service of the Department of Psychiatry, Siriraj Hospital. All subjects were diagnosed with depressive disorders according to ICD10 criteria (International Classification of Disease; F32-F34) and were given 10-125 mg/day of amitriptyline as a treatment.

The research protocol was approved by the ethics committee of Siriraj Hospital, Mahidol University and informed consent was obtained from all patients. Approximately 10 ml of blood were collected from all patients and stored in EDTA tube for DNA preparation. One hundred and fifty depressive patients were classified into 2 groups as case and control group by Naranjo's Algorithm. Each group consists of 75 patients, those having adverse drug reactions (ADRs) from amitriptyline were in case group and non ADRs were in control group. Both groups were evaluated ADR severity using visual analog scales of ADR questionnaire. There are 20 ADR symptoms listed from high incidence reported in the literature. Each ADR symptoms has 10 scales (1-10, each scale counting for 10 scores) allowing subjects to point which scale they were having that symptom. The example of questionnaire containing questions and scores are given in appendices.

Inclusion criteria

The patients who had all of those characteristics were enrolled in this study.

1. The patients must have age not less than 18 years with the diagnosis of depressive disorder.
2. A diagnosis was made according to diagnostic of DSM4 and ICD10 criteria (F32-34).
3. The patients were given amitriptyline for depressive treatment.
4. The patients were informed the study protocol and willing to enroll in this study.

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Exclusion criteria

The patients who had either one of these characteristics were excluded from this study.

1. The patients had addicted to drug or having drug abuse.
2. The patients had clinically relevant laboratory abnormalities eg. hepatitis (elevated of enzyme in liver such as AST and AGT).
3. The patients had severe illness not allow using tricyclic antidepressant (eg. severe epilepsy, glaucoma, or cardiovascular disease).
4. The patients during pregnancy or lactation.
5. The patients had taken drugs (imipramine, paroxetine, fluoxetine, haloperidol, perphenazine) or herbal (St. John's wort, and others) which could interfere CYP2D6 metabolizing function.
6. The patients who taking medicine that could give ADR symptoms as those from amitriptyline (such as anticholinergic symptoms; dry mouth, constipation, confused, blurred vision and others) were excluded.
7. The patients had known allergy or history of allergy to amitriptyline.
8. The patients who have severe mental abnormality other than depressive disorder (eg. dementia and schizophrenia) that will not be able to understand the detail of this study.

2. Materials

Apparatus and Instruments

1. Automatic pipette and tips
2. Bio imaging systems (Sungene®, Gengenius and tools match)
3. Centrifuge (Hermle ZK 380, Berthold Hermle AG, Germany)
4. Denaturing high performance liquid chromatography (DHPLC)
5. Fume hood
6. Horizontal electrophoresis set (I-MyRun.N®)

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7. Power supply (E-C®, EC250-90)
8. Needle no 21
9. Rubber bulb
10. Sterile pasteur pipette
11. Sterile glass ware (e.g. beaker, cylinder)
12. Shaking water bath
13. Sterile microcentrifuge tube
14. Sterile 15 ml screw-cap plastic tubes
15. Spectrophotometer, Nanodrop® ND-1000
16. Test-tube rack
17. 10, 20 ml syringe
18. Vortex mixture

Chemicals and Reagents

1. Absolute ethanol (chilled at -20°C)
2. 1% and 2% of LE agarose gel in TBE buffer
3. 24:1(v/v) Choloform-isoamyl alcohol (the latte is as a defoaming agent)
4. 0.5 M EDTA, pH 8.0
5. 70% Ethanol
6. 4 M NaCl solution
7. 2 mg/ml Proteinase K in TE 20-5 (prepared before use)
8. Phenol (equilibrated several times with 0.1 M Tris-HCL pH 8.0 and added 8-hydroxyquinoline, as an antioxidant to 0.1% w/v)
9. 1×Phosphate-buffered saline (PBS), pH 7.3 (137mM NaCl, 2.7mM KCl, 1.4 mM KH_2PO_4, 4.3 mM $Na_2HPO_4.7H_2O$)
10. 1×RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA)
11. Sterile deionized water
12. 10% SDS solution

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13. TBE(5x : 54 g Tris base, 27.5 ml boric acid, 20 ml 0.5 M EDTA, pH 8.0; used 0.5XTBE)
14. TE 20-5 (20 Mm Tris-HCL pH7.5 and 5Mm EDTA; prepare from 50×TE 20-5)
15. 1×TEA buffer (40 Mm Tris-acetate,1mM EDTA)
16. 1 M Tris-HCl, pH 8.0

3. Methods

3.1 Sample preparations

3.1.1 Blood samples

(a) Processing of blood samples

In routine genetic analysis, DNA is isolated from whole blood. The process of blood samples include blood collecting from individuals and isolation of nucleated blood cells (white blood cells) for DNA preparations. About 10 ml blood were collected from venous vessel and kept in 15 ml screwed-cap tube containing 200 μl of 0.5 M EDTA.

(b) Isolation of total WBC using RBC-lysis buffer (for DNA preparation)

Procedure

1. Centrifuge whole EDTA-blood sample in 15 ml screw-cap plastic tube at 3,000 rmp, 4°C for 5 minutes.
2. Discard plasma by using a pasture pipette.
3. Wash whole cells twice with 1X PBS; in each washing, centrifuge and discard PBS by using a pasture pipette.
4. Add 3-5 volumes of 1X RBC lysis buffer and incubate at room temperature or at 4°C for 10 minutes.
5. Centrifuge and discard RBC lysate.
6. Repeat steps 4-5 once or twice.
7. Wash WBC twice with PBS.

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8. Store WBC in 15 ml screw-cap tube at -20°C or -70°C for DNA preparation.

(c) DNA isolation from WBC

1. Added 4 ml of TE 20-5 (20 mM Tris-HCL pH 7.5and 5 mM EDTA) and resuspended WBC pellet by vigorous shaking until all clumps disappeared then added 200 µl of 20 mM Tris-HCl pH 7.5 and add 10 µl 10% SDS (final concentration is about 5%) and added 20 µl of 2 mg/ml proteinase K (final concentration is about 100 µg/ml). Incubated mixture at 37°C overnight.

2. Added ½ volumes of phenol and ½ volumes ofchloroform-isoamyl alcohol (24:1) to the mixture, and mixed gently but thoroughly (several times). Centrifuged at 2,500 rpm for 4°C, 10 minutes.

3. Inserted the pasteur pipette into the bottom layer and suctioned out organic phase. Repeated step 2 and 3 twice.

4. Added one volume of chloroform-isoamyl alcohol (24:1) then centrifuged, suctioned out the orangnic phase and repeated this step twice.

5. Added ½ volume of 4M NaCl and 2 volumes of chilled absolute alcohol for precipitating DNA out of aqueous phase. Collected DNA by centrifugedat 3,000 rpm for 10 minutes.

6. Decanted solution and washed DNA once with 5 ml of 70% ethanol then centrifuged at 3,000 rpm for 10 minutes, decanted, and left the tubes open at room temperature until dried.

7. Dissolved DNA in 0.5-1.0 ml of sterile distilled water, mix well.

8. Measured optical density (OD) at 260 and 280 nm.

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9. Calculated OD260/OD280 ratio to observe purity (the ratio of pure DNA is 1.8) and estimated concentration of DNA following this formula:

$$\text{DNA concentration in } \mu l/ml \text{ or } ng/\mu l = OD\ 260 \times 50 \times \text{dilution factor}$$

3.2 Detection of whole gene deletion and duplication from CYP2D6 gene

CYP2D6 gene is located in chromosome 22 lining in tandem with other 2 pseudogenes, *CYP2D8* and *CYP2D7*. In order to prevent the co-amplification of pseudogene, whole gene of *CYP2D6* should be specifically amplified first. In this study, multiplex long PCR were used with 2 pairs of primers detecting both *CYP2D6* gene and whole gene deletion (*CYP2D6**5). Then, secondary PCR can be performed to elucidate each SNP type using PCR amplified from the first reaction as a template.

3.2.1 Multiplex long PCR techniques

The whole gene of *CYP2D6* was amplified using Elongase® enzyme with primer A1, primer A2 and deletion gene used primer I3, and primer I4 in following PCR mixture.

Table 10 Mixture of 25 µl PCR reaction detecting *CYP2D6* whole gene and gene deletion

Mixture	Volume (µl)
Buffer A	1.25
Buffer B	3.75
2 mM dNTPs	2.50
10 pmol/µl of each primer A1and A2	1.20
10 pmol/µl of each primer I3 and I4	0.50
1 u/µl Elongase® (Tag polymerase mix)	0.50
100% DMSO	1.25
Sterile distilled water (SDW)	5.85
25 ng/µl genomic DNA	6.00
Total volume	25.00

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The PCR products showing 4.8 kb fragment containing all 9 exons of *CYP2D6* gene. Individual represents 3.2 kb fragment indicated the presence of the *CYP2D6**5 allele. Therefore, the PCR containing only 4.8 kb band indicated no gene deletion while PCR amplified showing both 4.8 and 3.2 kb band can be interpreted as hemizygous *CYP2D6**5. Homozygous deletion can be indicated if the electrophoresis band shows only 3.2 kb. The thermal profile consisted of initial denaturation at 94°C for 2 minutes, denaturation 94°C for 30 seconds, anealing 65°C for 30 seconds, extension 68°C for 5 minutes and final extension 68°C for 7 minutes for 30 cycles. The product was checked by 1% LE agarose gel electrophoresis. Amplicon 1 µl was diluted with sterile distilled water 400 µl and used this dilution as a template for further secondary PCR reactions.

3.2.2 The long-range PCR techniques

This techniques was used to detect the duplication of *CYP2D6* gene using Elongase® enzyme as Taq polymerase with primer 7S and primer set E (R)

Table 11 Mixture of 25 µl PCR reaction detecting CYP2D6 gene duplication

Mixture	volume (µl)
Buffer A	1.25
Buffer B	3.75
2 mM dNTPs	2.50
10 pmol/µl of primer 7S	1.00
10 pmol/µl of primer set E (R)	1.00
1 u/µl Elongase® (Tag polymerase mix)	0.50
100% DMSO	2.50
Sterile distilled water (SDW)	2.50
Genomic DNA	10.00
Total volume	25.00

The thermal profile consisted of initial denaturation at 94°C for 2 minutes, denaturation 94°C for 30 seconds, anealing 55°C for 30 seconds, extension 68°C for 6.30 minutes and final extension 68°C for 7 minutes for 30 cycles. The product was checked by 1% LE agarose gel electrophoresis

3.3 The analysis CYP2D6 single nucleotide polymorphisms (SNPs)

3.3.1 Tetraplex PCR

There are 10 SNPs (C100T, T843G, C1039T, G1661C, 1707delT, G1758A/T, G1846A, C2850T, C4155T, and G4180C) frequently reported in Asian population. They were detected from *CYP2D6* whole gene template. Tetraplex PCR was performed to provide templates for single base extension reaction. Four pairs of primers were used to amplify tetraplex-amplicons covering regions containing all 10 SNPs. The optimized PCR mixture is presented below and thermal cycles are as following: 94°C for 10 minutes, denaturation 94°C for 30 seconds, anealing 60°C for 30 seconds, extension 72°C for 1 minutes and final extension 72°C for 7 minutes for 30 cycles. The teraplex-amplicons were checked by gel electrophoresis using 2% LE agarose. If the tetraplex PCR is successful, ExoSAP® was used to inactivate the remained active enzyme in order to purify the PCR product before conducting the subsequent single base extension reaction.

Table 12 Mixture of 25 µl of tetraplex PCR mixture in PCR reaction

Mixture	Volume (µl)
10x ImmoBuffer	2.50
2 mM dNTPs	2.50
50 mM $MgCl_2$	1.00
10 pmol/µl of each primer B1, B2	0.80
10 pmol/µl of each primer D3, D4	0.40
10 pmol/µl of each primer F1, G2	0.40
10 pmol/µl of each primer H1, H2	0.40
5x Q-solution	5.00
5u/µl Immolase DNA Polymerase	0.15
Sterile distilled water (SDW)	8.35
Diluted PCR product	1.50
Total volume	25.00

3.3.3 Single base extension (SBE)

Single base extension was conducted by using single specific dideoxynucleotides triphosphate (ddNTPs) instead of deoxynucleotides triphosphate (dNTPs) in order to extend only one base from the primer. The primers were newly designed to hybridize adjacent to SNPs, thereby, one extension base from the primer was a base complementary to DNA template whether it contained SNP or wild type base. The extension primers were designed varying its length as to be eluted from DHPLC column in order. Basically, the shorter extension primer will be eluted before the longer one, however, it depends on the base composition (A, T, G and C) as well. Ten SBE reactions were separated into 2 tubes; each contained 5 extension primers detecting 5 SNPs. Name and sequence of primers are given in Table 15. SNPs in each SBE set of reaction and its mixture are as following and extension primer are given in Table 16.

Table 13 Extension primers

Set 1	C100T	T843G	C1039T	G1661C	1707delT
Set 2	G1846A/T	G1758A	C2850T	C4155T	G4180C

Table 14 Mixture of 20 μl of single base extension in PCR reactions

Mixture	Set 1 (μl)	Set 2 (μl)
1 mM ddNTPs	1.00	1.00
2u/μl Thermo sequenase	0.25	0.25
Thermo sequence enzyme dilution	0.67	0.67
Distilled water	8.08	8.58
Extension primers each*	1.00	1.00
ExoSAP® products	5.00	5.00
Total volume	20.00	20.00

Note: each of extension primer was 1.0 μl except only one primer (G1846A) used 0.5 μl in set 2.

The thermal profile consisted of initial denaturation at 96°C for 1 minute, denaturation 96°C for 15 seconds, annealing 50°C for 20 seconds and extension 60°C for 1 minute for 60 cycles. Extension products from SBE reaction were heated to denature and snapped cool in the ice immediately to produce single strands before inject to DHPLC column.

Table 15 Name and sequence of primers used in multiplex long PCR, 4-plex PCR and 5-plex PCR reactions

Primer	Sequence (5′……3′)
A1	GGCCTACCCTGGGTAAGGGCCTGGAGCAGGA
A2	CTCAGCCTCAACGTACCCCTGTCTCAAATGCG
B1	CCATTTGGTAGTGGAGCAGGTAT
B2	CCCCACTCGCTGGCCTGTTTCA
D3	GAGACTCCTCGGTCTCTCG
D4	TAATGCCTTCATGGCCACGCG
F1	GCTGGGGCCTGAGACTT
G2	CCCCTGCACTGTTTCCCAGA
H1	GAGACAAACCAGGACCTGCCA
H2	GCCTCAACGTACCCCTGTCTC
H3	CCAGCCACCATGGTGTCTTTG
DYS-F	TTGTCGGTCTCCTGCTGGTCAGTG
DYS-R	CAAAGCCCTCACTCAAACATGAAGC
LDL-F	TACAAGTGCCAGTGTGAGGAAG
LDL-R	GTGCAAAGTTCAGAGGATGAAACT
I3	CAGGCATGAGCTAAGGCACCCAGAC
I4	CACACCGGGCACCTGTACTCCTCA
7S	AAGGAGTGTCAGGGCCGGA
Set E(R)	CCTGTAGTGTCAGTGACTCAGAAGGCTG

Table 16 Primers designed for single base extension reactions

Primer	Sequence (5′-.......-3′)
C100T	TGGGCTGCACGCTAC
T843G	TTTCTTGTCAAGCCAGGATC
C1039T	T TTTTATTTTTTTGGGAACGCGGCCC
G1661C	TTTTTTATTTTTTTGCAGAGGCGCTTCTCCGT

Primer	Sequence (5′-.......-3′)
1707delT	TTTTTTTTATTTTTTTTTAAAGAAGTCGCTGGAGCAG
G1846A/T	TTTTTTTTTTATAATATTTTTTTTGCCTTCGCCAACCA CTCC
G1758A	TTTTTTTTAATTTTTTTTGCATCTCCCACCCCCA
C2850T	CAGCCACCACTATGC
C4155T	TT TTTAT TTCCG GCCCAGCCAC
G4180C	TTTTTTTTAAAGCTCATAGGGGGATGGG

3.2.4 Denaturing High Performance Liquid Chromatography (DHPLC)

Denaturing High Performance Liquid Chromatography (DHPLC) was used to analyze single stranded extension products from SBE reaction. The condition was completely denaturing (70 °C) which can resolve short DNA fragments according to size and base composition. The different properties (size and base composition) of single stranded extension product produce different hydrophobicity resulting in the different retention time of ss-DNA in DHPLC column. DHPLC peaks of wild type and SNPs can be easily interpreted.

3.2.5 Restriction fragment length polymorphisms (RFLP) and DNA sequencing

Since multiplex primer-extension analysis using DHPLC was newly developed, this new method should be validated with known techniques such as RFLP or DNA sequencing. RFLP was used to confirm the equivocal result from DHPLC peak. The template that used in restriction enzyme reaction obtained from secondary PCR which were amplified using the same primers as tetraplex PCR reaction but done separately for each primer. Then, restriction enzyme that can distinguish between wild type and SNPs were incubated with template overnight. The reaction mixture was given in Table17. DNA sequencing was also conducted to confirm the results by using MegBACE 1000 capillary sequencer using DYEamic™ ET dye terminator chemistry (Amersham Biosciences). Thus, this techniques use to detect SNPs of some subject who had unclear results of DHPLC.

Table 17 Conditions of restriction fragment length polymorphisms (RFLP)

Position	G1661C	C2850T	C4155T	G4180C
Primer	D3D4	G1G2	H1H2	H1H2
Enzyme	BsmAI	HhaI	NcoI	BstEII
BSA	-	+	-	-
Temperature (°C)	55	37	37	60
Buffer no. (2µl)	2	2	4	3
Cutting site	GTCTC(N1):	GCG:C	-	G:GTNACC
Inactivate	80°C, 20 min	65°C, 20 min	-	-
Note: Temperature (°C); incubate over night				

3.4 Semi-quantitative analysis of copy number of CYP2D6 gene rearrangement

Quantitative copy number determination could be analyzed by various quantitative detection techniques such as Real-time PCR. In this study, semi-quantitative analysis of 5-plex PCR by DHPLC was performed since it is convenient and high-throughput. Semi-quantitative measurement of copy number was made in the assumption of amplified product can relatively reflect the initial template copies. Twenty six cycles of PCR were selected to end the amplification since it is still in linear phase of amplification curve. The condition of 5-plex PCR reaction is optimized and the mixture is shown in Table 18 with following thermal cycles: denaturation 94°C for 10 minutes and then 94°C for 30 seconds, 60°C for 30 seconds, extension 72°C for 30 seconds and final extension 72°C for 7 minutes for 26 cycles.

Table 18 Mixture of 5-Plex PCR reactions

Mixture	Volume (µl)
10x ImmoBuffer	2.00
2 mM dNTPs	2.00
50 mM $MgCl_2$	0.6
10 pmol/µl of each primer LDL-F, LDL-R	0.56
10 pmol/µl of each primer DYS-F, DYS-R	0.56
10 pmol/µl of each primer G1, G2	0.36
10 pmol/µl of each primer D3, D4	0.84
10 pmol/µl of each primer H3, H2	0.56

Mixture (continued)	Volume (µl)
5x Q-solution	4.00
5u/µl Immolase DNA Polymerase	0.10
Sterile distilled water (SDW)	2.54
25 ng/ul gDNA	3.00
Total volume	20.00

4. Statistic analysis

The descriptive characteristics of subjects in both case and control group were presented with mean ± SD. Allele frequencies were estimated using SNPstats program and were compared between two group using Chi's square test. Haplotypes were made and compared between two group using HAPstats program. The input data were dose of amitriptyline, allele frequencies and ADR scores of each subject in both groups. Output values represented haplotype frequencies compared between two groups giving with 95% CI and p-value. Age and doses of amitriptyline of each subject were treated as environmental factor in the software to test whether it is a confounding or not. Descriptive statistics were calculated by Microsoft Excel and SPSS version 16. SNPstats program were used to test Heindy Weinberg Equillibium (HWE) and SNP effect for ADR scores. P-value less than 0.05 are considered statistically significant.

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CHAPTER IV

RESULTS

1. Characteristic of 150 depressive patients

A total of 150 out-patients diagnosed with depressive disorders were recruited. The study was designed as a matched case-control study, matching age range with doses of amitriptyline. Case and control groups each consist of 75 patients with baseline characteristic as shown in Table 19, the number of male and female patients included in this study were similar. The mean ± SD of age, dose (amitriptyline, mg/day) and the number of depression patients according to ICD10 criteria were the same. Drug allergy to antibiotics such as Penicillin is slightly different between control (13.3%) and case (5.3%) group. Concurrent drug used (e.g. acetaminophen, ibuprofen) is different in 2 groups; 28.0% in control and 42.7% in case group. However, no CYP2D6 inhibitors (such as quinidine, fluoxetin, paroxetin, etc) were co-administered recently or otherwise that case would be excluded.

Table 19 Baseline characteristic of the 150 depressive patients recruited in the study

		Control group (n=75)	Case group (n=75)
Gender			
	Male	15 (20%)	15 (20%)
	Female	60 (80%)	60 (80%)
Age (year)			
	Mean ± SD	53.40 ± 14.10	49.10 ± 11.40
	Min	23	23
	Max	89	75
Dose (mg/day)			
	Mean ± SD	28.90 ± 26.97	37.00 ± 26.60
	Min	10	10
	Max	125	125
Depression; ICD 10 Criteria (n)			
	F32	44 (58.7%)	49 (65.3%)
	F33	8 (10.6%)	7 (9.40%)
	F34	23 (30.7%)	19 (25.30%)

Abbreviations; F32 = Depressive episode, F33 = Recurrent depressive disorder, F34 = Persistent mood disorder, n = number of patients and percent distribution is in parenthesis.

2. CYP2D6 Genotyping

CYP2D6 gene contains a large number of SNPs and also various types of gene rearrangement including gene duplication, multiplication, deletion and different allele duplication in tandem (*CYP2D6**36-*10B). Moreover, another two pseudogenes in tandem (*CYP2D8P* and *CYP2D7P*) which contain very high sequence similarity (~ 97%) should be taken cautiously when design to genotyping. Hence, *CYP2D6* gene should be specifically amplified first and then secondary PCR can be performed from whole gene amplicon to subsequently detect SNPs therein. In this study, multiplex long PCR were used to simultaneously detect *CYP2D6* gene and whole gene deletion (*CYP2D6**5). For gene rearrangement detection, 5-plex PCR was developed combined with quantitatively analyzed by DHPLC. Those with duplication detected with quantitative DHPLC were further analyzed the upstream segment of duplicated allele using long PCR. Finally, Ten SNPs were determined from SBE analyzed with DHPLC in 4-plex PCR of whole gene and also duplicating segment of long PCR. All analyzing results taken together can be assigned for *CYP2D6* genotype.

2.1 Results of multiplex long PCR detecting *CYP2D6**5 and long PCR detecting duplication

CYP2D6 whole gene and *CYP2D6**5 was successfully detected using primer A1/A2 without co-amplification of in tandem pseudogenes producing 4.8 kb while primer I3/I4 produce 3.2 kb amplicon only if the template contain gene deletion as shown in Figure 15. The allele frequency of *CYP2D6**5 is 4.67% which only heterozygous deletion were detected.

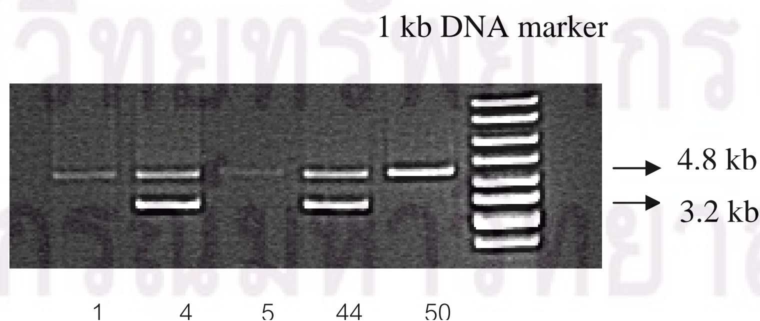

Figure 15 Whole gene was shown in 4.8 kb fragment contained entire 9 exons of *CYP2D6* gene and 3.2 kb fragment indicated the presence of the *CYP2D6**5 allele.

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Upstream segment of duplicated allele was successfully amplified using long PCR as shown in Figure 16. There could be two sizes of product indicating two types of duplication; 6.5 kb indicated *CYP2D6**36x2 or *CYP2D6**36-*10 and 4.9 kb indicated *CYP2D6**2, *10 or other- associated duplication. However, some samples were failed to amplified duplicated allele since DNA integrity was poor but quantitative copy number data can relatively be used together with its SNP results to infer the genotype.

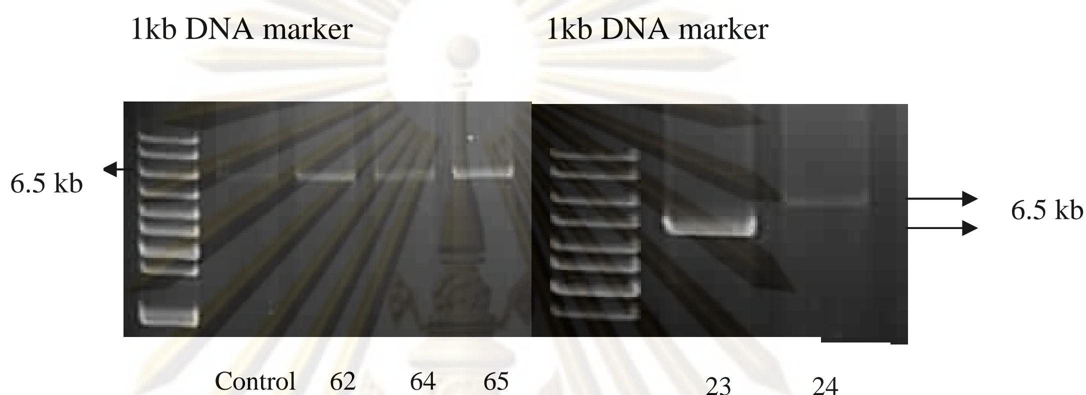

Figure 16 Duplication of *CYP2D6* gene were 6.5 kb indicated as *CYP2D6**36×N allele (number 62, 64, 65 and 24) and 4.9 kb indicated as *CYP2D6**2×N or *CYP2D6**10×N allele or other type (number 23).

2.2 Semi-quantitative analysis of *CYP2D6* gene rearrangement by DHPLC

In order to detect gene rearrangement type (duplication, deletion and gene conversion), 5-plex PCR was developed from genomic DNA coupled with quantitative DHPLC analysis. Three regions from *CYP2D6* gene were selectively amplified including exon 3-4, exon 5-6 and exon9 which primers anneal only wild-type sequences but conversed sequences. Another two genes were used as an internal control; LDL gene is autosomal gene presenting 2 copies in everyone and dytrophin gene is X-linked gene presenting 1 copy in male. Quantitative copy number analysis was made in the assumption of amplified product can relatively reflect the initial template copy using cycle numbers (n=26) during the linear phase of amplification curve. The results are shown in Figure 17. Demonstrating various type of gene rearrangement.

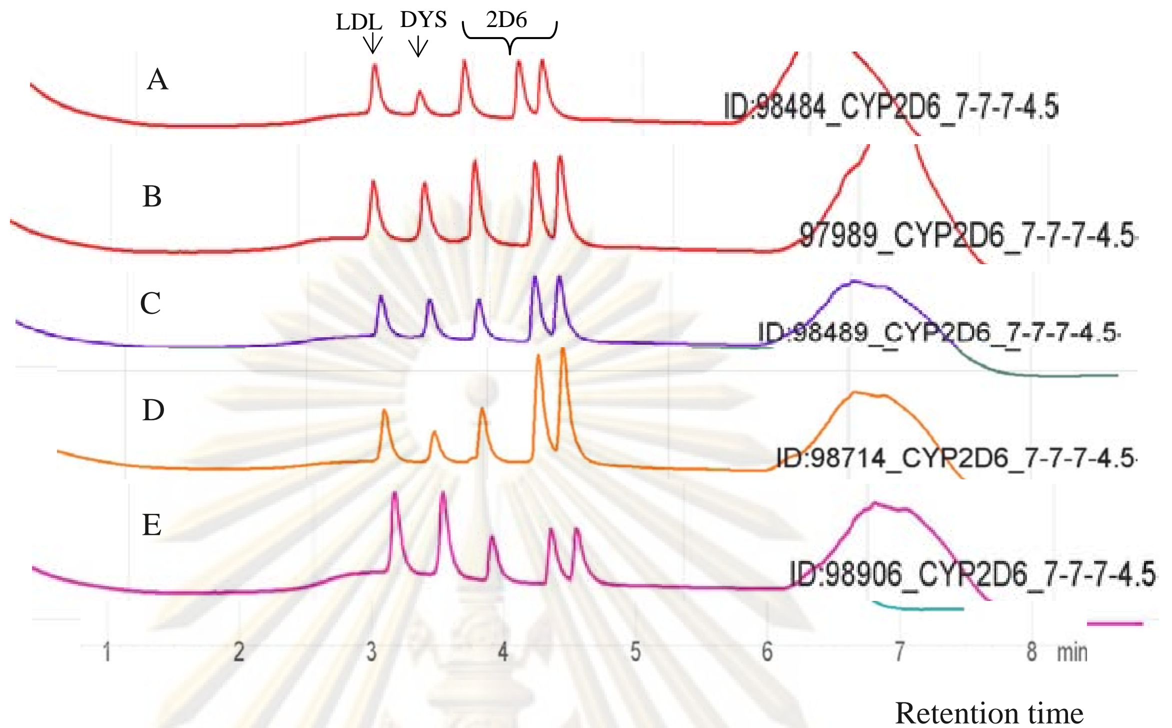

Figure 17 Semi-quantitative DHPLC profile; pattern A is normal, B, C and D is duplication with 1.5-fold (3 copies), 1.5-fold with gene conversion and 2.0-fold (4 copies) copy numbers with 2 gene conversion, respectively, and E is heterozygous deletion. LDL = *LDL* gene, DYS = dystrophin gene. 2D6 = *CYP2D6* gene in the last 3 peaks.

Relative peak height ratio between LDL gene (2 copies) and *CYP2D6* gene (exon3-4, exon 5-6) were compared whether it contains one, two, three and higher copy numbers. In exon 9, the primers were designed to selectively anneal only wild-type sequence, therefore; the presence of product in exon 9 indicates no gene conversion if the peak height is as high as of the last 2 peaks, exon 3-4 and exon 5-6. The number of gene conversion can be obtained from the deduction of copy number in exon 9 from copy number in exon 3-4 or exon 5-6 as shown in Figure 17. The distribution of gene duplication or deletion between two groups is shown in Table 23 no significance of deviation tested with Chi's Square.

2.2 Analysis of CYP2D6 single nucleotide polymorphisms (SNPs)

Ten SNPs that are commonly found in Asians were selected to determine in this study. Tetra-plex PCR which was developed covering all 10 variant sites as shown in Figure 18 were used as a template for SBE reaction and subsequently analyzed with DHPLC as shown in Figures 19-20. Five SNPs in one injection can be eluted in order corresponding to its size and can be interpret straightforward as presenting base or have to convert to opposite base depending on initial forward or backward primer design.

100 bp DNA marker

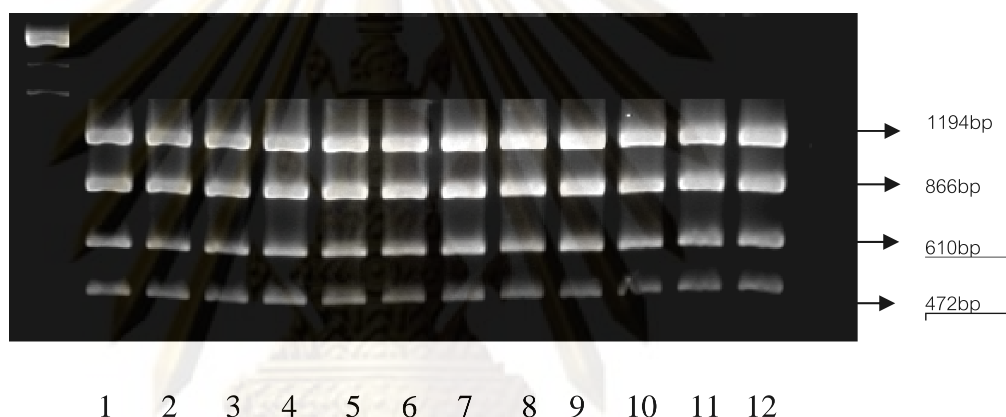

Figure 18 This tetraplex-PCR was amplified covering all 10 SNPs in order to be a template of SBE reaction and further injected to DHPLC for SNPs analysis.

2.3.1 DHPLC profile detecting SNPs

DHPLC profiles analyzed from a primer extension-based method detecting 10 SNPs shows a clear results of homozygous and heterozygous pattern. Percent allele frequencies are shown in Table 24. Allelic variants detected from 150 out-patients were classified into genotype groups follow the report of the Human CYP Allele Nomenclature web-site (http:www.imm.ki.se/CYP2d6.html).

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SNP = C100T T843G C1039T G1661C 1707delT

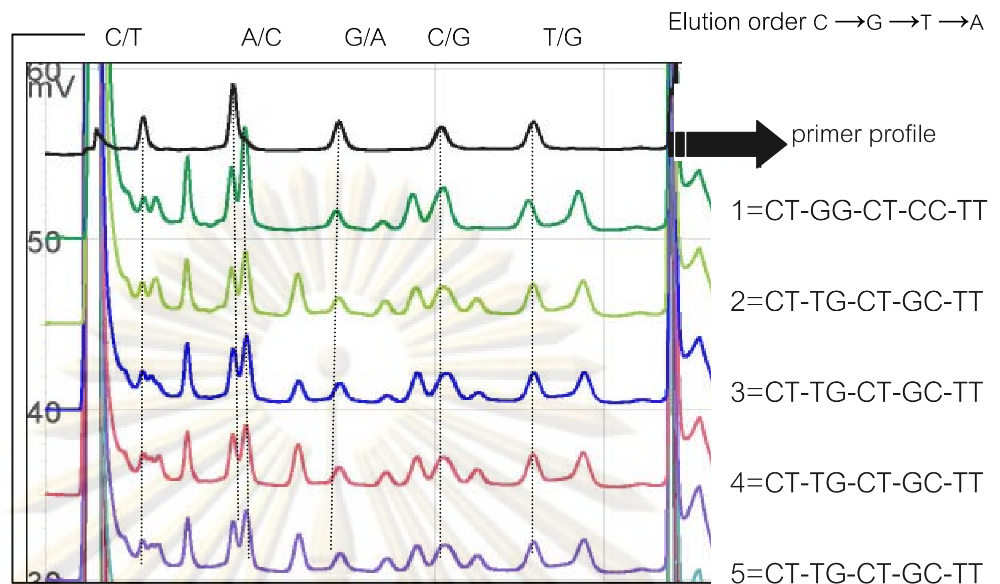

Figure 19 DHPLC profiles of 5 primers (set1) detecting 5 SNPs. Right panel shows SNP interpretations (no 1-5) compare to the profile of primer.

C2850T C4155T G4180C G1846A G1758A/T Elution order C ⟶G ⟶T ⟶A

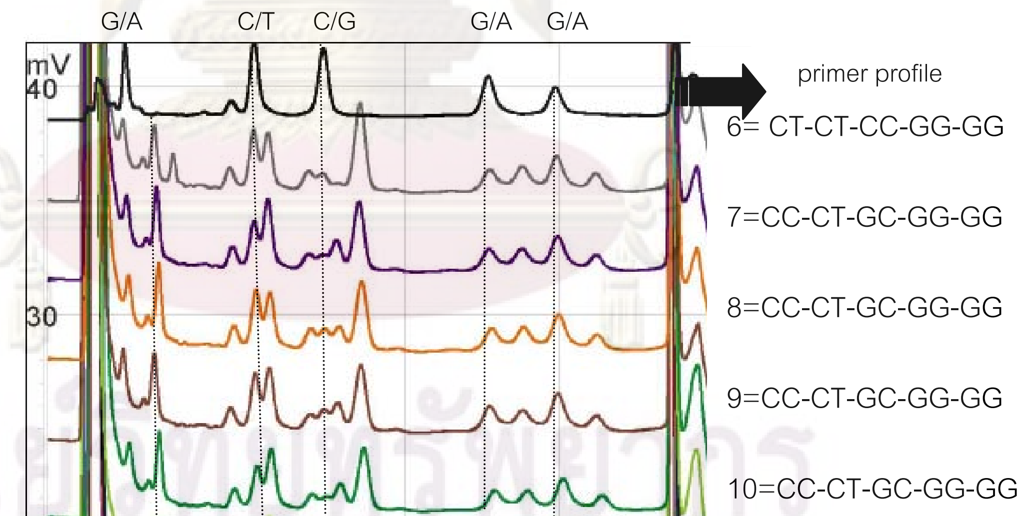

Figure 20 DHPLC profiles of 5 primers (set2) detecting 5 SNPs. Right panel shows SNP interpretations (no 6 -10) compare to the profile of primer.

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2.3.2 Restriction fragment length polymorphisms (RFLPs)

This technique was used to confirm unclear results from DHPLC. SNP profiles showing in Figures 21-22.

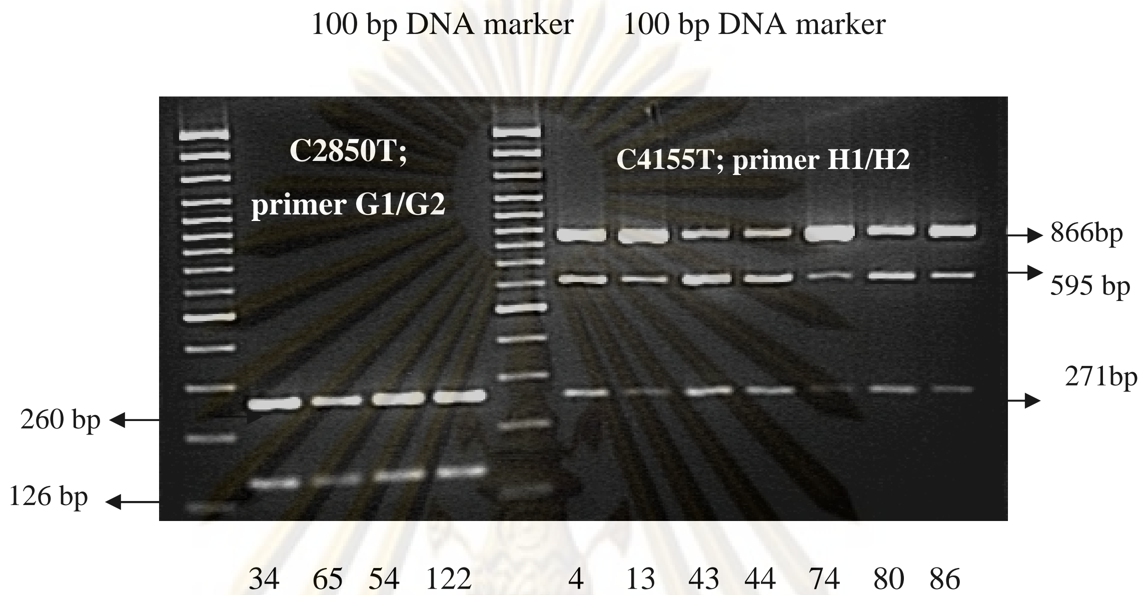

Figure 21 For C2850T, restriction band number 34, 65, 54 and 122 are mutant fragments that restricted with *Hha*I (wild type = 386 bp). C4155T restriction band number 4, 13, 43, 44, 74, 80 and 86 are heterozygous restricted with *Nco*I containing 3 fragments (866, 595 and 271 bp).

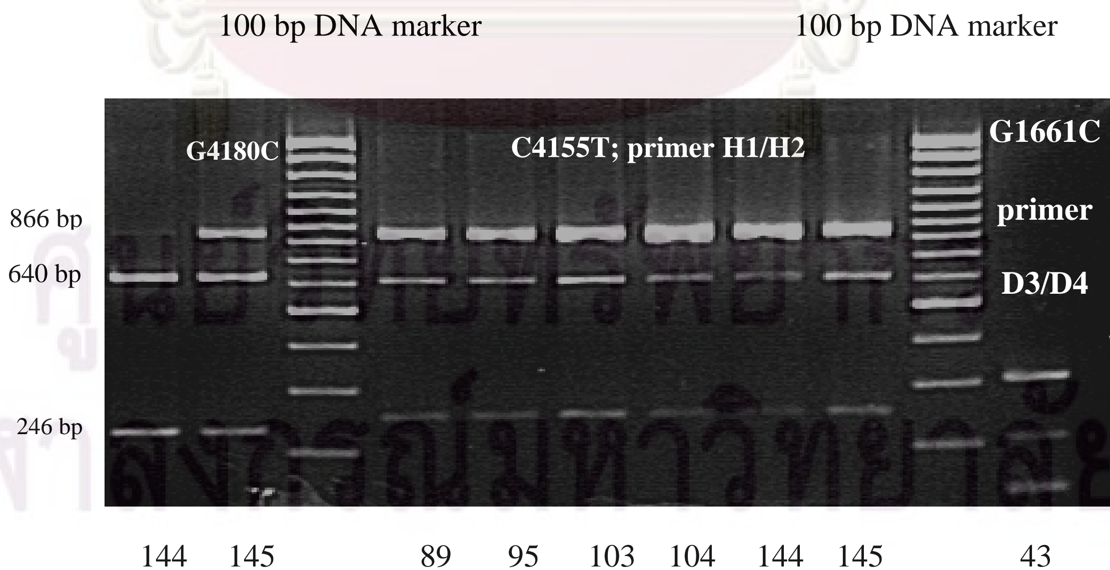

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Figure 22 G4180T restriction band number 144 is mutant fragment and number 145 is heterozygous band that restricted with *BstE*II (wild type = 866 bp) G1661C restriction band number 43 is mutant that restricted with *Bsm*AI (wild type = 311/156 bp).

One hundred and fifty depressive patients were genotyped for 10 SNPs; C100T, T843G, C1039T, G1661C, 1707 delT, G1758T/A, G1846A, C2850T, C4155T, and G4180C. Three SNPs were not found in this studied subjects which are 1707 delT, G1758T/A, and G1846A. SNP frequencies were calculated and the outcome was slightly different between control and case group. The frequency of determined SNPs in sample of N individual was estimated by (2nx/x + nx/-)/2N where nx/x is a number of individuals homozygous for x, and nx/- is a number of individuals heterozygous for the x. The following SNPs were observed from high to low frequencies ranging as G4180C, G1661C, T843G, C100T, C1039T, C4155T and C2850T, respectively, as shown in Table 20. As expected, these SNPs were reported with high frequencies in Asians since they were commonly found in association with *CYP2D6**10. On the contrary, those three SNPs that were not detected in this studied subjects are in association with *CYP2D6**4, *6 and *14 and they were found mostly in Caucasians.

The conbination of these detected SNPs can be assigned as *CYP2D6* genotype according to the standard nomenclature (http:www.imm.ki.se/CYP2d6.html). In this study, the combination of SNPs that is not corresponding to the standard nomenclature is presented as unclassified allele (UA). All detected SNP combinations can be classified into 15 haplotypes using the HAPstats program as shown in Table 21. Haplotype ranging from high to low observed frequencies are as following; *CYP2D6**1, *CYP2D6**36, *CYP2D6**2, *CYP2D6**10B and *CYP2D6**10A, respectively as shown in Table 23. Seven haplotypes out of fifteen is considered to be significant different in frequency between case and control group.

Genotypes were inferred from data of SNPs and gene copy number altogether. One hundred and fifty depressive patients presented with following genotypes; *1/*1, *1/*10B, *10B/*10B, *1/*2, *2/*10B, *10A/*10B, *10B/*36, *36-10B/*10A, *36-10B/*10B and *36-10B/*36-10B. While genotypes of *CYP2D6**4, *CYP2D6**6 and *CYP2D6**8 which are commonly found in Caucasian are not detected in this recruited subjects as shown in Table 23. As expected, allele frequencies of *CYP2D6* were found in agreement with those found in other Asians such as Japanese and Chinese [49]. For those who had deletion, duplication and both deletion and duplication are excluded

from haplotype and SNPs frequency calculations. The frequency of *CYP2D6* rearrangement type is shown in Table 23.

Genotype distribution among case and control group is shown in Table 24. The observed genotype frequencies that tend to be significantly different between two groups were selected to conduct the Chi's square test. Genotypes that are considered to cause normal metabolism such as *CYP2D6**1/*1 and *2/*10B were used to test for frequency difference versus other observed genotype in both groups. *CYP2D6**1/*1 represented the same numbers of observed frequency in both group which lead to non-significant difference versus any genotypes as in Table 24 In contrary, *CYP2D6**2/*10B were observed differently in two groups that cause significantly different in frequency distribution when tested with *CYP2D6**10A/*10B, *CYP2D6**36-10B/*10A and *CYP2D6**36-10B /*36-10B. This result demonstrates the tendency of having ADR in whom posses IM genotypes without duplication more than those with extensive genotype or IM with additional in tandem duplication when getting amitriptyline.

When consider deletion and duplication, there is no homozygous *CYP2D6**5 but the most common deletion types are *CYP2D6**1/*5 and *2/*5. The duplication rearrangement type that found at high frequency are *36-*10B/*10A and *36-*10B/*10B which are the same type as reported in Japanese and Chinese [47, 77]. Gene rearrangement including duplication, in tandem duplication, and deletion is not statistical different when tested with Chi's square even though there is quite a number of deletion in control higher than case group. Interestingly, 4 copies with 2 copies of gene conversion is higher in control group as shown in Table 22.

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Table 20 Frequencies of SNPs of *CYP2D6* in 97 depressive patients (47 in control and 50 in case) and for those having gene rearrangement were excluded from the calculation.

SNPs	Genotype	SNP distribution		Allele frequency		OR (95% CI)	P-Value
		Control (%)	Case (%)	Control	Case		
C100T	C/C	20 (42.5%)	18 (36%)	45.74 %	47.00%	1.00	
	C/T	11 (23.4%)	17 (34%)			1.72 (0.64 - 4.62)	0.51
	T/T	16 (34.0%)	15 (30%)			1.04 (0.40 – 2.69)	
T843G	G/G	19 (40.4%)	22 (44%)	56.38%	60.00%	1.00	
	T/G	15 (31.9%)	16 (32%)			0.92 (0.36-2.35)	0.91
	T/T	13 (27.7%)	12 (24%)			0.80 (0.29-2.16)	
C1039T	C/C	18 (38.3%)	17 (34%)	39.36%	47.00%	1.00	
	C/T	21 (44.7%)	19 (38%)			0.96 (0.39 – 2.38)	0.43
	T/T	8 (17%)	14 (28%)			1.85 (0.62 - 5.53)	
G1661C	C/C	18 (38.3%)	22 (44%)	60.63%	59.00%	1.00	
	G/C	21 (44.7%)	15 (30%)			0.58 (0.24 – 1.45)	0.28
	G/G	8 (17%)	13 (26%)			1.33 (0.45 – 3.91)	
C2850T	C/C	32 (68.1%)	38 (76%)	19.14%	13.00%	1.00	
	C/T	12 (25.5%)	11 (22%)			0.77 (0.30 – 1.98)	0.47
	T/T	3 (6.4%)	1 (2%)			0.28 (0.03 - 2.83)	
C4155T	C/C	20 (42.5%)	19 (38%)	28.72%	31.00%	1.00	0.65
	C/T	27 (57.5%)	31(62%)			1.21 (0.54-2.72)	
G4180C	C/C	22 (46.8%)	26 (52%)	61.72%	63.00%	1.0	
	G/C	14 (29.8%)	11 (22%)			0.66 (0.25 – 1.76)	0.68
	G/G	11 (23.4%)	13 (26%)			1.00 (0.37 – 2.67)	

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Table 21 Frequencies of haplotype of *CYP2D6* polymorphism in 97 depressive patients excluded those having gene rearrangement

Inferred Allele	Haplotype	Haplotype Frequencies		Standard Error	Z-Statistic	P-Value
		Control	Case			
*CYP2D6**1	0000000	0.3085	0.3194	0.0476	6.4760	0.0000*
*CYP2D6**36	1111011	0.2660	0.2705	0.0367	7.5645	0.0000*
*CYP2D6**2	0101101	0.1064	0.0591	0.0229	4.1275	0.0000*
*CYP2D6**10B	1111001	0.0851	0.1183	0.0248	4.5254	0.0000*
*CYP2D6**10A	1101101	0.0638	0.0200	0.0165	2.8724	0.0041*
*CYP2D6**2	0000001	0.0532	0.0503	0.0183	3.2241	0.0013*
*CYP2D6**2	0001000	0.0319	0.0000	0.0102	1.7420	0.0815
*CYP2D6**2	0001011	0.0106	0.0000	0.0059	1.0019	0.3164
UA	0011000	0.0106	0.0000	0.0059	1.0019	0.3164
*CYP2D6**2	0100000	0.0106	0.0303	0.0117	2.0154	0.0439*
*CYP2D6**10B?	0111101	0.0106	0.0100	0.0083	1.4196	0.1557
UA	1000000	0.0106	0.0000	0.0059	1.0019	0.3164
UA	1000001	0.0106	0.0000	0.0059	1.0019	0.3164
*CYP2D6**10B	1111000	0.0106	0.0000	0.0059	1.0019	0.3164
*CYP2D6**36?	1111111	0.0106	0.0190	0.0102	1.7420	0.0815

Note: UA= unidentified allele, question mark = could be inferred as presented allele, * = P-value (2-tailed) significant levels for two sides.
UA = Unclassified Allele; 0 = wild type; 1= mutation of single base nucleotide

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Table 22 Frequencies of wild type, duplication and deletion *CYP2D6* polymorphism in 150 depressive patients

			Control (n=74)	Case (n= 69)
Wild type (n=89)				
	Wild type		41	40
	Wild type with 1 copy of gene conversion		3	4
	Wild type with 1 copy of gene conversion and deletion		1	0
Duplication (n=46)				
	Duplication 2.0-fold (4 copies)			
		with 2 copies of gene conversion	7	3
		With 3 copies of gene conversion	0	3
	Duplication 1.5-fold (3 copies)			
		No gene conversion	0	1
		With 1copy of gene conversion	16	11
		With 2 copies of gene conversion	1	4
Deletion (n=7)			5	2
Duplication and deletion (n=1)			0	1

Abbreviations; Control group = without ADR; Case group = with ADR.

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Table 23 Frequencies of *CYP2D6* genotype in 150 depressive patients

CYP2D6 Genotype	Control (n=75)	Case (n=75)	Combined (n=150)
*1/*1	10 (13.33%)	10 (13.33%)	20 (13.33%)
*1/*10B	9 (12.00%)	9 (12.00%)	18 (12.00%)
*10B/10B	6 (8.00%)	11 (14.67%)	17 (11.33%)
*1/*2	7 (9.33%)	6 (8.00%)	13 (8.67%)
*2/*10B	3 (4.00%)	9 (12.00%)	12 (8.00%)
*10A/*10B	7 (9.33%)	3 (4.00%)	10 (6.67%)
*10B/*36	3 (4.00%)	3 (4.00%)	6 (4.00%)
*2/*2	3 (4.00%)	0	3 (2.00%)
*10A/*36	1 (1.33%)	1 (1.33%)	2 (1.33%)
Duplication			
*1/*2x2	0	1 (1.33%)	1 (0.67%)
*36x2/*2	0	1 (1.33%)	1 (0.67%)
*36x2/*2x2	1 (1.33%)	0	1 (0.67%)
Deletion			
*2/*5	0	1 (1.33%)	1 (0.67%)
*1/*5	1 (1.33%)	1 (1.33%)	2 (1.33%)
*5/*10B	2 (2.67%)	0	2 (1.33%)
Gene arrengments			
*36-10B/*10A	7 (9.33%)	2 (2.67%)	9 (6.00%)
*36-10B/*10B	4 (5.33%)	4 (5.33%)	8 (5.33%)
*36-10B/*36-10B	6 (8.00%)	2 (2.67%)	8 (5.33%)
*36-10/*2	2 (2.67%)	2 (2.67%)	4 (2.67%)
*10B/*36X2	1 (1.33%)	2 (2.67%)	3 (2.00%)
*36-10B/*1	1 (1.33%)	1 (1.33%)	2 (1.33%)
*36-10B/*36X2	0	2 (2.67%)	2 (1.33%)
*36-10B/*36	0	1 (1.33%)	1 (0.67%)
*36-10B/*2	0	1 (1.33%)	1 (0.67%)
Unclassified Allele	1 (1.33%)	2 (2.67%)	3 (2.00%)

Abbreviations; 0 = no data; UA = Unclassified Allele

Table 24 Chi's Square test for the difference of genotype distribution between control and case group.

CYP2D6 genotype	Chi's Square, df = 1	P-Value
*1/*1 vs *10A/*10B	1.09	0.297
*1/*1 vs *10B/*10B	0.810	0.368
*1/*1 vs *2/*10B	0.194	0.163
*2/*10B vs *10A/*10B	4.45	0.035*
*2/*10B vs *10B/*10B	0.348	0.555
*2/*10B vs *36-10B/*10A	5.74	0.017*
*2/*10B vs *36-10B /*36-10B	4.85	0.028*

* = P-value (2-tailed) significant levels for two sides.

Fifty two out of 150 patients that have gene deletion or duplication were excluded from the association analysis by SNPstats and HAPstats program but it was analyzed for deviation between two groups using Chi's square test. The analysis of the association between genetic polymorphisms and side effect scores allows identifying susceptibility genotype by using SNPstats and HAPstats.

SNPstats (Statistical analysis of SNPs-disease association)

Analysis of single SNPs under Hardy-Weinberg Equilibrium (HWE) for its effects to ADRs are determined by multiple inheritance models, co-dominant, dominant, recessive and over-dominant (data not shown), but it was non-significant different.. Analysis of association is based on a linear or logistic regression (OR = odds ratios, 95%CI = 95% confidence intervals) according to the group status (control and case group). Data showed no SNPs effects to ADRs group since SNPs frequencies are not different between two groups.

HAPstats (Statistical analysis of haplotype-disease association)

In the analysis of multiple SNP genotype or haplotypes, descriptive statistics displays the estimated relative frequency for each haplotype. Haplotype determination designed to analyze the individual genotype at the ten most common polymorphic positions. Eighty eight from ninety seven (47 in controls and 41 in cases) were analyzed under Hardy-Weinberg

equilibrium. The analysis of only gene effect that compared between control and case group showed non-significant differences

In this study, the factor of ages and doses between case and control group were checked in HAPSTATs whether both factors are confounding in the association or not. Only the SUM scores of amitriptyline adverse reactions found with statistical difference between control and case groups (P<0.05) because they were already classified as ADRs and non-ADRs from the beginning. The association analysis of the SNPs into haplotypes as additive model of haplotype effects under Hardy-Weinberg Equilibrium are shown in Table 26. Analysis of haplotype-environment interactions through regression modeling found no statistical differences between control and case groups Thus, haplotype with environment (age, dose and SUM scores of amitriptyline adverse reactions) are not co-response during the occurrence of adverse reaction.

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Table 25 Association between haplotype with control and case group by effects (environment; age, dose and SUMADR) under Hardy-Weinberg equilibrium Tolerance: 0.0001 Maximum iterations: 500, Subjects: 97/88 (Cases: 41/50, Controls: 47) (Dominant model)

		Estimate	Standard Error	Z-Statistic	P-Value
Effects					
Environment					
	Age	0.0262	0.0302	0.8667	0.3861
	Dose	0.0032	0.0133	0.2439	0.8073
	SumADR	0.0438	0.0107	4.0805	0.0000*
Frequencies (association)					
Gene					
	0000000 (*1)	2.2493	1.7630	1.2759	0.2020
	Age*0000000 (*1)	-0.0438	0.0314	-1.3927	0.1637
	Dose*0000000 (*1)	-0.0223	0.0156	-1.4301	0.1527
	SumADR*0000000 (*1)	0.0021	0.0030	0.6967	0.4860

Haplotype frequencies under Hardy-Weinberg equilibrium; case group (Log-Likelihood -174.7739, interactions 106), combined group (Log-Likelihood -350.8229, interactions 61).Additive effects under Hardy-Weinberg equilibrium; Log-Likelihood: -319.2941, interaction 7
Abbreviations;* = P-value (2-tailed) significant levels for two sides.
UA = Unclassified Allele; 0 = wild type; 1= mutation of single base nucleotide.

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Table 26 Summary of adverse reactions from amitriptyline in 75 depressive patients of control group (without of amitriptyline adverse reactions)

No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
n	30	60	20	12	22	4	9	16	9	17
Min-Max	0.1-19.76	0.1-45.7	0.1-20.9	0.1-10.7	0.4-22.0	3-19.7	0.2-13.2	0.1-22	0.1-12	0.3-14.8
SUM	130.28	495.76	66.42	35.00	96.02	32.01	37.22	48.07	31.19	65.41
Mean	4.3427	8.2627	3.3210	2.9167	4.3645	8.0025	4.1356	3.0044	3.4656	3.8476
SD	5.3027	11.3080	5.4035	2.9664	5.8851	7.8565	4.6865	5.2966	4.4512	4.0796
No.	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
n	2	5	5	7	4	3	2	4	6	12
Min-Max	1.57-3.00	0.20-10.0	0.44-4.50	1.70-9.80	0.91-21.00	0.20-44.70	0.20-2.70	0.20-3.60	1.12-23.00	0.80-3.00
SUM	4.57	16.9	11.94	31.09	34.61	45.50	2.90	7.10	56.92	23.11
Mean	2.2850	3.3800	2.3880	4.4414	8.6525	15.1600	1.4500	1.7750	9.4867	1.9258
SD	1.0111	3.8343	1.8702	3.1461	8.7142	25.5770	1.7677	1.4930	9.9383	0.7957

Abbreviations; 1=Blurred vision, 2= Dry mouth, 3=heart burn, 4= Urinary retention, 5= Constipation, 6= Ataxia, 7= tremors, 8= Dizziness, 9= fatigue, 10= tachycardia, 11=hypotension orthostatic, 12= Nausea/Vomiting, 13= Anorexia, 14=hypo/hypertension, 15= Peculiar taste, 16= Excessive sweating, 17= skin rash / Photosensitization, 18= Insomnia, 19= Headache, 20= others (Weight gain/loss, Alopecia)

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Table 27 Summary of adverse reactions from amitriptyline in 75 depressive patients of case group (with of amitriptyline adverse reactions)

No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
N	15	70	6	7	44	7	15	52	17	19
Min-Max	1.90-85.71	3.81-93.34	4.76-80.0	4.76-56.19	0.95-100.0	1.9-85.71	0.95-51.49	0.95-87.61	1.9-85.71	0.95-71.42
SUM	287.60	3595.37	179.04	116.86	1424.36	185.70	231.06	1013.19	557.18	556.46
Mean	19.1733	51.3624	29.8400	16.6943	32.3718	26.5286	15.4040	19.4844	32.7753	29.2874
SD	23.4900	28.5430	34.7310	18.1680	28.4670	30.6960	16.2210	20.1890	28.5790	26.2007
No.	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
n	13	6	11	5	7	10	3	9	20	15
Min-Max	4.76-76.20	3.81-23.81	2.86-80.95	5.02-58.57	5.71-60	4.76-100	1.71-69.52	2.85-88.57	0.95-79.04	9.3-88.57
SUM	336.07	63.07	276.18	149.3	119.17	250.48	83.61	379.02	438.56	465.34
Mean	25.8515	10.5117	25.1073	29.8600	17.0243	25.0480	27.8700	42.1133	21.9280	51.0227
SD	21.6100	69.2182	25.7571	23.3310	19.1220	29.0170	36.4623	32.2880	23.2640	30.1260

Abbreviations; 1=Blurred vision, 2= Dry mouth, 3=heart burn, 4= Urinary retention, 5= Constipation, 6= Ataxia, 7= tremors, 8= Dizziness, 9= fatigue, 10= tachycardia, 11=hypotension orthostatic, 12= Nausea/Vomiting, 13= Anorexia, 14=hypo/hypertension, 15= Peculiar taste, 16= Excessive sweating, 17= skin rash / Photosensitization, 18= Insomnia, 19= Headache, 20= others (Weight gain/loss, Alopecia)

SUM ADR scores of Amitriptyline adverse reaction

700
600
500
400
300
200
100
0

With ADR

Without ADR

Figure 23 SUM ADR scores of amitriptyline adverse reaction

Amount of patients each group; n =75

SUM scores of amitriptyline adverse reactions

One hundred and fifty depressive patients were classified into group with Naranjo's algorithm questionnaires. Both control and case group were evaluated with amitriptyline adverse reactions using questionnaire with 20 symptoms (adverse reactions) and the scale summary of frequency are shown in Table 27 and 28.

From the summary of frequency, when considering summation of amitriptyline adverse reaction scores in both control and case group, it was found that case groups have remarkably high scores than control group in the same symptoms of amitriptyline adverse reactions. Especially, anticholinergic effects were firstly pronounced and the most common reactions are dry mouth, constipation and blurred vision, respectively. For CNS, neuromuscular and cardiovascular were common reactions such as drowsiness, headache, fatigue or weakness, arrhythmias, and orthostatic hypotension. Other symptoms were rare in both groups; there were ataxia, heart burn, hypo/hypertension, peculiar taste, skin rash, photosensitization and nausea/vomiting, respectively. As shown in the summation of amitriptyline adverse reactions scores are displayed in the graph comparing of case and control group.

CHAPTER V

DISCUSSION AND CONCLUSION

One hundred and fifty depressive patients were characterized for *CYP2D6* genotypes in order to find the association with amitriptyline adverse reactions conducting at Siriraj Hospital. Gene rearrangement and ten SNPs were investigated in developed tested panel. Gene copy numbers were analyzed by DHPLC and multiplex long PCR were performed for simultaneously amplified whole gene and gene deletion. SBE was subsequently performed coupled with DHPLC analysis to determine SNPs therein. From tested panel, seven SNPs were found in these depressive patients and three SNPs (1707delT, G1758T/A and G1846A) were not observed (non-polymorphic). It is not surprised that those three SNPs were not detected in these recruited subjects since they are likely found in Caucasian more than in Asians. *CYP2D6* genotype especially *CYP2D6**36 and *10-related genotype are generally in agreement with those reported previously in other Asians, especially Chinese [47, 49 and 77]. Moreover, allele frequency is quite similar which is as high as up to 60 % and it is considered to be an intermediate metabolizer. *CYP2D6**36 in tandem rearrangement located upstream of *CYP2D6**10B was the most common type of gene rearrangement in present study and only one patient presented with *CYP2D6**2x2 (duplication). *CYP2D6**36 contain major cause of six-amino acid difference in exon 9 which resulted in the decreased functional gene [1, 47, 49, 77 and 78]. It is one of the most common alleles in Asian populations [49]. Because the exon 9 conversion to *CYP2D7* has been found almost exclusively on a *CYP2D6**10 background that explains the high frequency of the *CYP2D6**10 allele (30-50%) [22, 47 and 49].

The observed frequency of *CYP2D6**2/*10B is significantly different when compared with *CYP2D6**10A/*10B, *CYP2D6**36-10B/*10A and *CYP2D6**36-10B /*36-10B. This result obviously shows the different genotype between control and case groups. It is noticeable that tandem duplication as commonly found in *CYP2D6**36-10B/*10A were found at a larger number in control than those in case group. This could be explained that one more copy of gene can increase metabolism capacity although the duplicated one is also decreased functional allele. This data reasonably demonstrates the tendency of having ADR in whom posses those IM/IM genotypes without duplication more than extensive genotype or IM genotypes having duplication when taking amitriptyline.

Fifty two samples (34.67%) containing gene rearrangement (deletion, duplication and conversion) were detected with higher frequencies compared to those formerly reported in Thais and Asians [47]. This probably resulted from the improved accuracy of detection panel to clarify its complexity. There is only one patient presenting both deletion and duplication in different allele, 30% having duplication and/or in tandem duplication and 4.67% with deletion. Furthermore, only hemizygous deletion (*CYP2D6**5) was found which is considered as normal metabolic status since it cause no impact on enzyme activity. It is never found any relationships between adverse reactions with hemizygous deletion but significantly shown in homozygous deletion [68-70, 74-76]. However, it was found significantly higher in a number of in tandem duplication genotypes mentioned above in control group when tested versus *CYP2D6**2/*10B.

Several studies have investigated for the association between genetic polymorphism of *CYP2D6* with various measurable phenotypes such as plasma drug concentrations, drug responses and adverse reactions [1, 13, 21-24]. The studies that designed to measure plasma concentration of interested CYP2D6 substrates mostly established the high correlation between genotype and phenotype. In contrary, the association studies between drug response/ADRs and genotypes were reported inconsistently [1, 26-31 and 33]. The problems regarding the contradictory of correlation findings probably arises from the different genotypes tested in various ethnics and sample sizes. In addition, the accuracy of genotype calling should be also taken into consideration especially the complexity of gene rearrangement mostly found in Asians [77-85]. However, there was a report showing only dysfunctional *CYP2D6* alleles (poor metabolizer) correlate with severe adverse reactions [68-71]. There are a number of results demonstrated that severe side effects are experienced from those who had undergone gene deletion [75, 76]. As documented earlier, only homozygous deletion can cause absent enzyme activity but hemizygous deletion seems to cause no effect on enzyme function. Unfortunately, this study exhibits none of homozygous deletion candidate to be tested for association with the ADRs. It is noteworthy that the gene-drug response/ADRs association is found correlated only in Caucasians. One of the reasons is that Caucasians possess significantly higher numbers of poor metabolizers than of Asians and this lead to more pronounced effects of ADRs. In Asian populations, there are only a few studies conducting the association regarding *CYP2D6* genotype and ADRs and never reported the associated case. Furthermore, it is interesting to find a concentration of active metabolite (nortriptyline) but not its parent (amitriptyline) compound correlated significantly with adverse event and this observed correlation are probably relevant to dosage only

[1, 13, 22-24 and 26-27]. This may lead to non-significant association findings in this study since only low doses (10-25 mg/day) of amitriptyline were administered to patients and, hence, serious adverse reactions were not observed. One more concern, the tested substrates can have other elimination pathway. The main metabolism can shift to functional enzymes instead of the decreased functional one if they have more than one enzyme involved. This could be the reason why we scarcely see the pronounced effects neither adverseness nor responses in some drug treatments.

In conclusion, this matched case-control study of amitriptyline ADRs and *CYP2D6* genotypes association does not support the impact of *CYP2D6* genotype on the susceptibility to adverse reactions of amitriptyline at Odd ratio 2.5 in these 150 depressive patients. Non-significant association may result from none of poor metabolizer presented and low doses of drug challenging. If the Odd ratio is reduced together with larger sample size can be obtained, the significance of genotype and ADR association is possibly found. Moreover, amitriptyline can be metabolized also by CYP2C19 giving an active metabolite (nortriptyline) so that polymorphism of CYP2C19 might have an effect on elimination. Therefore, the study of CYP2C19 combine with *CYP2D6* genotype might increase to the significance of association. However, *CYP2D6**36-*10B associated-allele tend to be a factor contributing to higher metabolizing capacity than those posses no duplication. Nonetheless, this is the first study providing *CYP2D6* genotypes in depressive patients with some significance of genotype difference in non-ADR patients.

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APPENDICES

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Naranjo's algorithm

ประกอบด้วยคำถาม 10 ข้อ (เวลา 5 นาที)

โดยมีคะแนนกำกับไว้ ในกรณีตอบว่า "ใช่" "ไม่ใช่" หรือ "ไม่ทราบ" นำคะแนนที่ตอบได้มารวมกันแล้วจัดลำดับคะแนนดังนี้

คะแนน	ระดับการประเมิน
มากกว่า / เท่ากับ 9 -7 ขึ้นไป	ใช่แน่ (highly probable)
5-8	น่าจะใช่ (probable)
1-4	เป็นไปได้ (possible)
0	ไม่น่าใช่ (doubtful, unlikely)

Naranjo's Algorithm เพื่อการประเมิน การเกิดอาการไม่พึงประสงค์ (Adverse drug reactions : ADRs)

		Yes ใช่	No ไม่ใช่	Do not know ไม่ทราบ	Score คะแนนรวม
1.	Are there previous conclusive reports on this reaction? เคยมีการสรุปหรือรายงานปฏิกิริยานี้กับยาชนิดนี้มาแล้ว	+1	0	0	
2.	Did the adverse event appear after the suspected drug was administered? เกิดอาการไม่พึงประสงค์ขึ้นหลังจากได้รับยา	+2	-1	0	
3.	Did the adverse reaction improve when the drug was discontinued or a specific antagonist was administered? อาการไม่พึงประสงค์ดีขึ้นเมื่อหยุดยาที่สงสัย หรือเมื่อ ให้ยาต้านที่จำเพาะเจาะจง	+1	0	0	
4.	Did the adverse reaction reappear when the drug was readmistered?	+2	-1	0	

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รหัสโครงการ 109/2550
วันที่รับรอง 29 พ.ค. 2550

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	อาการไม่พึงประสงค์เกิดขึ้นอีกเมื่อเริ่มใช้ยาใหม่				
5.	Are there alternative causes (other than the drug) that could on their own have caused the reaction? ปฏิกิริยาที่เกิดขึ้นสามารถเกิดจากสาเหตุอื่นนอกเหนือจากยาที่สงสัย	-1	+2	0	
6.	Did the reaction reappear when a placebo was given? ปฏิกิริยาดังกล่าวเกิดขึ้นได้อีกเมื่อให้ยาหลอก	-1	+1	0	
7.	Was the drug detected in the blood (or other fluids) in concentrations known to be toxic? สามารถตรวจวัดระดับยาในเลือดหรือของเหลวอื่นได้ความเข้มข้นของยาในระดับที่เป็นพิษ	+1	0	0	
8.	Was the reaction more severe when the dose was increased, or less severe when the dose was decreased? ปฏิกิริยารุนแรงขึ้นเมื่อเพิ่มขนาดยาหรือลดลงเมื่อลดขนาดของยา	+1	0	0	
9.	Did the patient have a similar reaction to the same or similar drugs in any previous exposure? ผู้ป่วยเคยมีปฏิกิริยาคล้ายกันนี้มาแล้วเมื่อได้รับยาครั้งก่อน	+1	0	0	
10.	Was the adverse event confirmed by any objective evidence? อาการอันไม่พึงประสงค์ได้รับการยืนยันโดยมีผลปฏิบัติการหรือผลจากการตรวจสอบอื่น ๆ ยืนยัน	+1	0	0	

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รหัสโครงการ 109/2550
วันที่รับรอง 2 9 พ.ค. 2550

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แบบบันทึกข้อมูลผู้ป่วย (Patient profile) จำนวน 10 ข้อ (5 นาที)

1. ข้อมูลทั่วไปของผู้ป่วย (Demographic Data)

1.1 เพศ...........................(โปรดระบุ)

อายุ....................ปี ..... (โปรดระบุ)

น้ำหนัก...............กิโลกรัม.........ส่วนสูง............เซนติเมตร...... (โปรดระบุ)

ความดัน..............................มิลลิเมตรปรอท................(โปรดระบุ)

1.2 การศึกษาสูงสุด

- ☐ ต่ำกว่าหรือเท่ากับประถมศึกษา
- ☐ ปริญญาตรี
- ☐ มัธยมศึกษา
- ☐ ระดับปริญญาตรีขึ้นไป
- ☐ อนุปริญญา หรือ ปวส.
- ☐ ไม่ได้ศึกษา
- ☐ อื่นๆ(โปรดระบุ).....................

1.3 อาชีพ

- ☐ รับราชการ
- ☐ รัฐวิสาหกิจ
- ☐ ลูกจ้างเอกชน (ทุกระดับ)
- ☐ ค้าขาย
- ☐ เกษตรกรรม
- ☐ ไม่ประกอบอาชีพ
- ☐ อื่นๆ(โปรดระบุ)....... ....

1.4 สวัสดิการการเข้ารักษา

- ☐ ประกันสังคม
- ☐ บัตรประกันสุขภาพ
- ☐ ข้าราชการ หรือ พนักงานของรัฐ
- ☐ จ่ายเอง
- ☐ สวัสดิการอื่นๆ (โปรดระบุ)................................

1.5 โรคประจำตัว หรือโรคอื่นๆนอกเหนือจากโรคซึมเศร้า (โปรดระบุ)

1...............................

2...............................

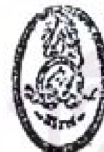
3...............................

1.6 ประวัติการแพ้ยา (โปรดระบุ)

1...............................

2...............................

3...............................



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1.7 การใช้ยาอื่นร่วมกับยา amitriptyline(โปรดระบุ)

1...............................

2...............................

3...............................

4...............................

5...............................

1.8 ความร่วมมือในการใช้ยา

- ☐ สม่ำเสมอ เช่น รับประทานยาครบตามแพทย์สั่ง
- ☐ ไม่สม่ำเสมอ เช่น ลืมทานยาในบางครั้ง
- ☐ ไม่ร่วมมือ เช่น ไม่รับประทานยาเลย

1.9 ความร่วมมือในการมาพบแพทย์

- ☐ สม่ำเสมอ เช่น มาพบแพทย์ตามวันนัด
- ☐ ไม่สม่ำเสมอ เช่น มาพบแพทย์ในบางครั้งคราว
- ☐ ไม่ร่วมมือ เช่น ไม่มาพบแพทย์อีกเลย

1.10 ผลการตรวจทางห้องปฏิบัติการ เช่น การทำงานของเอนไซม์ในตับ

- ☐ เกณฑ์ปกติ
- ☐ เกณฑ์ผิดปกติ
- ☐ ไม่ทราบ/ไม่มีข้อมูล

รับรองโดย คณะกรรมการจริยธรรมการวิจัยในคน
คณะแพทยศาสตร์ศิริราชพยาบาล
รหัสโครงการ 109/2550
วันที่รับรอง 2 9 พ.ค. 2550

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

แบบสอบถามอาการไม่พึงประสงค์จากการใช้ยา amitriptyline จำนวน 20 ข้อ (10 นาที)

คำสั่ง ผู้ถูกวิจัยจะต้องกากบาททับในแบบวัด (scale) ที่กำหนด หลังจากถามคำถามในแต่ละข้อ

ข้อ	แบบวัดผล (scale) การเกิดอาการไม่พึงประสงค์
1. ตาพร่ามองไม่ชัด	0 ———|——— 10
2. ปากแห้ง, คอแห้ง	0 ———|——— 10
3. แสบยอดอก	0 ———|——— 10
4. ปัสสาวะคั่ง (ไม่ออก, ลำบาก)	0 ———|——— 10
5. ท้องผูก	0 ———|——— 10
6. การทรงตัว, เคลื่อนไหวผิดปกติ	0 ———|——— 10
7. อาการสั่นผิดปกติ	0 ———|——— 10
8. ภาวะมึนงง	0 ———|——— 10
9. ภาวะอ่อนเพลีย, อ่อนแรง	0 ———|——— 10
10.หัวใจเต้นเร็วผิดจังหวะ	0 ———|——— 10
11.ความดันต่ำเมื่อเปลี่ยนอิริยาบท	0 ———|——— 10
12.คลื่นไส้, อาเจียน	0 ———|——— 10
13.เบื่ออาหาร	0 ———|——— 10

รับรองโดย คณะกรรมการจริยธรรมการวิจัยในคน
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14.ความดันโลหิตต่ำ/สูงจากปกติ	0 ——————————— 10
15.การรับรสเปลี่ยนไป	0 ——————————— 10
16.เหงื่อออกมากกว่าปกติ	0 ——————————— 10
17.มีผื่นแดงที่ผิวหนัง, ไวต่อแสงมากกว่าปกติ	0 ——————————— 10
18.นอนไม่หลับ	0 ——————————— 10
19.ปวดศีรษะ	0 ——————————— 10
20.อื่นๆ	0 ——————————— 0

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Haplotype	Controls	Cases	Combined
0000000	0.3085	0.3194	0.3142
1111011	0.2660	0.2705	0.2676
0101101	0.1064	0.0591	0.0823
1111001	0.0851	0.1183	0.1031
1101101	0.0638	0.0200	0.0412
0000001	0.0532	0.0503	0.0518
0001000	0.0319	0.0000	0.0155
0001011	0.0106	0.0000	0.0103
0011000	0.0106	0.0000	0.0052
0100000	0.0106	0.0303	0.0206
0111101	0.0106	0.0100	0.0103
1000000	0.0106	0.0000	0.0050
1000001	0.0106	0.0000	0.0053
1111000	0.0106	0.0000	0.0052
1111111	0.0106	0.0190	0.0156
0000100	0.0000	0.0000	0.0000

Tolerance 0.000001 Iterations 2000

Assumptions

▾ Hardy-Weinberg equilibrium

Samples

☑ Cases

☑ Controls

☑ Combined

Calculate

Analyted Genotype 16Sep08 Final p

Subjects:	88/97
Cases:	41/50
Controls:	47
SNPs:	7
Covariates:	-3

```
Dominant effects under Hardy-Weinberg equilibrium
Log-Likelihood  Iterations  Status
-319.2941       7           Success.

Haplotype frequencies under Hardy-Weinberg equilibrium
Sample   Log-Likelihood  Iterations  Status
Cases    -174.7739       106         Success.
```

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	Estimate	Standard Error	Z-Statistic	P-Value
Effects				
Environment				
Age	0.0262	0.0302	0.8667	0.3861
Dose	0.0032	0.0133	0.2439	0.8073
SumADR	0.0438	0.0107	4.0805	0.0000
Gene				
0000000	2.2493	1.7630	1.2759	0.2020
0000000*Age	-0.0438	0.0314	-1.3927	0.1637
0000000*Dose	-0.0223	0.0156	-1.4301	0.1527
0000000*SumADR	0.0021	0.0030	0.6967	0.4860
Frequencies				
0000000	0.3671	0.0462	7.9508	0.0000
0000001	0.0541	0.0168	3.2146	0.0013
0001000	0.0162	0.0093	1.7405	0.0818
0001011	0.0054	0.0054	1.0016	0.3165
0011000	0.0054	0.0054	1.0016	0.3165
0100000	0.0216	0.0107	2.0130	0.0441
0101101	0.0866	0.0211	4.1074	0.0000
0111101	0.0108	0.0076	1.4188	0.1560
1000000	0.0054	0.0054	1.0016	0.3165
1000001	0.0054	0.0054	1.0016	0.3165
1101101	0.0433	0.0151	2.8657	0.0042
1111000	0.0054	0.0054	1.0016	0.3165
1111001	0.1028	0.0228	4.4990	0.0000
1111011	0.2543	0.0342	7.4432	0.0000

Tolerance 0.0001 Iterations 500

Assumptions

- Dominant model
- Hardy-Weinberg equil

Effects

Environment
- Age
- Dose
- SumADR

Gene
- 0000000
- 0000000*Age
- 0000000*Dose
- 0000000*SumADR

Calculate

Analyted Genotype 16Sep08 Final p

Subjects:	88/97
Cases:	41/50
Controls:	47
SNPs:	7
Covariates:	3

Haplotype frequencies under Hardy-Weinberg equilibrium

Sample	Log-Likelihood	Iterations	Status
Controls	-162.2380	58	Success.

Dominant effects under Hardy-Weinberg equilibrium

Log-Likelihood	Iterations	Status
-319.2941	7	Success.

eady.

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Siriraj Ethics Committee

Certificate of Approval COA no.Si 175/2007

Protocol Title : THE RELATIONSHIP BETWEEN AMITRIPTYLINE ADVERSE REACTIONS AND GENETIC POLYMORPHISM OF CYP2D6 IN THAI PSYCHIATRIC PATIENTS AT SIRIRAJ HOSPITAL.

SiEC number : 109/2550

Principal Investigator/Affiliation : Miss. Pranee Suechareon / Pharmaceutical Department Chulalongkorn University

Research site : Faculty of Medicine Siriraj Hospital

Approval includes :

1. EC Submission form
2. Participant information sheet
3. Informed consent form
4. Questionnaire

Approval date : May 29, 2007

Expired date : May 28, 2008

This is to certify that Siriraj Ethics Committee is in full Compliance with International Guidelines For Human Research Protection such as Declaration of Helsinki, The Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Prof. Shusee Visalyaputra, M.D.
Vice- chairperson for Chair Person Prof. Sumalee Nimmannit, M.D.

June 1, 2007
date

(Clin. Prof. Piyasakol Sakolsatayadorn)
Dean of Faculty of Medicine Siriraj Hospital

June 1,2007
date

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Curriculum vitae

Miss Pranee Suecharoen was born in Karnjanaburi on October 1, 1981, Thailand. She spent and learnt her childhood in Khonkaen. She graduated with Bachelor Degree in Pharmaceutical Sciences in 2005 from Faculty of Pharmaceutical Sciences, Rangsit University. She had been enrolled in the Master Degree Program of Pharmaceutical Sciences, Department of Pharmacology, Chulalongkorn University in the year 2005.

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