EFFECTS OF *CYP2D6* AND *UGT2B7* POLYMORPHISMS ON TAMOXIFEN PHARMACOKINETICS AND TREATMENT OUTCOMES IN THAI BREAST CANCER PATIENTS

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ผลของภาวะพหุสัณฐานของยีน *CYP2D6* และ *UGT2B7* ต่อเภสัชจลนศาสตร์และผลการรักษาของยาทาม็อกซิเฟนในผู้ป่วยมะเร็งเต้านมชาวไทย

นางสาวณัฏฐดา อารีเปี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
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Thesis Title	EFFECTS OF *CYP2D6* AND *UGT2B7* POLYMORPHISMS ON TAMOXIFEN PHARMACOKINETICS AND TREATMENT OUTCOMES IN THAI BREAST CANCER PATIENTS
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ณัฏฐดา อารีเปี่ยม: ผลของภาวะพหุสัณฐานของยีน *CYP2D6* และ *UGT2B7* ต่อเภสัชจลนศาสตร์และผลการรักษาของยาทาม็อกซิเฟนในผู้ป่วยมะเร็งเต้านมชาวไทย. (EFFECTS OF *CYP2D6* AND *UGT2B7* POLYMORPHISMS ON TAMOXIFEN PHARMACOKINETICS AND TREATMENT OUTCOMES IN THAI BREAST CANCER PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ดวงจิตต์ พนมวัน ณ อยุธยา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.นพ.นรินทร์ วรวุฒิ, 83 หน้า.

ทาม็อกซิเฟนเป็นยาต้านฮอร์โมนเอสโตรเจนที่ใช้สำหรับการรักษาเสริมในผู้ป่วยมะเร็งเต้านมที่มีตัวรับเอสโตรเจนเป็นบวก ทาม็อกซิเฟนถูกเปลี่ยนแปลงเป็นเอนด็อกซิเฟนซึ่งเป็นเมแทบอไลต์ที่ออกฤทธิ์จับกับตัวรับเอสโตรเจนได้ดีกว่าทาม็อกซิเฟนประมาณ 100 เท่าโดยเอนไซม์ CYP2D6 และถูกกำจัดออกจากร่างกายผ่านกระบวนการ glucuronidation โดยกลุ่มเอนไซม์ UGT ซึ่งมี UGT2B7 เป็นหนึ่งในเอนไซม์ที่ทำหน้าที่นี้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของภาวะพหุสัณฐานของยีนที่ควบคุมการทำงานของเอนไซม์ CYP2D6 และ UGT2B7 ต่อเภสัชจลนศาสตร์ของยาทาม็อกซิเฟน โดยพิจารณาจากระดับยาทาม็อกซิเฟนและเอนด็อกซิเฟน มีผู้ป่วยหญิงที่เป็นมะเร็งเต้านมเข้าร่วมการศึกษาทั้งหมด 59 ราย ผู้ป่วยมีอายุเฉลี่ย 50±9.3 ปี ร้อยละ 76 อยู่ในช่วงยังไม่หมดประจำเดือนและร้อยละ85 เป็นมะเร็งเต้านมที่มีตัวรับเอสโตรเจนเป็นบวก

พบภาวะพหุสัณฐานของยีน *CYP2D6* ชนิด **10* และยีน *UGT2B7* ชนิด **2* ในสัดส่วน 0.53 และ 0.28 ตามลำดับ ผู้ป่วยที่มียีน *CYP2D6 *10/*10* มีระดับเอนด็อกซิเฟนต่ำกว่าเมื่อเปรียบเทียบกับผู้ป่วยที่มียีน *CYP2D6* แบบ **1/*10* และ **1/*1* (14.7±14.7, 17.9±9.8 และ 22.4±12.8 นาโนกรัม/มิลลิลิตร ตามลำดับ, p = 0.045) ภาวะพหุสัณฐานของยีน *UGT2B7* ไม่ส่งผลต่อความแตกต่างของระดับเอนด็อกซิเฟน แต่เมื่อพิจารณาเฉพาะในผู้ป่วยที่มีภาวะพหุสัณฐานของยีนชนิด *CYP2D6 *10/*10* (จำนวน 20 ราย) ผู้ป่วยที่ภาวะพหุสัณฐานของยีน *UGT2B7 *2/*2* มีแนวโน้มจะมีระดับเอนด็อกซิเฟนสูงกว่าผู้ป่วยที่มียีน *UGT2B7 *1/*1* และ *UGT2B7 *1/*2* (27.2±7.2 ng/ml, 9.03±4.9 และ 15.6±19.7 ng/ml, ตามลำดับ, p= 0.073)
ผลการรักษาของยา
ทาม็อกซิเฟนอาจสัมพันธ์กับระดับเอนด็อกซิเฟน ผู้ป่วยที่มีระดับเอนด็อกซิเฟนต่ำกว่า 15.3 นาโนกรัม/มิลลิลิตร มีผลตรวจแมมโมแกรม BI-RADS ระดับ 3 ขึ้นในสัดส่วนสูงกว่าผู้ป่วยที่มีระดับเอนด็อกซิเฟนสูงกว่า (ร้อยละ 41.2 และ ร้อยละ 19) และพบเหตุการณ์ไม่พึงประสงค์ในผู้ป่วยที่มีระดับเอนด็อกซิเฟนสูงกว่า 15.3 นาโนกรัม/มิลลิลิตรบ่อยกว่าในผู้ป่วยที่มีระดับเอนด็อกซิเฟนต่ำกว่า (ร้อยละ 26.7 และ ร้อยละ 3.4, OR 10.18, p= 0.034)

โดยสรุป การศึกษานี้ยืนยันผลของภาวะพหุสัณฐานของยีน *CYP2D6* และ *UGT2B7* ที่มีต่อเภสัชจลนศาสตร์ของยาทาม็อกซิเฟน และพบว่าระดับเอนด็อกซิเฟนอาจสัมพันธ์กับผลการรักษาและสัมพันธ์กับเหตุการณ์ไม่พึงประสงค์จากการรักษาด้วยยานี้ในผู้ป่วยมะเร็งเต้านมชาวไทย

ภาควิชา เภสัชกรรมปฏิบัติ ลายมือชื่อนิสิต.....
สาขาวิชา การบริบาลทางเภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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NUTTHADA AREEPIUM: EFFECTS OF *CYP2D6* AND *UGT2B7* POLYMORPHISMS ON TAMOXIFEN PHARMACOKINETICS AND TREATMENT OUTCOMES IN THAI BREAST CANCER PATIENTS. ADVISOR: ASSOC. PROF. DUANGCHIT PANOMVANA NA AYUDHYA, Ph.D., CO-ADVISOR: ASSOC. PROF. NARIN VORAVUD, M.D., 83 pp.

Antiestrogen tamoxifen (TAM) is used as adjuvant treatment in estrogen receptor positive breast cancer. TAM is a prodrug which is converted to endoxifen (END), an active metabolite with approximately 100 times higher affinity with estrogen receptor than TAM, by CYP2D6 then is excreted via glucuronidation by UGT2B7, one of the enzymes in UGTs family. The objectives of this study were to evaluate the impacts of enzyme polymorphisms on TAM pharmacokinetics using TAM and END plasma concentrations. Fifty-nine female breast cancer patients were included in the study. Average age was 50±9.3 years old, 76% of them were premenopausal and 85% had estrogen receptor positive breast cancer.

Allele frequency of *CYP2D6*10* and *UGT2B7*2* were 0.53 and 0.28, respectively. Patients with *CYP2D6*10/*10* had lower END concentrations compare to *CYP2D26*1/*10* and *CYP2D6*1/*1* (14.7±14.7 vs 17.9±9.8 and 22.4±12.8 ng/ml, respectively, p = 0.045). Polymorphisms of *UGT2B7* alone did not have any impact on TAM metabolism, however, among patients with *CYP2D6 *10/*10* (n=20), one with *UGT2B7*2/*2* tended to have higher END concentrations compared to patients with *UGT2B7*1/*1* and *UGT2B7*1/*2* (27.2±7.2 ng/ml vs 9.03±4.9 and15.6±19.7 ng/ml, respectively, p= 0.073). Regarding treatment outcomes, low END concentrations that might be related to worse results shown in mammography screening. In patients with END concentration less than 15.3 ng/ml, the percentage of mammogram result as BI-RADS≥3 was higher when compared to patients with higher END concentrations (41.2 % vs 19%). Adverse events were also found more frequent in patients with higher END concentration (26.7% vs 3.4%, OR 10.18, p = 0.034).

In summary, this study confirmed impacts of *CYP2D6* and *UGT2B7* polymorphisms on pharmacokinetics of TAM. END concentrations tended to be related to treatment outcomes of Thai breast cancer patients.

Department : Pharmacy Practice Student's Signature .....

Field of Study : Pharmaceutical Care Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AEs	=	adverse events
AIs	=	Aromatase inhibitors
BC	=	Breast cancer
BI-RADs	=	Breast imaging reporting and data system
CYP	=	Cytochrome P450
END	=	endoxifen
ER	=	estrogen receptor
HPLC	=	High-pressure liquid chromatography
NDMT	=	N-desmethytamoxifen
TAM	=	Tamoxifen
UGT	=	UDP-glycoronosyl transferase

CHAPTER I

INTRODUCTION

1.1 Rational and Background

Breast cancer (BC) is the most common cancer among females and the leading cause of cancer related death. [1] About two-third of patients with BC is classified as estrogen receptor (ER) positive of which their tumor growths are stimulated by estrogen. Adjuvant hormonal therapy reduces almost half of BC recurrence. In postmenopausal BC patients, Aromatase inhibitors (AIs) are preferably option, while in pre- and peri-menopausal patients which their ovary still function in estrogen production, Tamoxifen (TAM) is almost only one choice. [2]

TAM is one agent among antiestrogens which has been used for the past 3 decades. According to the literature, it saves over half a million of BC patients' lives worldwide, when given as 5 years-adjuvant hormonal therapy after completing BC treatment. TAM works by interfering with estrogen binding at the ER and consequently exerts estrogen antagonistic effect in breast tissue, while in other organs for instance, bone, uterus, pituitary and liver, its interaction with the ER produces estrogen agonistic effects. [2-4]

TAM is a prodrug that is required metabolism to be active metabolites, of which 4-hydroxy-*N*-desmethyltamoxifen or endoxifen (END) is the most potent one. The biotransformation of TAM to END is processed through cytochrome P450 (CYP450) enzymes. CYP3A4 and CYP3A5 are considered the most important enzyme for the demethylation, while CYP2D6 is the most important enzyme for the hydroxylation reactions. Sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs) are

important for increasing the solubility and facilitating the excretion of TAM and it metabolites. [2-4]

There are high inter-individual variation among patients receiving TAM treatment and result in 30% to 50% BC recurrence after adjuvant hormonal treatment. Multiple factors are contributed to TAM treatment failure, and one of important factor is the polymorphisms in drug metabolizing enzymes responsible for TAM metabolism. The *CYP2D6* gene is a highly polymorphic gene. More than 60 functional variants currently identified resulted in abolished, decreased, normal and ultrarapid enzyme activities. *CYP2D6*4* and **5* are the most important null alleles, while *CYP2D6*10, *17* and **41* are most common for severely reduced enzyme activity. Ultrarapid enzyme activity caused by duplication or multiduplications of active *CYP2D6*1* (wild type) gene (*CYP2D6*1* x N, $N \geq 2$). [5-10]

There are differences in prevalence of variant alleles among ethnic. Caucasians are likely to contain more non-functional *CYP2D6 *4* and *CYP2D6 *5* alleles (12-21% vs < 5% in Asians), while prevalence of reduced function *CYP2D6*10* alleles is much higher among Asians (up to 70%). [8, 10]

Results of these genotypes impacted on enzyme activity and classified into 4 groups as poor metabolizer (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizers (UM). PM contains homozygous null alleles of *CYP2D6* gene, while IM contains heterozygous null alleles or homozygous reduced function alleles of *CYP2D6* gene. UM contains duplicate or multiple copies of wild type alleles. [7, 8, 10]

Several studies have reported the association between *CYP2D6* genotype and clinical outcomes in women treated with adjuvant TAM. Evidence from two studies suggested that women with CYP2D6 PM or IM who were treated with TAM had a significantly shorter time to recurrence and recurrence-free survival (but not overall

survival) as compared to EMs. [11-13] Borges' group reported an updated analysis of their prospective TAM pharmacology cohort by assessing the combined effect of 33 different *CYP2D6* alleles on the plasma concentrations of TAM and its metabolites. They found that patients with heterozygous reduced function (*e.g.*, *CYP2D6 *10*) and null function (*e.g., CYP2D6 *4*) allele (*i.e.*, individuals typically classified as IM) had similar END concentrations as compared to PM. This indicated the importance of comprehensive *CYP2D6* genotypes which accounted for the variability in END plasma concentrations. [14]

TAM and its metabolites including END are eliminated by glucuronidation. It has been suggested that glucuronidation within target tissues, like the adipose tissue of the breast, may also be important in terms of TAM metabolism and the overall TAM activity. *N*-glucuronidation occurs for TAM and 4-hydroxytamoxifen (4-OH-TAM), whereas *O*-glucuronidation occurs for 4-OH-TAM and END. [15,16] *In vitro* studies have shown that the hepatic UGT1A4 is the only active enzyme responsible for the *N*-glucuronidation of TAM and 4-OH-TAM, whereas UGT2B7 and, to a lesser extent, UGT1A1 are the major hepatic enzymes involved in the *O*-glucuronidation of the *trans* isomers of 4-OH-TAM and END. [17] From *in vitro* study, active hepatic UGTs, the *UGT2B7*^{268Tyr} *(*2)* variant exhibited significant 2- and 5-fold decreased in activity against the *trans* isomers of 4-OH-TAM and END, respectively. In studies of 111 human liver microsomal specimens, the rate of *O*-glucuronidation against *trans* 4-OH-TAM and *trans*-END was 28% and 27% lower, respectively, in individuals homozygous for the *UGT2B7 Tyr*^{268}*Tyr (*2/*2)* genotype compared with subjects with the *UGT2B7His*^{268}*His (*1/*1)* genotype, with a significant trend of decreasing activity against both substrates with increasing numbers of the *UGT2B7*^{268His} *(*2)* allele. [17,18] These results suggested that functional polymorphisms in TAM metabolizing *UGTs*, including *UGT2B7* and potentially *UGT1A8*, may be important in inter-individual variability in TAM metabolism and responsible for TAM therapy.

Ethnic differences also found in polymorphisms of *UGT2B7 C802T (His^{268}Tyr)*. [18] Results from study of 91 Australians demonstrated that 25% proportion of **2/*2* genotypes, whereas Japanese study demonstrated that only 5% of *UGT2B7 *2/*2* were found. [21] These differences might have some implication on TAM and its metabolites' concentrations, especially the active one, END.

In Thai, most frequent variant was *CYP2D6 *10* (approximately 60-70%) which was not different from other reports on Asian population. [19,20] Patients with homozygous *CYP2D6 *10/*10* or IM may have lower TAM and END concentrations. However, prevalence of *UGT2B7 *2* which is reduced enzyme activity are not known since the study of *UGT2B7* polymorphisms in Thai population is not available. Moreover, there is no study on the impact of *UGT2B7* polymorphisms on TAM metabolism in Thai BC patients. Our study is the first investigation that intended to evaluate effects of different genotypes and phenotypes polymorphisms of *CYP2D6 and UGT2B7* on pharmacokinetics of TAM and treatment outcomes of Thai BC patients.

1.2 Hypothesis

Polymorphisms of *CYP2D6* and/or *UGT2B7* may influence the metabolisms and pharmacokinetics of TAM and its metabolites. The effects of these polymorphisms could be detected by differences in the concentrations of TAM and its metabolites in patients' plasma. The concentrations of TAM and its metabolites of patients with polymorphic genes would be different from wild type genotypic patients.

1.3 Objectives

1.3.1 To compare pharmacokinetics of TAM in patients with different genotypes and phenotypes of *CYP2D6 (*1,*10)*.

1.3.2 To compare pharmacokinetics of TAM in patients with different genotypes and phenotypes of *UGT2B7 (*1,*2)*.

1.3.3 To investigate the influence of *CYP2D6* and *UGT2B7* polymorphisms in treatment outcomes (efficacy and safety) of TAM in Thai BC patients.

1.4 Expected Outcomes

This study should provide information on the effects of *CYP2D6* and *UGT2B7* polymorphisms on pharmacokinetics of TAM along with their influences on the treatment outcomes in Thai BC patients. Information abstracted from the results of this study may potentially lead to more appropriate treatment selection in patients with BC in order to provide good clinical outcomes.

CHAPTER II

LITERATUERE REVIEWS

2.1 Breast cancer: disease and treatment overview

Cancer of the breast is the most common tumor found in females. About a million new patient cases were identified worldwide.[1] Numerous studies addressing BC in various aspects, particularly in cancer molecular biology along with the history of this disease. Insights from those studies have improved understanding in tumorgenesis and lead to the creation of many safe and effective treatment options that extend patients survival. Despite survival rate of BC patients is relatively high compare to that of other cancers, approximately 60% of patients survive longer than 5 years without disease recurrence, while about 40% have refractory diseases with limited options to control (*e.g.*, triple negative BC) resulting in poor prognosis.[22]

Treatment of BC patients was planned according to the prognostic and predictive factors. Several of prognostic factors (*e.g.*, tumor size, number of node involvement, histological subtype and grade, ER and/or Progesterone receptor (PR) status , extent of proliferative factor Ki-67, HER-2/*neu* amplification) along with patient's characteristics (*e.g.*, age, number and type of underlying disease) as well as patient's preferences are utilized to design the combination of treatment for each individual.[23]

Tumor size, lymph node involvement and/or the presence of distant metastases (or TNM) are indicative for BC staging. T is the size of tumor that can be categorized as T1 (< 2 cm), T2 (2-5 cm), T3 (> 5 cm) and T4 which indicated tumor spreading to chest wall (4a), skin (4b), and both chest wall and skin (4c). Patients are classified as T4d if they have inflammatory carcinoma which described as red, swollen and painful lesions

while touching tumor. N is classified as N0 to N3, N0 is defined as no cancer cell found in nearby nodes while in N1, cancer cells are found in the upper level of axillary lymph node. In N2, cancer cells invade into surrounding tissues of axillary lymph node. N3 is described as cancer cells are present in lymph node below or above the collarbone or in lymph node under breast bone. TNM system can be used to categorize BC cancer into stage I to IV. Early BC is found in patients with stage 0 (carcinoma *in situ*) to stage II (presence of cancer cells in lymph node), while advanced BC can start from stage III (larger tumor with extensive node involvement) to stage IV where there is a presence of distant metastases (M1). Five-year survival rates are highly correlated with tumor stage as following, 99-100% for stage 0, 95-100% for stage I, 86% for stage II, 57% for stage III, and less than 20% for stage IV. This prognostic information can be used to guide physicians in selecting therapeutic options.[24]

The local treatment for BC consists of surgery and radiation, while chemotherapy, hormonal therapy, and targeted therapy are considered as systemic treatment to eradicate cancer cells that already micrometastases. Whether hormonal and/or targeted therapies are given to patients is based on pathological information. In case of ER and/or PR positive cancer, hormonal therapy should be given to patient to improve disease free survival (DFS) and overall survival (OS) by preventing disease recurrence. Anti-HER-2 targeted therapy also should be added if patients have overexpression of HER-2/*neu* receptor.

Two-third of BC patients expressed ER positive status. These patients are the candidates for hormonal therapy. There are two pharmacological classes of hormonal treatment naming antiestrogens and aromatase inhibitors (AIs). Antiestrogens are the only option for pre- and peri-menopausal patients since their ovary still produce estrogen while post-menopausal patients can use either antiestrogens or AIs. Later clinical evidences strongly support the use of AIs in post-menopausal patients over antiestrogens as it can lower recurrence rate and improve survival. There are some

different in toxicities profile between antiestrogen and AIs that influence the decision to use in BC patients. Patients used antiestrogens are at increased risk of endometrial hyperplasia, ovarian cancer and venous thromboembolism, while patients on AIs are at risk of fracture from osteoporosis. Other toxicities from hormonal therapy such as hot flash and vaginal bleeding or spotting from anti-estrogen and arthralgia from AIs are also found but otherwise well tolerated. In most cases, 5 years treatment duration can be accomplished without compromising patients' quality of life. [25-27]

2.2 Tamoxifen; pharmacology, pharmacokinetic and clinical evidences

Currently, oral antiestrogen which is approved for BC treatment is solely limited to TAM.[27] TAM or Tamoxifen citrate is approved for metastatic breast cancer and adjuvant therapy following surgery, radiation, and/or anthracycline-based chemotherapy in both pre- and post-menopausal women with ER-positive disease.

TAM is a nonsteroidal triphenylethylene derivative that binds to the estrogen receptor. It has both estrogenic and antiestrogenic actions, depending on the specific target tissues. It is a strongly antiestrogenic on mammary epithelium, therefore it is used in both the prevention and treatment of breast cancer. TAM was originally screened in a drug development program toward discovering new contraceptive agents. Although it was effective in rats as contraceptive but it was not useful in women. It was not until the early 1970s when it was shown to be clinically useful for palliation of advanced breast cancer. Subsequent animal studies performed in rats induced with dimethylbenzanthracene (DMBA) and nitrosomethylurea as carcinogens, showed that TAM was highly effective in preventing the development of breast cancer. These results had also been validated in the cancer induced mouse model in which murine mammary tumor virus was utilized as carcinogen. [28-29]

The mechanism of action of TAM is somewhat complex. Theoretically, its principal mechanism of action is mediated by its binding to the estrogen receptor and blocking of the proliferation of mammary epithelium. A proposed mechanism for this antiproliferative action is the synthesis of the cytokine transforming growth factor-β (TGF-β), which acts as a negative autocrine regulatory molecule. Furthermore, it has also been shown that TAM can induce synthesis of TGF-β in estrogen receptor-negative cells, such as fetal fibroblasts. In addition, immunohistochemical studies have shown that TAM induces the synthesis of TGF-β in the stromal (mesenchymal) compartment of breast cancers, suggesting a paracrine as well as autocrine mechanism of action, independent of an interaction with the estrogen receptor. [30] Other studies in accord with these observations are the findings that TAM can lower the circulating levels of insulin-like growth factor I (IGF-I) in breast cancer patients. IGF-I is a potent mitogen for breast cancer cells and may act through endocrine, paracrine, and autocrine routes to stimulate the tumor growth. [31]

Following a single oral dose of 20 mg TAM, an average peak plasma concentration of 40 ng/mL (range from 35 to 45 ng/mL) occurred approximately 5 hours after administered. The decline in plasma concentrations of TAM is biphasic with a terminal elimination half-life of about 5 to 7 days. The average peak plasma concentration of N-desmethyl TAM (NDMT) is 15 ng/mL (range from 10 to 20 ng/mL). The average steady-state plasma concentrations of TAM and NDMT after administration of 20 mg TAM once daily for 3 months are 122 ng/mL (range from 71 to 183 ng/mL) and 353 ng/mL (range from 152 to 706 ng/mL), respectively. After initiation of therapy, steady state concentrations for TAM are achieved in approximately 4 weeks and steady-state concentrations for NDMT are achieved in about 8 weeks, suggesting a half-life of approximately 14 days for this metabolite. [32]

TAM is extensively metabolized by liver after oral administration. NDMT is the major metabolite found in plasma. The biological activity of NDMT appears to be similar

to that of TAM. 4-HydroxyTAM (4-OH-TAM) and a side chain primary alcohol derivative of TAM including 4-OH-NDMT or END have been identified as minor metabolites in plasma but exert 100 times more potency compare to that of their parent drug. TAM is a substrate of CYP3A, CYP2C9 and CYP2D6, and is an inhibitor of P-glycoprotein. [32]

Studies in women receiving 20 mg of ^{14}C-TAM have shown that approximately 65% of the administered dose was excreted and eliminated over a period of 2 weeks in feces as the primary route of elimination. The drug is excreted mainly as polar conjugates, unchanged drug and unconjugated metabolites for less than 30% of the total fecal linked radioactivity. [32]

TAM undergoes extensive phase I and phase II metabolisms in the human hepatic as shown in figure 1. The bioconversion of tamoxifen involves N-oxidation, N-demethylation, and hydroxylation. Formation of the major metabolite NDMT is primarily catalyzed by CYP3A4 and CYP3A5, with minor contributions by CYP2D6, CYP1A1, CYP1A2, CYP2C19, and CYP2B. NDMT is subjected to hydroxylation to produce the major clinically active metabolite END. The conversion of NDMT to END is catalyzed almost exclusively by CYP2D6. [3]

Another important active metabolite is 4-OH-TAM, which is catalyzed by a number of CYPs, including CYP2D6, CYP3A4, CYP2C9, CYP2B6, and CYP2C19. Compared with END, the steady-state concentrations of 4-OH-TAM are lower, ranging from 1.15 ng/ml to 6.4 ng/ml. With the exception of END and 4-OH-TAM, no other highly active metabolites have been described thus far. [3]

TAM and its metabolites are eliminated and detoxified by sulfation and glucuronidation. TAM is excreted predominantly through the bile primarily by conjugation to glucuronic acid. Most of the 4-OH-TAM found in the bile of TAM-treated

patients as a glucuronide conjugate. TAM glucuronides have also been identified in the urine and serum of TAM-treated patients. It has been suggested that glucuronidation within target tissues like the adipose tissue of the breast may also be important in terms of TAM metabolism and overall TAM activity. N-glucuronidation occurs for both TAM and 4-OH-TAM, whereas O-glucuronidation occurs for 4-OH-TAM and END. *In vitro* studies have shown that the hepatic UGT1A4 is the only active enzyme responsible for the N-glucuronidation of TAM and 4-OH-TAM, whereas UGT2B7 and, to a lesser extent, UGT1A1 are the major hepatic enzymes involved in the O-glucuronidation of the *trans* isomers of 4-OH-TAM and END. UGT2B7 exhibited higher levels of activity against the *trans* isomers of 4-OH-TAM and END. The extrahepatic UGTs, UGT 1A10 and UGT1A8 are expressed in target tissues, including breast, and were also shown to be highly active against isomers of 4-OH-TAM and END *in vitro*.[33]

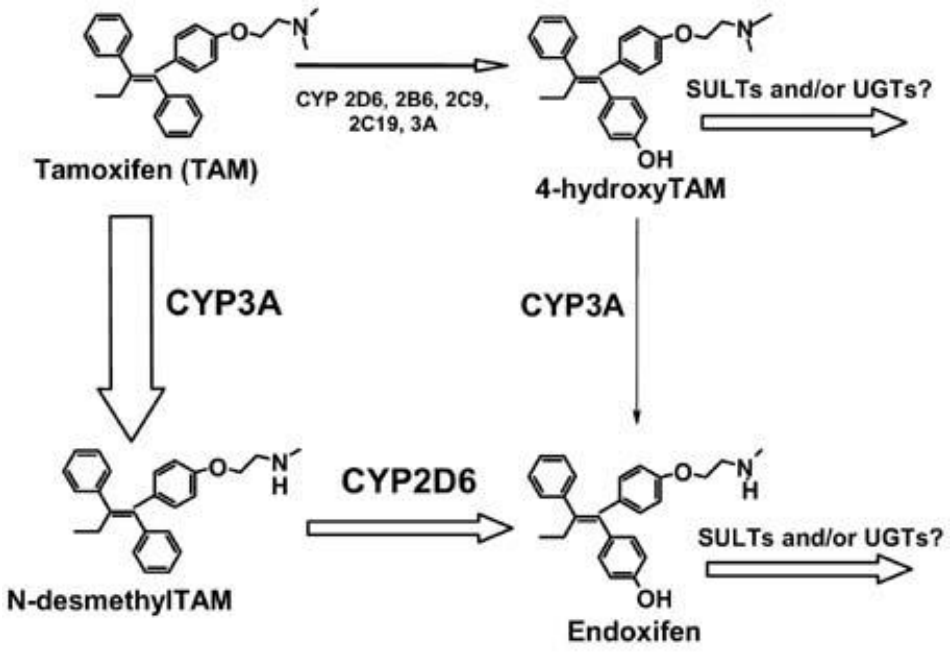

Figure 1. Tamoxifen metabolism pathway [34]

There are many clinical trials supported the use of TAM. Results from The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) study showed benefit of adjuvant TAM treatment in the 20 mg per day dose for 5 years in women with ER positive or unknown BC receiving 1 year or less, 2 years or about 5 years of TAM, the proportional reductions in mortality were 12%, 17%, and 26%, respectively (two-sided significance [2p] < 0.003). The corresponding reductions in BC recurrence were 21%, 29% and 47% (two-sided significance [2p] < 0.00001).[35] These results were similar to other prospective studies (ECOG-1178, NATO) [36-37] using TAM adjuvantly as a single agent demonstrated an improved DFS following total mastectomy and axillary dissection for postmenopausal women with positive axillary nodes compared to placebo/no treatment controls. The NATO study also demonstrated an overall survival benefit. [37] In node negative BC setting, NSABP B-14, a prospective, double-blind, randomized study, compared TAM with placebo in women with axillary node-negative, ER positive ($\geq$ 10 fmol/mg cytosol protein) BC (as adjuvant therapy, following total mastectomy and axillary dissection, or segmental resection, axillary dissection, and breast radiation). After five years of treatment, there was a significant improvement in DSF in women receiving TAM. This benefit was apparent both in women under age 50 and in women at or beyond age 50.[38]

Clinical evidences have been demonstrated benefit of using TAM outweigh the risk for TAM treatment in prevention of relapse and recurrence of BC in various types of patients. In high risk to develop BC, TAM is also approved for use as chemoprevention. [39] When taken all data together, it can be roughly concluded that TAM can prevent recurrence or development of BC for about 50%. Other up to 50% TAM treatment failure was consolidated from various factors. Even in ER positive patients, estrogen is not the only stimulating or aggregating factor for BC development or recurrence. An important factor should be taken to consider for individual not responding to TAM treatment is the polymorphisms of drug metabolizing enzymes responsible for TAM metabolism. Advances in pharmacogenomics and molecular biology help us to understand

interindividual variation of enzyme activities which could impact both level of TAM and its metabolites as well as relationship of polymorphisms of enzymes and TAM treatment associated outcome.

2.3 *CYP2D6* Polymorphisms and the impact on tamoxifen therapy

The CYP2D6 enzyme is an important phase I drug enzyme involved in the metabolism of up to 25% of all drugs. More than 48 different drug substrates for this enzyme have been identified, include drugs from the following classes: beta-blockers, antidepressants, antiarrhythmics and antidepressants. [10]

The *CYP2D6* gene is highly polymorphic with more than 100 major alleles known currently.[3] These are associated with increased, decreased, or abolished function of the final gene product. The *CYP2D6* phenotypes associated with these different alleles include poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultrarapid metabolizers (UM). Some of the most common and important variant alleles distributed in different ethnic groups are listed in table 1.

Table 1 Major *CYP2D6* alleles, effect on enzyme metabolism, and allele frequencies in selected populations [10, 48]

Major variant alleles	Consequence in enzyme activity	Allele frequencies (%)			
		Caucasians	Black African	Asians	Thai
*CYP2D6*2xn*	Increased	1-5	2	0-2	3.6
*CYP2D6*4*	Inactive	12-21	2	1	1.8
*CYP2D6*5*	None	27	4	6	5.4
*CYP2D6*10*	Decreased	1-2	6	51	37.8
*CYP2D6*17*	Decreased	0	20-35	0	0.01
*CYP2D6*41*	Decreased	8-10	11-14	0-2	NA

Carriers of any two of approximately 20 known null alleles are the phenotypic poor metabolizers, representing 7-10% of the European and North American Caucasian population. One of the most important functionally altered null variants is *CYP2D6*4* (15-21% in Caucasians). Major alleles associated with reduced enzyme activity include *CYP2D6*10* (upto 57% in Asians) and *CYP2D6*17* (20-34% in African and Africans Americans). [10]

TAM is converted to NDMT by CYP3A, then second step conversion from NDMT to END which is more potent metabolite by CYP2D6. TAM is also directly converted to 4-OH-TAM in the less extent but which is eqipotent to END by CYP2D6. Theoretically, difference in enzyme activity of CYP2D6 due to gene polymorphisms could affect TAM and its metabolites plasma concentrations. In general, long term treatment up to 5 years is recommended for BC patients. Difference in safety and efficacy in TAM adjuvant therapy might have been impact from CYP2D6 polymorphism.[7,11,40]

Jin's group reported statistically lower concentration of END in patients with homozygous variant (Vt/Vt) *CYP2D6* (20.0 nM/ml) as compared to patients with heterozygous variant (Wt/Vt) and homozygous wild type (Wt/Wt) (43.1 and 78.0 nM/ml, respectively). While TAM and other metabolites (NDM, 4-OH-TAM) concentrations were not statically different among other genotypes.[41] Similarly, Madlensky 's study reported END concentration in PM was only 5.6 ng/ml compared to 8.1, 15.9, and 22.8 ng/ml in IM, EM and UM, respectively. [42] Latest results from TAM dose adjustment per patients' genotype by Kiyatoni's group also demonstrated in the similar pattern. Before dosing adjustment, all patients received TAM 20 mg/day, END concentration of patients with *CYP2D6* genotype *1/*10 of 15 ng/ml compared to that of 10 ng/ml ($p<0.001$) in *CYP2D6*10/*10 patients. These studies confirmed the impact of *CYP2D6* gene polymorphisms on TAM metabolisms in term of different END concentrations.[43]

Nevertheless, conflicting data on the association of *CYP2D6* genotypes and clinical or treatment outcomes have been reported for past decade. As summarized in the review article reported by Kiyatoni team [44]. More than 10 studies had been performed to evaluate association of *CYP2D6* polymorphism and outcomes including disease free survival (DFS) or relapse free survival (RFS). Approximately 50% of these results are significantly different in improved treatment outcomes while other half failed to report significantly outcome differences. These studies were designed heterogenously with varied patient population. Most of studies enrolled a small number of patients (50-200). Only one study (by Schroth's group) [12] included 1,325 patients showed statically significant difference in RFS among studied genotypes and phenotypes. RFS in wt/wt patients was significantly improved as compare to patients with heterozygous IM or PM, (HR 1.40 (1.04-1.90), $p < 0.03$). In comparison of patients with wt/wt vs homozygous PM, HR was reported to increased to 1.90 (1.10-3.28), $p<0.02$.

To date, it is still controversial whether genotype analysis of CYP2D6 is warranted for patients treated with 2-5 year of TAM. Conflicting data lead to the acquisition of a better well designed study to generate more clinically important tool. Hypothetically, availability of information on enzyme activity as well as therapeutic drug monitoring of TAM's active metabolites *e.g.*, END or 4-OH-TAM should be able to guide physicians to make decision with better plan and monitoring strategic for individual patients with hormonal treatment.

2.4 *UGT2B7* polymorphism and its potential role in TAM metabolism.

The UGTs are a superfamily of enzymes located primarily in the endoplasmic recticulum of cells that detoxify a diverse range of xenobiotics, as well as, endogenous compounds, through their conjugation to glucuronic acid in a reaction with the hydrophilic co-substrates, UDPGA. The conjugated sugar alters the biological

properties of the compound to enhance its excretion in the urine or bile.
Usually, it converts substrates into less pharmacologically active products. Based on differences in sequence homology, three main families of UGTs have been identified. Each contains several UGT genes with high homology in their $COOH^-$ end.[16]

UGT2B7 is one of the most important hepatic UGTs that metabolize a vast set of clinically, physiologically and toxically important compounds. The UGT2B7 protein is also found in the brain, kidney, pancreas, mammary gland, lung, gastrointestinal tract and several additional tissues. A cytosine to thymine polymorphism at base pair 802, leading to a histidine(H)^{268} to tyrosine(Y) amino acid substitution (UGT2B7*2) has been identified. Data from large genotyping studies suggested that approximately one-third of the Caucasian population expresses, the variant *UGT2b7*2/*2* genotype. The prevalence of the *UGT2B7*2* allele appears to be lower in Asians individuals with only 5% of the Japanese population being homozygous for the *UGT2B7*2/*2* genotype.[18] Polymorphic variations of *UGT2B7*2* among different ethnic were shown in table 2.

Table 2 Frequency of *UGT2B7*2* among different ethnics [18, 45-47]

Population characteristics	Allelic frequency (%)	
	Wild type	$H^{268}Y$ (Variant)
Asian (Japanese)	73	27
Asian (Korean)	61	39
Asian (Chinese)	44	56
Caucasian (American)	46	54
Caucasian (Australian)	33	67

One of the major UGTs involved in the glucuronidation of TAM and its metabolites. The hepatic enzyme, UGT1A4 catalyzes the formation of a quarternary ammonium-linked glucuronide with TAM's and 4-OH-TAM's N,N-dimethylaminoalkyl side

chain. In addition to UGT1A4, UGTs 1A1, 1A3, 1A8, 1A9, 2B7, and 2B15 overexpression appeared to exhibit a detectable activity against 4-OH-TAM. In a comprehensive characterization and kinetic analysis of the glucuronidating enzymes responsible for O-glucuronidation of TAM metabolites, it has shown that UGTs 2B7 ≈ 1A8 > 1A10 exhibited the highest overall activity against trans-4-OH-TAM. As determined by V_{max}/K_M, with the hepatic enzyme, UGT2B7, exhibiting the highest binding affinity and lowest K_M (3.7 micromole). UGTs 1A10 ≈ 1A8 > 2B7 exhibited the highest overall glucuronidating activity as determined by V_{max}/K_M for trans-END, with the extrahepatic enzyme UGT1A10 exhibiting the highest binding affinity and lowest K_M (39.9 micromole), but UGT2B7 demonstrated the highest activity of hepatic UGTs. These data suggested that several UGTs could play an important role in the metabolism of 4-OH-TAM and END.[16]

UGT polymorphisms appeared to affect TAM glucuronidation activities observed in UGT-overexpressing cell lines. In UGT2B7 kinetic analysis, the result demonstrated that significantly higher glucuronidation activities were observed for the wild-type $UGT2B7^{268His}$ *(*1)* as compared to $UGT2B7^{268Tyr}$ *(*2)* variant against the trans isomers of both 4-OH-TAM and END. This was manifested by higher K_M (2.4-fold) and lowers V_{max}/K_M (2.4-fold) for 4-OH-TAM, as well as a lower V_{max} (5.5-fold) and lower V_{max}/K_M (5.0-fold) for END. [17]

UGT2B7 expression has been detected in a variety of tissues including liver, gastrointestinal tract, and breast. The variations in *UGT2B7* function or expression could potentially impact individual response to drugs or chemotherapeutic agents. The data demonstrated that O-glucuronidation of both *trans*-4-TAM and *trans*-END in HLM was significantly associated with UGT2B7 genotype. The lower activities correlated with increasing number of *UGT2B7*2* allele. [16]These data were consistent with the observation of HEK293 cells that overexpressed the *UGT2B7*2* variant which exhibited

lower activity *in vitro* against both TAM metabolites (4-OH-TAM and END) as compared to cell expresseing wild-type *UGT2B7*1.* [17]

Similar to what is described in *CYP2D6*, functional SNPs in *UGT2B7* potentially could affect overall treatment response to TAM. Additional studies to examine the effect of *UGT1A8* and *UGT2B7* genotypes (e.g., breast microsomal glucuronidation activity against TAM metabolites, plasma TAM metabolites levels in women taking TAM, and overall patient response to TAM) are needed to further examine the role of UGT polymorphisms on the therapeutic efficacy of TAM.

From the previous pharmacogenomics study in Thai population [19-20] , more than 50% of subjects are *CYP2D6**1 (extensive metabolizers) and prevalence of variant polymorphisms is mostly *CYP2D6**10 (approximately 40%) which is defined as reduced enzyme function. For *UGT2B7*, there is no data on prevalence of variant polymorphisms in Thai population and very limited data of variants *UGT2B7*2* prevalence among Asians. No studies have been performed to examine the effects on pharmacokinetics and treatment outcomes of both *CYP2D6* and *UGT2B7* polymorphisms on TAM in Thai breast cancer patients. Therefore, this study is intended to investigate the effect of difference on genotypes of *CYP2D6 and UGT2B7* on pharmacokinetics of TAM and treatment outcomes of Thai breast cancer patients.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Study Patients

This study was conducted from February 2011 to January 2012 at Outpatient Department, Pramongkutkloa Hospital.

Study Samples

The subjects of this study were enrolled from Thai BC patient. The study protocol was reviewed and approved by the institutional review board of Royal Thai Army Medical Department. Written inform consent were obtained from each individual participating in this study following intensive explanation of the aims, methods, objectives and potential harms of the study at time before undertaking and study-related procedures. Fifty nine subjects completing the following criteria were recruited in this study. The criteria for enrollment included:

Inclusion criteria:

1. Currently on TAM 20 mg once daily for at least 6 weeks.
2. Age $\geq$ 20 years old.
3. Normal liver function (AST and ALT $\leq$ 2 x UNL).
4. Normal renal function (serum creatinine < 1.2 mg/dL).
5. No history of venous thromboembolism (deep vein thrombosis, pulmonary embolism, cerebrovascular accident and transient ischemic attack).
6. Agree to participate in the study by agreeing to sign the inform consent.

Exclusion criteria:

1. Use concomitant drugs that could affect (inhibition or induction) on CYP2D6.
2. Non-adherence or not comply with the time schedule of TAM administration.

Sample size determination

Sample size calculation was based on probability to random patients in each genotype group. Given probability of patients in the *CYP2D6 *10* was 0.38 according to data from the study of Tassaneeyakul group [48], sample size were calculated as below

Formula $n = \frac{p(1-p)(Z_{\alpha/2})^2}{E^2}$ ($\alpha = 0.05$, $Z_{\alpha} = 1.96$, E(error) = 0.1)

$$n = \frac{0.38(0.62)(1.96)^2}{(0.1)^2}$$

$$n = 91$$

Sample sizes should be at least 91 cases in order to include patients with *CYPD2D6*10* enough for comparison.

For *UGT2B7*, probability of patients in variant *UGT2B7*2* in Japanese was 0.27.[45] Sample calculation with the same formula with *CYP2D6* as written above, number of subject should be at least 76 cases in order to include patients with *UGT2B7*2* enough for comparison

In conclusion, sample sizes in this study should be not less than 91. However, with time constraint and limited time and resources and (*e.g.*, manpower for including patients into the study), we decided to enroll last patient in January 2012 to get 59 patients in the study.

3.2 Methods

Study design and procedures

This study was the experimental study in Thai BC patients at Pramongkutklao Hospital. Demographic data, clinical findings, laboratory results, treatment regimen and adverse events and treatment outcomes were recorded. Information obtained from the subjects and laboratories were recorded in case record forms as shown in Appendix A.

Patients were prescribed TAM as hormonal therapy for their BC and returned to follow up at outpatient department, Pramongkutklao hospital were approached to participate in this study by investigators. After receiving thoroughly explanation about study objectives, methodology and possibilities of harm, patients who agreed to be enrolled in the study would sign in the inform consent.

Patients were interviewed by investigator for demographic details as well as TAM administration, adherence to TAM treatment regimen, experience of TAM adverse effects. Other treatments for their underlying diseases as well as other concomitant complimentary and alternative medicines that used with TAM was also recorded. Blood samples were collected at outpatient department and subsequently centrifuged to get plasma. DNA were thereafter collected from Buffy coated layer. All samples were stored at -20°C until analysis. Medical record from each patient was reviewed extensively by research team for recording clinical relevant data.

Primary outcome measure:

- Genotype distributions of gene *CYP2D6* and *UGT2B7*
- Steady state plasma concentrations of TAM and its two detectable metabolites; NDMT, and END
- Difference of plasma concentrations of TAM, NDMT and END among patients with different genotypes of *CYP2D6* and *UGT2B7*

Secondary outcome measure:

- Association of genotypes and treatment outcomes in terms of adverse drug events and related disease progression surveillance investigation report (*e.g.*, mammogram result, if any)

3.3 TAM, NDMT and END analysis

Patients' blood samples were collected utilizing EDTA tubes. Plasma was subsequently separated within 1 hour of blood collection by centrifugation at 2060 *g*. All samples (plasma and whole blood) were transferred to cryogenic vials, and were stored at -20°C until analysis.

Validation of Reversed phase High-Pressure Liquid Chromatography for TAM, NDMT and END quantification analysis

Minor modification of HPLC method of Zhu's group [49] was used to quantify TAM and its metabolites.

Chemicals and reagents

Standard TAM, NDMT, END, mexilitine (internal standard) and Triethylamine (TEA) were purchased from Sigma (St.Louis, MO, USA). HPLC grade, methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (USA).

Standard solutions

Standard solutions of TAM and NDMT were prepared by dissolving free-base of each compound 5 mg in 10 ml of MeOH. Dilutions of the standard stock solutions for TAM and NDMT were made in methanol range from 5 to 750 ng/ml to prepare for the standard curve and quality control (QC) samples. END

concentrations ranging from 1 to 150 ng/ml. The internal standard (IS) mexilitine, was prepared by dissolving 25 mg free-base in 5 ml methanol. All solutions were stored at -20°C.

Standard curves

Six-point standard curves were prepared by adding known concentrations of TAM, NDMT and END in to drug-free plasma covering the range expected in BC patients. END was added at 1, 5, 10, 50, 100, 150 ng/ml, while concentrations of NDMT and TAM were 5 times higher than END (25, 50, 125, 250, 500, 750 ng/ml). All solutions were stored at -20°C until analysis.

Sample preparation

After plasma samples were thawed at room temperature. One milliliter of plasma was placed into clean centrifuge tube, then 5 microlitre of IS was added and mixed. Acetonitrile 1.5 ml was added and mixed on a vortex mixer for 1 minute before centrifugation at 3000 rpm for 20 minutes. Supernatant 1 ml was transferred to clear ampoule and left in UV lamp hood which set wave length at 375 nm for 20 minutes and then 20 microlitres of sample were injected into the HPLC column.

Instruments and chromatography conditions

HPLC was accomplished by using an Agilent 1200 series liquid chromatography with a binary pump, on-line degasser, autosampler, column heater and fluorescence detector.

An Agilent Extend C_{18} chromatography column (150 mm x 4.6 mm, 5 micron, Agilent, USA), combined with double endcapping process that protects dissolution of silica from up to pH 11 was used with HPLC condition consisted of 1%TEA (aqueous solution pH 11 : MeOH 18:82 v/v). The system was run at flow rate of 1.1 ml/min with controlled column temperature at 35°C.

The fluorescent detector was set at an excitation wavelength of 260 nm and emission wavelength of 375 nm. Peak areas of each compound were generated from computerized software Agilent EZchrome Elite (Agilent, USA).

The chromatographic data were processed using IS method of plotting peak area ratios of analytes/IS vs the relative concentration followed by least square regression of these data.

Selectivity

In condition described, TAM, NDMT and END exhibited good chromatogram with baseline resolution of each compound. There are no foreign peaks interfered with analytes and IS at the retention times. The retention times for TAM, NDMT, END and IS were 16, 11.6, 3.8 and 2.5 minutes, respectively.

All chromatograms were shown in Appendix.

Linearity

Calibration curves were determined by least square linear regression analysis. Linear regression calibration curves based on six data points, constructed for each compound plotting peak area ratio of the compound to IS peak versus the concentration of plasma standard of each compound. The results were expressed as the regression equations.

The calibration curves were linear from 1 to 150 ng/ml for END, from 25 to 750 ng/ml for TAM and NDMT. The mean values of regression equation of the analytes in plasma were shown in table 3.1.

Table 3.1 Regression Equation of TAM, NDMT and END

Analytes	Regression Equation	r^2	n
TAM	y = 0.0047x – 0.0748	0.9940	5
NDMT	y = 0.0056x + 0.0124	0.9942	5
END	y = 0.0141x - 0.013	0.9975	5

Extraction efficiency

The extraction efficiencies of TAM, NDMT and END were obtained by comparing the extracted standard curves to an unextracted standard.

Table 3.2 Extraction efficiency (% average ± SD)

TAM (ng/ml)	Efficiency (%)	NDMT (ng/ml)	Efficiency (%)	END (ng/ml)	Efficiency (%)
25	49.2 (±1.9)	25	59.5 (±5.0)	1	55.3(±21.1)
250	89.1 (±6.4)	250	93.2 (±6.0)	50	97.2(±6.6)
750	57.6 (±2.1)	750	69.2 (±5.5)	100	99.2(±7.7)

Assay accuracy and precision

Standard in plasma were extracted and analysed to assess inter-day variability of the method. Accuracy and precision (C.V.) throughout the standard curve are summarized in table 3.3. The lowest standard for TAM exhibited the largest variation (27%). In general, the C.V.s were less than 10% for the standard concentrations for each compound, which illustrated a precise assay. The least precision were found in the lowest concentration of END (20%). In summary, the accuracy of the reported method was about 100%, which is acceptable for quantitative assay.

Limit of quantification

The limit of quantification (LOQ) was the lowest calibration standard for each compound (1 ng/ml for END, 25 ng/ml for TAM and NDMT). The LOQ allowed successful measurement of therapeutic plasma concentration of TAM, NDMT and END.

Table 3.3 Inter-day accuracy and precision for TAM, NDM and END

Analyte	Standard concentration (ng/ml)	Calculate measure Concentration (ng/ml)	Accuracy (% Bias)	Precision (%R.S.D)
TAM	25	27.5±1.2	27	3.6
	100	98.4± 5.1	-1.6	5.6
	500	534.8± 44.6	-1.1	8.3
NDMT	25	25.6±0.9	2.3	3.7
	100	102.5± 10.2	2.5	2.5
	500	496.1± 5.6	-0.7	1.1
END	2.5	2.7±0.5	9.5	20
	10	11.1± 0.3	10.8	2.46
	50	46.1± 5.3	-7.9	11.6

HPLC with fluorescence detector was used in TAM and its metabolites analysis. Fifty nine blood samples were divided in to 3 batches (approximately 20 patients' sample per batch; one working day was used for extraction and HPLC run for each batches). Peak area ratio (PAR) from HPLC chromatograms were used to calculate TAM, NDMT and END concentrations of each samples.

3.4 Genotyping analysis

QIAamp DNA blood Mini Kits (Qiagen, Gene Plus, Thailand) were used to extract genomic DNA from the leukocyte portion of whole blood and used the DNA to genotype variant allele of *CYP2D6* *10 (100C>T; rs1065852) and *UGT2B7*2* (802C>T; rs7439366). Assays and Master Mix for allele determination were bought from Applied Biosystem (Thailand). Real time polymerase chain reaction (RT-PCR) processes were done twice for all 59 samples, first for *CYP2D6*10* genotyping analysis and second for genotype analysis of *UGT2B7*2*.

To prepare the reaction components for one reaction refer to the table 3.4. The StepOnePlus™ Real time PCR Systems (Applied Biosystems Inc.,Foster City, CA USA). The reaction mixtures contained TaqMan Drug Metabolism Genotyping Assay Mix, TaqMan Universal PCR Master Mix, No AmpErase UNG, and DNase-free water. The final reaction volume per well is 20μL in a 96-well plate as shown in table 3.4.

Table 3.4 Allelic Discrimination PCR Reaction

Reaction Components	Volume/Well (20 μL volume reaction) *	Final concentration
TaqMan® Universal PCR Master Mix (2 X)	10 μL	1 X
20 X TaqMan® Drug metabolism Genotyping Assay Mix	1 μL	1 X
Genomic DNA (10 ng/μL) **	2 μL	-
dH_2O	7 μL	-
Total	20 μL	-

* If different reaction volumes are used, amounts were adjusted accordingly.

** 3-20 ng of genomic DNA per well. All wells on a plate should have equivalent amounts of genomic DNA.

Table 3.5 Thermal Cycler Conditions

Times and Temperatures		
Initial Steps	Denature	Anneal/Extend
HOLD	40 CYCLES	
10 min 95 ^{0}C	15 sec 92 ^{0}C	90 sec 60 ^{0}C

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3.5 Statistical analysis

All data analysis were performed by using the SPSS for windows version 17.0 and analyzed by descriptive statistics and inferential statistics. Independent t-test and ANOVA were used in data with normal distribution, while Wilcoxon signed-rank test and/or Kruskal-Wallis test were used for comparison of END, NDMT, TAM, ratio END/NDMT, ration NDM/TAM among the different genotypes. Chi-square was used for association analysis for categorical data. A p-value of less than 0.05 was considered to be statistically significant for all analyses.

3.6 Ethical consideration

This study was complied with the standard for gathering subjects' information for confidential in every process since data collection, analysis, conclusion and publication. All data collected from patients were coded in order to protect their confidentiality. There had no record any details that led to identify the subjects. Results from this study may be published in scientific journals or presented at medical meetings but subjects were not been personally identified.

CHAPTER IV

RESULTS

4.1 Study patients

This study was conducted from February 2011 to January 2012 at the outpatient department of Pramonkutklao hospital. Sixty breast cancer patients were initially included but one patient was excluded due to nonadherence (confirmed by very low level of TAM from HPLC analysis and 3 months duration lost of follow-up before resume TAM treatment). At the end of study, data from 59 patients were genotyped and analyzed for plasma concentrations of TAM and metabolites.

Average age of BC patients in this study was 50$\pm$9.3 years old. Weight, height, body mass index (BMI) and body surface area (BSA) were, 58.3$\pm$9.8 kg, 156.6$\pm$5.5 cm, 22.8$\pm$3.8 kg/m^2 and 1.56 m^2 , respectively.

Most of patients were in their fourth decade of age (24 cases, 40.7%). Approximate 10% had chronic underlying diseases (*e.g.*, type 2 DM, hypertension, dyslipidemia). Majority were in early BC cases (70 % of cases had T1 and T2 tumor, and 50% were N0). Metastatic BC were found in 4 patients (6.8%). Sixty-nine percent (41 out of 59) had received chemotherapy. For history of radiation, 9 cases were unclear medical records, while 8 cases had records of no radiation therapy. There were 3 cases diagnosed as recurrence BC.

Duration of TAM treatment was in the range of 1.5 months to 79 months (median 26 months). Most of patients (86%) were ER positive BC.

Table 4.1 Patients' characteristics

Patient's characteristics	Mean±SD/ No of patients (%)
Mean age (years old)	50±9.3
Mean BMI	22.8±3.8 kg/m^2
Underlying diseases	
- DM Type 2	8 (13.6)
- hypertension	11 (18.6)
- dyslipidemia	7 (11.9)
- other underlying diseases	10 (16.9)
ER	
- positive	51(86.4)
- negative	4 (6.8)
- unknown	4 (6.8)
PR	
- positive	50 (84.7)
- negative	7 (11.9)
- unknown	2 (3.4)
HER-2	
- positive	5 (8.5)
- negative	45 (76.3)
- unknown	5 (8.5)
Menopausal status	
- pre/peri-menopause	45 (76.3)
- post-menopause	14 (23.7)
TNM Stage of BC	
-T1	24 (40.6)
-T2	18 (30.5)

Table 4.1 Patients' characteristic (cont)

Patient's characteristic	Mean±SD/ No of patients (%)
-T3	4 (6.8)
-T4	3 (5.1)
-N0	33 (55.9)
-N1	7 (11.9)
-N2	9 (15.3)
-M0	56 (94.9)
-M1	4 (6.8)
Unknown	10 (16.9)
Recurrence case at diagnosis	3 (5.1)

4.2 Genotypes

*CYP2D6*10* and *UGT2B7*2* allele frequencies and distribution of genotypes of both genes were shown in Table 4.2. The frequencies of both *CYP2D10* and *UGT2B7* were within Hardy-Weinberg equilibrium.

Table 4.2 Genotype frequency of *CYP2D6* and *UGT2B7*

	Allele frequency		Genotype n (%)		
CYP2D6	*1	*10	*1/*1	*1/*10	*10/*10
	0.47	0.53	16 (27.1)	23 (39)	20 (33.9)
UGT2B7	*1	*2	*1/*1	*1/*2	*2/*2
	0.72	0.28	31 (52.5)	23 (39)	5 (8.5)

Selected patient's characteristics of each genotype were presented in table 4.3 and 4.4.

Table 4.3 Patients characteristic for each *CYP2D6* genotypes

Patient's characteristics	Mean±SD/ No of patients (%)			
CYP2D6 Genotype	*1/*1 n = 16	*1/*10 n = 23	*10/*10 n = 20	P-value
Mean age (years old)	47.3±7.2	50.8±10.6	51.1±9.2	0.412
Mean BMI (kg/m^2)	21.3±7.1	21.6±6.6	18.9±2.1	0.519
TAM duration (months)	21.0±13.4	29.6±17.1	24.3±21.3	0.309
Disease-specific information	*1/*1 n = 16	*1/*10 n = 23	*10/*10 n = 20	
ER-positive	16 (100)	21 (95.6)	20 (95)	
Pre-menopause	14 (87.5)	17 (73.9)	14 (70.0)	
Presence of node involvement	4 (25)	5 (21.7)	7 (30.0)	

Table 4.4 Patients characteristics for each *UGT2b7* genotypes

Patient's characteristic	Mean±SD/ No of patients (%)			
UGT2B7 Genotype	*1/*2 n = 31	*1/*2 n = 23	*2/*2 n = 5	P-value
Mean age (years old)	49.8±9.9	49.8.2±8.1	54.4±10.8	0.526
Mean BMI (kg/m^2)	21.2±5.8	19.7.6±10.1	21.7±2.5	0.775
TAM duration (months)	21.0±13.4	29.6±17.1	24.3±21.3	0.544
Disease-specific information	*1/*1 n = 31	*1/*2 n = 23	*2/*2 n = 5	
ER-positive	28 (90.3)	23 (100)	4 (80)	
Premenopause	18 (77.4)	18 (78.3)	3 (60.0)	
Presence of node involvement	7 (22.6)	6 (26.1)	3 (60.0)	

None of statistical differences in demographic profiles were found among different *CYP2D6* and *UGT2B7* genotypes.

4.3 Concentrations of TAM and its metabolites

Range, mean±SD and median concentrations of TAM and its metabolites at steady state of all 59 patients were presented in table 4.5.

Table 4.5 Concentrations of TAM and its metabolites

Concentration	Range (ng/ml)	Mean (±SD) (ng/ml)	Median (ng/ml)	Inter-individual variation (%C.V.)
TAM	28.12-714.56	367.09 ± 146.33	336.51	39.9
NDMT	87.19-1355.71	532.50 ± 236.63	532.70	44.4
END	1.88 – 66.15	18.5 ±12.61	15.33	68.2

4.4 Impacts of *CYP2D6* polymorphism on TAM and its metabolites concentrations

Average TAM and its metabolites concentrations in different CYP2D6 genotypes were shown in table 4.6. Both mean and median were presented for an END concentration because of data distribution was not normal.

Table 4.6 TAM concentrations in different *CYP2D6* genotypes

Concentration (ng/ml)	**1/*1*	**1/*10*	**10/*10*	*P-value*
TAM	323.6±79.8	336.3±151.1	437.3±161.2	0.027*
NDMT	458.7±129.8	481.6±213.5	650.1±287.4	0.020*
END (mean)	22.4±12.8	17.9±9.8	14.7±14.7	0.191
END (median) [a]	21.55	15.67	9.62	0.045*

* significant difference P < 0.05

The most potent metabolite which approximately 100 times higher estrogen affinity than TAM is END. Median END concentration in this study was 15.3 ng/ml. Patients were classified as low or high END concentrations according to their END concentration lower or higher than 15.3 ng/ml. Association of *CYP2D6* polymorphisms and lower END concentrations was demonstrated in Table 4.7.

Table 4.7 Association of *CYP2D6*10/*10* and lower END concentrations

Genotype	n in genotype	n in group that END < 15.3 ng/ml	Odds ratio and 95% CI	*P value*
*CYP2D6 *1/*1 or *1/*10*	39	15		
*CYP2D6 *10/*10*	20	14	3.73 (1.18-11.83)	0.0252*

* significant difference P < 0.05

Box plots of TAM and its metabolites mean concentrations in different genotypes were illustrated in Figures 4.1-4.3.

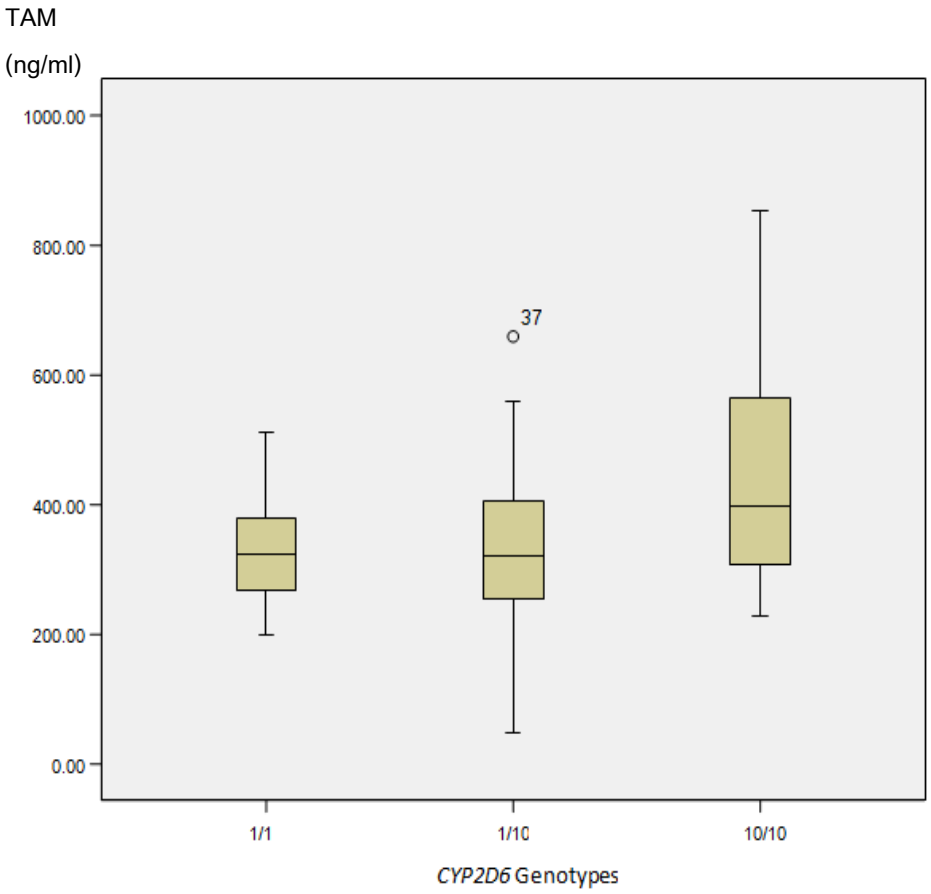

Figure 4.1 TAM concentrations in different *CYP2D6* genotypes

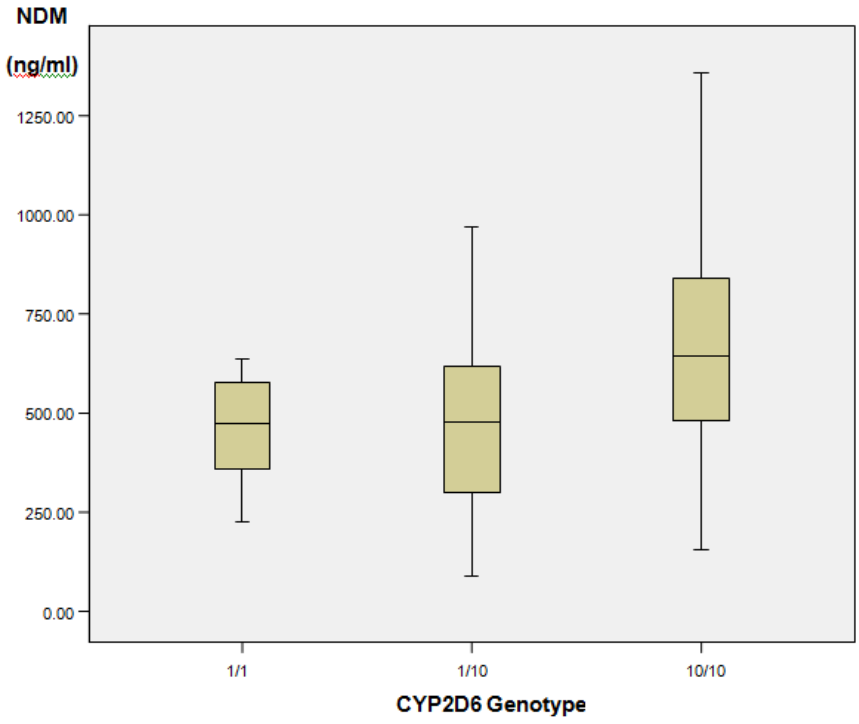

Figure 4.2 NDMT concentrations in different *CYP2D6* genotypes

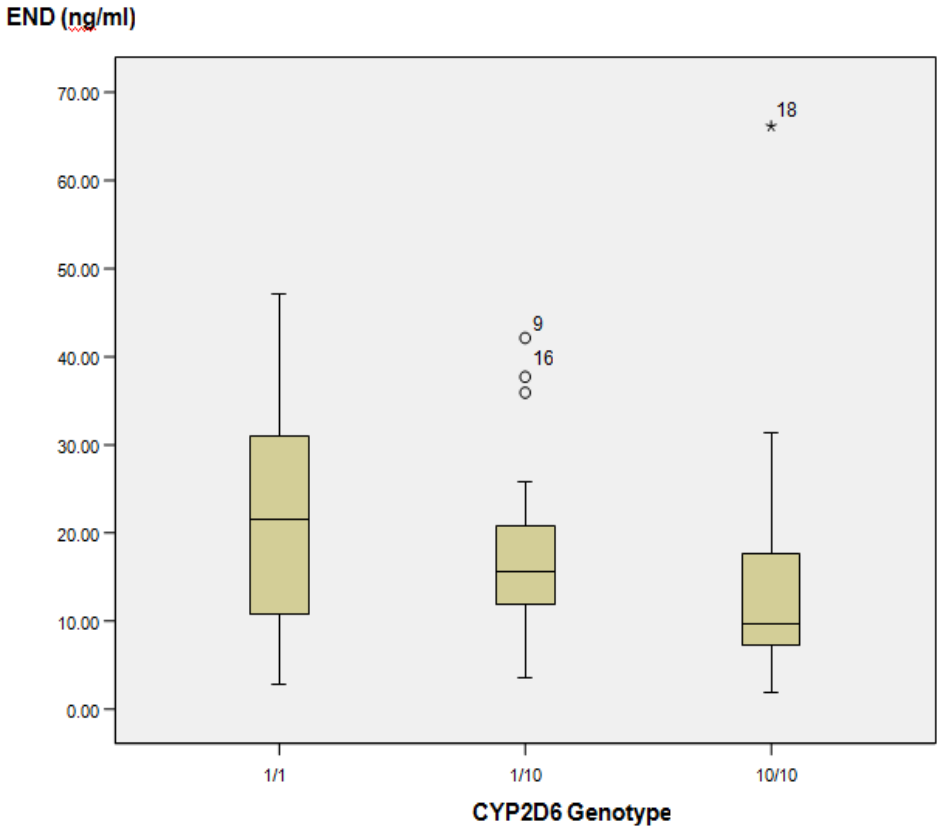

Figure 4.3 END concentrations in different *CYP2D6* genotypes

4.5 Impacts of *UGT2B7* polymorphism on TAM metabolism

Mean±SD of TAM and its metabolites among different *UGT2B7* genotypes were presented in Table 4.8. Both mean and median were presented for END concentrations because of data distribution were not normal. Association of polymorphisms of *UGT2B7*2* and END concentrations was demonstrated in Table 4.9. Figure 4.4 showed the box plots of mean END concentrations in different *UGT2B7* genotypes.

Table 4.8 TAM and its metabolites concentration in different *UGT2B7* genotypes

Concentration (ng/ml)	*1/*1 n = 31	*1/*2 n = 23	*2/*2 n = 5	*P-value*
TAM	362.3±158.8	360.5±130.0	427.3±153.3	0.613
NDMT	538.8±253.9	507.5±208.7	608.1±279.0	0.682
END (mean)	17.2±11.8	18.0±14.1	23.3±10.0	0.630
END (median)	14.12	15.33	28.74	0.503

Table 4.9 Association of *UGT2B7* genotypes and END concentrations

Genotype	n in genotype	n in group that END < 15.3 ng/ml	Odds ratio and 95% CI	*P value*
UGT2B7*1/*2 and *2/*2	28	16	2.11 (0.75-6.0)	0.1593
UGT2B7*1/*1	31	12		

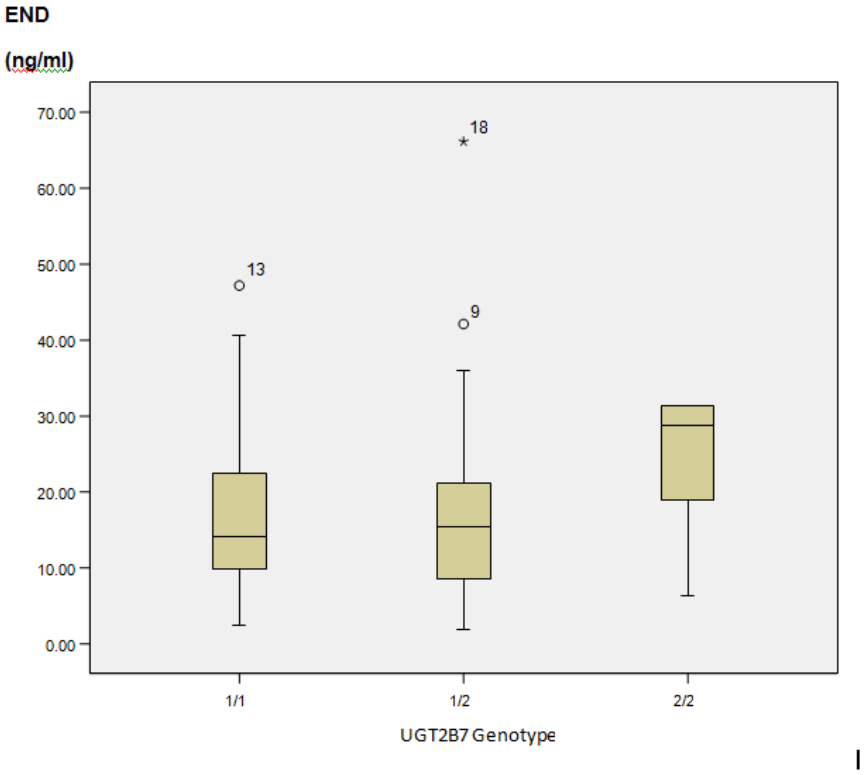

Figure 4.4 END concentrations in different *UGT2B7* genotypes

There were no statistically differences of TAM and its metabolites concentrations among homozygous wild type (*1/*1), heterozygous variant (*1/2) and homozygous variant *UGT2B7*.

4.6 Combined effects of *CYP2D6* and *UGT2B7* polymorphisms on TAM and its metabolites concentrations

Genotypes of *CYP2D6* and *UGT2B7* in same patients were listed in Table 4.10. END concentrations in each groups were presented within the same table.

Most patients were *CYP2D6 *1/*10 and UGT2B7 *1/*1* (20%). None of patient had polymorphisms as *CYP2D6 *1/*10 and UGT2B7 *2/*2*.

Figure 4.5 is box plot of mean END concentrations in each *CYP2D6* and *UGT2B7* genotype and figure 4.6 focused only in the group that *CYP2D6* were **10/*10.*

Table 4.10 *CYP2D6* and *UGT2B7* Genotype distribution

Genotype	*CYP2D6 *1/*1 and UGT2B7 *1/*1*	*CYP2D6 *1/*1 and UGT2B7 *1/*2*	*CYP2D6 *1/*1 and UGT2B7 *2/*2*
n (%)	11 (18.6%)	3 (5.1%)	2 (3.4%)
Mean END (ng/ml)	25.1±13.6	15.9±7.8	17.5±15.8
			P value = 0.499
Genotype	*CYP2D6 *1/*10 and UGT2B7 *1/*1*	*CYP2D6 *1/*10 and UGT2B7 *1/*2*	*CYP2D6 *1/*10 and UGT2B7 *2/*2*
n (%)	12 (20.3%)	11 (18.6%)	none
Mean END (ng/ml)	15.5±9.1	20.5±10.2	N/A
			P value = 0.234
Genotype	*CYP2D6 *10/*10 and UGT2B7 *1/*1*	*CYP2D6 *10/*10 and UGT2B7 *1/*2*	*CYP2D6 *10/*10 and UGT2B7 *2/*2*
n (%)	8 (13.6%)	9 (15.3%)	3 (5.1%)
Mean END (ng/ml)	9.03±4.9	15.6±19.7	27.2±7.2
			P value = 0.073

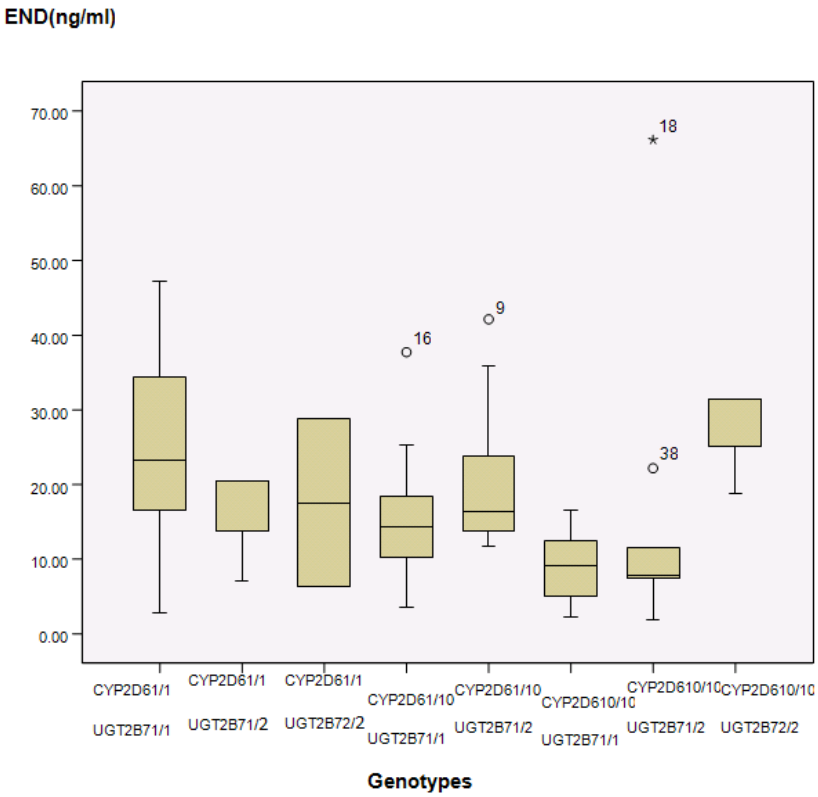

Figure 4.5 END concentrations in *CYP2D6* and *UGT2B7* genotypes

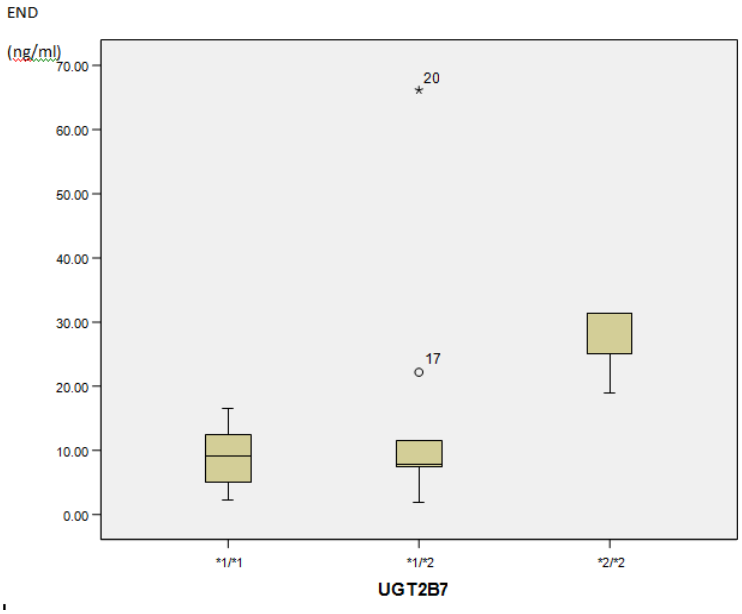

Figure 4.6 END concentrations in patients with *CYP2D6*10/*10* and different genotypes of *UGT2B7*

4.7 Association of *CYP2D6* and *UGT2B7* polymorphisms, TAM and its metabolites and treatment outcomes

Major clinical endpoint of TAM treatment for BC was relapse or recurrence. This required long term patient follow-up, while BC patients in this study on TAM treatment for only 2 years (median 26 months). However, breast imaging (every 6 month or 1 year) was used to monitor BC relapse and recurrence at least one time in 39 patients. Results of last breast imaging in each patient were used as surrogate marker of treatment outcomes. There was no association in *CYP2D6* and/or *UGT2B7* polymorphisms and breast imaging results, but there was some trend of association between END concentrations and breast imaging results.

The results of breast imaging were reported in BI-RADS ranged from 0 to 6. BI-RADS-0, 1, 2 are incomplete, negative and benign finding, respectively. BI-RADS-3 indicated probably benign findings. BI-RADS-4 and 5 were suspicious and highly suggestive malignancies. BI-RADS 6 was known biopsy proven malignancy.

Trend in association between END concentrations and breast imaging results was presented in table 4.11

Table 4.11 END concentrations and breast imaging results

END concentration	n in group BI-RADS result = 3	n in Total BI-RADS reported	Odds ratio and 95% CI	P-value
END < 15.3 ng/ml	7	17	2.975 (0.69-12.76)	0.1421
END $\geq$ 15.3 ng/ml	4	21		

Adverse events (AEs) were reported in nine cases (15.3%), all AEs were mild symptoms. AEs were found in this study such as fatigue, insomnia, and muscle weakness. High END concentrations were associated to AEs as described in Table 4.12

Table 4.12 END concentrations and AEs

END concentration	n of patients with AEs	n of all patients	Odds ratio and 95% CI	P-value
END < 15.3 ng/ml	8	30	10.18 (1.2-87.6)	0.034*
END ≥ 15.3 ng/ml	1	29		

* significant difference P < 0.05

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CHAPTER V

DISCUSSION

5.1 Patient Characteristics

Fifty-nine patients were included into the study during February, 2011 to January, 2012. Most of their characteristics of 59 patients were similar to the other studies of adjuvant TAM treatment. [11,12,14,41,42,50-55] Majority of patients were in their forties, in pre-menopause status with ER positive BC. BMI was less than that found in Caucasian studies [54] but similar to other studies in Asians.[53] Median TAM treatment duration was 26 months (range from 1.5 to 79 months). All patients' demographic data among different *CYP2D6* and *UGT2B7* genotypes were not different. Therefore, the differences in TAM and its metabolites concentrations were resulted from other factors than demographic data. Differences in genotypes might be one of the important factors.

5.2 TAM and its metabolites concentration in all patients

Mean ($\pm$ SD) of plasma TAM, NDMT and END concentrations were 367.09 $\pm$ 146.33, 532.5 $\pm$ 236.63 and 18 $\pm$ 12.61 ng/ml, respectively. Large coefficient of variation (C.V.) of TAM and its metabolites indicated highly inter-individual variation among patients in this study. END, the most potent metabolite and lowest concentration in plasma, expressed the largest interindividual variation for almost 70%, while interindividaul variation of prodrug TAM was approximately 40%. No demographic of data was correlated to the concentrations of TAM and its metabolites. The result of large interindividual variation in TAM and its metabolite concentrations in this study was similar to the study of Lim's group [53] which reported about 24-fold variation in END and 11 to 20 fold-variations of TAM, NDMT and 4-OH-TAM. These also similar to the results

of Barginear's group which reported about 5 to 20 fold variation in TAM and its metabolites [54]

5.3 Impact of *CYP2D6*10* Polymorphism on TAM and its metabolite concentrations

Variant polymorphism of *CYP2D6*10* was found in 53% of BC patients in our study group which was not different from general healthy population.[19,48] Heterozygous variants *CYP2D6*10* was the highest proportion (39%), while homozygous variants *(*10/*10)* was found in higher percentage than homozygous wild type as 34%, and 27%, respectively. The genetic distribution of *CYP2D6*10* was consistent with to other TAM studies that focus on effects of *CYP2D6*10* polymorphism on TAM metabolism and/or treatment outcomes in Asian population. [53,56-60] This indicated that approximately 70% of Thai BC patients had reduced function of enzyme CYP2D6 which can lead to low END concentrations and might relate to ineffectiveness of TAM treatment.

Impact of *CYP2D6* polymorphism on TAM metabolisms was confirmed in this study since significant difference in END concentrations was found among genotypes. Patients with *CYP2D6*10/10* had lowest END concentration at 14.7$\pm$14.7 ng/ml compared to 17.9$\pm$9.8, and 22.4$\pm$12.8 ng/ml in heterozygous and homozygous wild type, respectively. The differences of END concentration among *CYP2D6* polymorphisms were in the same pattern with Kiyatoni's group study which found the lowest END concentration of 7.9 ng/ml in *CYP2D6*10/*10*, while patients with *CYP2D6*1/*10* and *CYP2D6*1/*1* had higher END concentration of 19.9 and 18.1 ng/ml, respectively.[56] As same as the data reported by Lim's group [53] which found END concentrations of 8.03, 19.74 and 19.55 ng/ml in *CYP2D6*10/*10, CYP2D6*1/*10* and *CYP2D6*1/*1*, respectively.

Since END was converted from NDMT, the opposite direction was found for NDMT concentration. NDMT concentrations in patients with *CYP2D6 *1/*1, *1/ *10* and **10/*10*, were 458.7±129.8, 481.6±213.5, and 650.1±287.4 ng/ml, respectively (p value = 0.020). Similar pattern as NDMT was found for TAM, patients contained homozygous *CYP2D6*10* had highest TAM concentrations, (437.3±161.2 ng/ml) compared to 323.6±79.8 and 336.3±151.1 ng/ml in patients with heterozygous and homozygous wild type *CYP2D6,* respectively (p value = 0.027).

END is one of the most potent antiestrogenic metabolite of TAM. It is about 100 times more potent than TAM. END concentration was considered to be important for effectiveness of TAM treatment. Association of *CYP2D6*10/*10* and below median END concentrations (15.3 ng/ml) was found in this study. Odds ratio of carry homozygous *CYP2D6*10* alleles and had low END concentration was 3.73 (p = 0.0252)

5.4 Impact of *UGT2B7* Polymorphism on TAM and its metabolite concentrations

Allele frequency of *UGT2B7* variant *2 was found to be 28 %, yield in 8.5 % (5 cases) of homozygous variant *UGT2B7*2* in this patient population. The homozygous and heterozygous wild type patients were 52.5% and 39%, respectively, which indicated normal enzyme function in majority of patients in this study. These findings were similar to those were found in Japanese and Chinese population.[45-47]

Polymorphism of *UGT2B7*2* should result in higher END concentrations since this should result in slower excretion. From several experiments in cell lines, variant allele containing reduced enzyme function and result in approximately 5-times lower elimination rate of END, the active and potent metabolite.[16,17] However, the impact of *UGT2B7* polymorphism alone on TAM metabolism and/or END concentration in BC patients in this study was not statistically significant, even though concentrations of

END, in patients with homozygous *UGT2B7*2* which was reduced enzyme function were higher (23.3±10.0 ng/ml) when compared to those found in homozygous and heterozygous wild type, 17.2±11.8 and 18.0±14.1 ng/ml, respectively. Small number of patients carrying homozygous variant (5 cases) might be the reason for not finding statistically significant results. Odds ratio of polymorphism *UGT2B7*2* and concentrations of END was not significant either.

5.5 Combined Impacts of *CYP2D6*10* and *UGT2B7*2* on TAM and its metabolites concentrations

The most frequent combined pattern of *CYP2D6* and *UGT2B7* was found in this study had genotypes as *CYP2D6*1/*10* and *UGT2B7*1/*1* (20%), the second rank was *CYP2D6*1/*10* and *UGT2B7*1/*2* (18.6%). This suggested that most of BC patients in this study contained reduced function of enzyme CYP2D6 to convert TAM and NDMT to END, while they had enzyme UGT2B7 with fully function to change the END to its glucuronide form, which easier to be excreted.

When the effect from polymorphisms of both *CYP2D6* and *UGT2B7* genotypes were combined, the impact was more obvious; trend of differences in END concentrations among different genotypes of *UGT2B7* were found in subgroup of patients with *CYP2D6*10/*10* genotype (9.03±4.9 ng/ml in *UGT2B7* homozygous *1 and 15.6 ng/ml in heterozygous*2 and 27.2 ng/ml in homozygous *2, respectively, P = 0.073). This indicated the possibility of *UGT2B7* to have important role in END elimination.

In patients with homozygous variants both in *CYP2D6* and *UGT2B7*, the mean END concentration was as high as 27.2±7.2 ng/ml, which almost equivalent to 25.1±13.6 ng/ml in patients with both homozygous *CYP2D6*1/*1* and *UGT2B7*1/*1*. Same trend was found in patients with heterozygous *CYP2D6*10/*10*, mean END

concentrations in patient with one variant allele *UGT2B7*2* was higher in patients with homozygous wild type (20.5±10.2 vs 15.5±9.1ng/ml, respectively)

Since the concentrations of TAM and its metabolites (NDMT and END) that we monitored are affected by series of enzymes which are influence by both *CYP2D6* and *UGT2B7*, the impact of polymorphism of *CYP2D6* may confound the impact of polymorphism of *UGT2B7*, and vice versa. Besides polymorphisms, the concentrations of TAM, NDMT and END and therefore several other factors may have some impacts and confounds our results. Studies with higher number of patients are definitely required to evaluate the impact of *UGT2B7*2* polymorphisms before any strong conclusion should be made.

5.6 *CYP2D6* and *UGT2B7* polymorphisms, END concentrations and treatment outcomes

The major objectives of this study were to analyze the impact of *CYP2D6* and *UGT2B7* pharmacokinetics of TAM and its metabolites, while the secondary objectives were focused on the association of genotypes and treatment outcomes. Major treatment outcome of TAM for BC is to reduce the rate of relapse or recurrence of the disease which require long period up to 10 years of monitoring and follow up. Majority of patients in this study received adjuvant TAM treatment at the early stage BC. Therefore, the possibility of treatment outcomes which might be detected in this study was the results of breast imaging (mammogram) monitoring of some patients which was recorded in the medical records.

The effects of both *CYP2D6* and *UGT2B7* polymorphisms to treatment outcome could not be detected directly. However, higher concentration of TAM's most active metabolite, END, might associate with better (lower) BI-RADS score. When median concentration of END, 15.3 ng/ml, was used as the cut-off points. END concentration

less than 15.3 ng/ml (low END concentration) had tendency to associate with BI-RADS score as 3 or above. Odds ratio of low END concentration and higher BI-RADS score was 2.975 (95% CI 0.69-12.76, p = 0.1421). Seven of 17 patients (41%) with low END concentration had high BI-RADS, while only 4 out of 21 patients (19%) with high END concentration had high BI-RADS.

Although the results of breast imaging as BI-RADS number or score had not been approved to be used as indicator for monitoring the adjuvant TAM treatment outcomes and the analysis of differences of data from this study did not show statically significant, some interesting information about the association of END concentration and BI-RADS score was observed in this study.

In terms of treatment safety, significant association was found between high concentration of END and the presence of AEs in TAM treated patients, nine cases out of 59 patients were noted with possible AEs caused by TAM treatment (*e.g.*, hot flashes, insomnia) in their medical records, resulted in AEs rate of 15.3%. Severity of all AEs was mild to moderate. Out of these 9 cases, 8 patients had END concentrations higher than 15.3 ng/ml. Odds ratio of high END concentrations and AEs was 10.18 (95% CI 1.2-87.6, p = 0.034). This was similar to study of Lorizio's study.[61] who found that median END concentrations in patient with side effects was higher than in patients without any side effects (9.36 vs 7.29 ng/ml, respectively)

CHAPTER VI

CONCLUSION

This was the first study which determined the impacts of both *CYP2D6* and *UGT2B7* polymorphisms on TAM and its metabolites concentration in Thai BC patients. Fifty – nine patients received TAM treatment at outpatient department of Pramongkutkloa hospital were included into the study. *CYP2D6* and *UGT2B7* genotypes analysis was performed in concurrent with the analysis of plasma TAM and its metabolites concentrations.

Allele frequency of variant gene *CYP2D6*10* and *UGT2B7*2* as well as genotypic distribution in this study patients were similar to other Asian population. Majority of patients had genotypes of those reported for heterozygous *CYP2D6*10* and homozygous wild type *UGT2B7*1*.

Patients with homozygous *CYP2D6*10* showed significant impact on TAM metabolism as lower END and higher NDMT concentrations. Polymorphism in *UGT2B7*2* did not show significant impact on TAM and END concentrations when all 59 patients were included into the analysis. However, when patients with homozygous *CYP2D6 *10* only were analyzed, patients carry *CYP2D6 *10/*10* and *UGT2B7*2/*2* tended to have higher END concentrations as compared to patients carry *CYP2D6 *10/*10* and other *UGT2B7* genotypes. Importance of *UGT2B7* polymorphism became more obvious when coexist with slow metabolized polymorphism of *CYP2D6*.

Neithers *CYP2D6* nor *UGT2B7* alone were associated to treatment outcomes. Because the period of study was too short to evaluate TAM treatment outcomes base on recurrent rate of BC, which may take 10-15 years. However, surrogate marker as breast imaging result was use to determine different of TAM treatment. END concentration

which was below or higher than its median (15.3 ng/ml) was seemed to show some association to treatment outcomes, both efficacy and side effects.

Even though the direct association of treatment outcomes with *CYP2D6* and *UGT2B7* polymorphisms could not be confirmed. Since END concentration tend to show some impact on treatment outcomes, while *CYP2D6* and *UGT2B7* polymorphisms showed significant impact on END concentration, we concluded that polymorphisms of *CYP2D6* and *UGT2B7* have some important role on TAM and its metabolites concentrations and in turn, on clinical outcomes. Evidences from this study support the significant of *CYP2D6* genotype analysis in patient treated with TAM. Moreover, some data show necessity of TAM therapeutic drug monitor in some high risk patients in order to prevent toxicity, as well as, dosage adjustment to achieve the best benefit from TAM treatment.

In conclusion, concentrations of TAM and its metabolites were influenced by polymorphisms of *CYP2D6*, reduced function enzymes resulted in lower END concentrations. For *UGT2B7* polymorphism, its impact was more obvious in those who also carry homozygous variant, *CYP2D6*10/10*, resulted in higher END concentrations. Therapeutic monitoring, including genotyping analysis, might be an advantage in TAM treated patients espcially who have very high risk of disease relapse and/or recurrence.

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APPENDICES

Appendix A

Case Record Forms: TAM PG/PK project

Blood sample taking date	
Time for medication taken	

Demographic data

DOB…… Age…….

Height……cm Weight……kg

BMI……kg/m^2 BSA……m^2

Menopausal status ( ) premenopause ( ) perimenopause ( ) postmenopause

Underlying diseases:

Disease	Onset	Treatment

Social History:

Smoking……

Drinking……

IVDU/other drug abuse……

Breast cancer work-up and treatment related data

Tumor type: ( ) invasive ductal ( ) invasive lobular ( ) other……

Tumor size……

Tumor stage……

No. of positive lymph node……..

Estrogen receptor expression ( ) positive ( ) negative

Progesterone receptor expression ( ) positive ( ) negative

Ki-67……

HER 2/*neu* status: ( ) 1+ ( ) 2+ ( ) 3+ ( ) 4+

Treatment history:

- Surgery……
- Chemotherapy……
- Radiation……
- Start date for tamoxifen……
- Tamoxifen's adverse events……..
  ……
- conventional and/or alternative medicine……...
  ……

TAM's ADRs:

Symptoms	Onset

Part III: Levels of tamoxifen's metabolites

Tamoxifen.....ng/ml NDM.....ng/ml Endoxifen.....ng/ml

<u>Part IV: Polymorphism of CYP2D6 andUGT2B7</u>

CYP2D6 allele : *……/*…….

UGT2B7 allele : *……/*…….

Appendix B

Chromatograms of TAM, NDMT and END

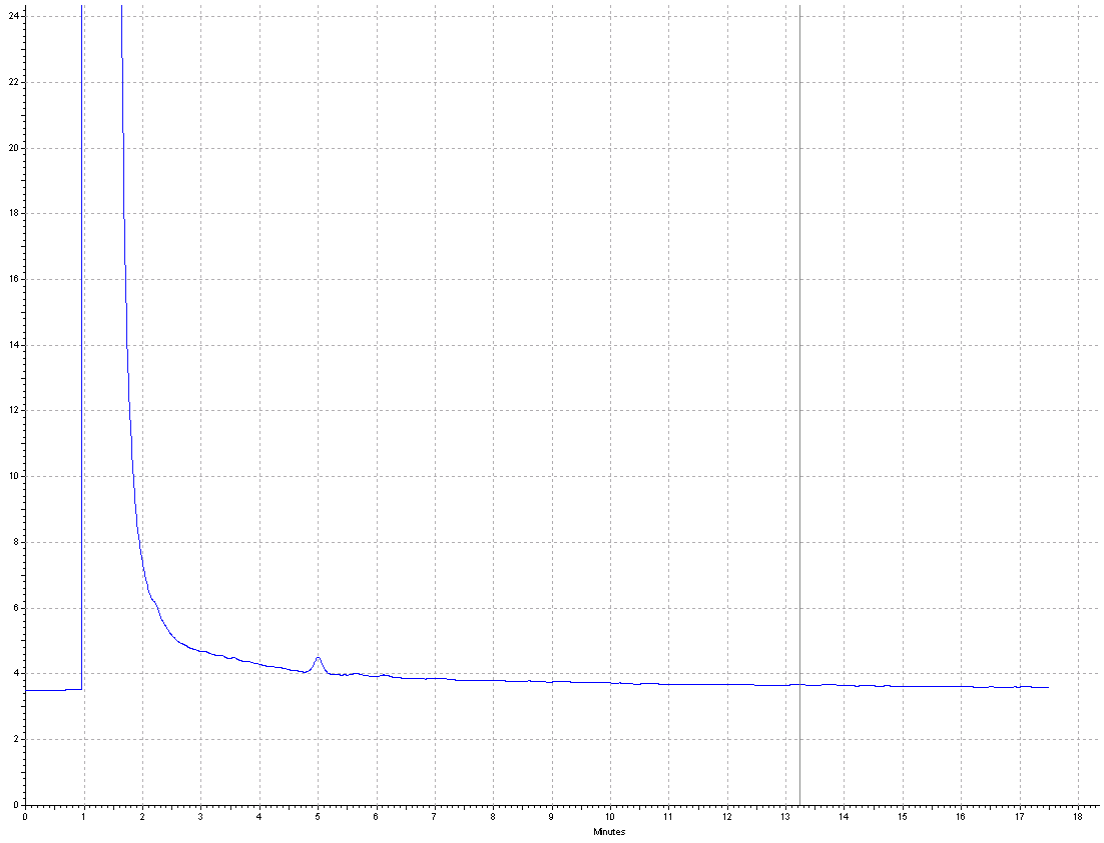


Figure 1: Chromatogram of drug-free-plasma.

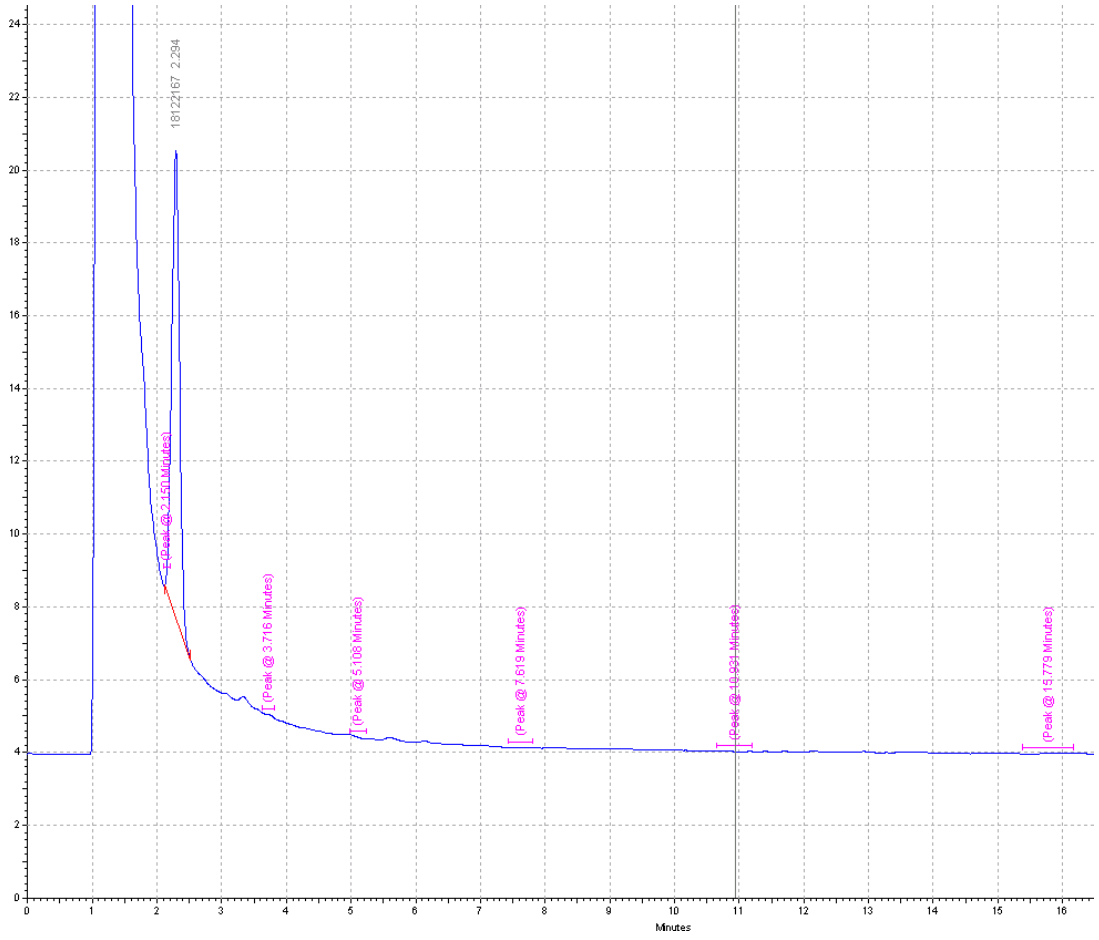


Figure 2: Chromatogram of drug-free-plasma with internal standard. Retention time (RT) of Internal Standard (IS) = 2.51 minutes

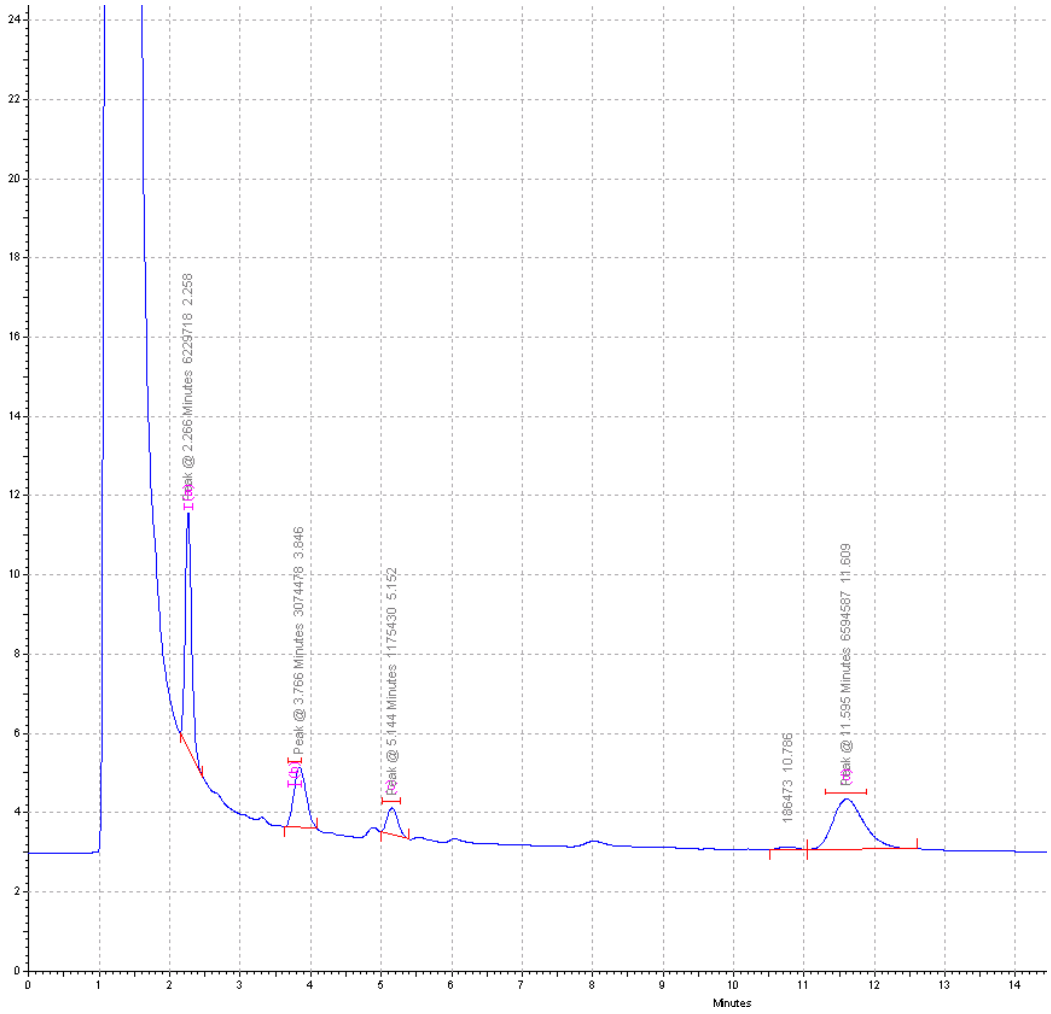


Figure 3: Chromatogram of drug-spiked-plasma (END 50 ng/ml, NDMT and TAM 250 ng/ml, respectively).

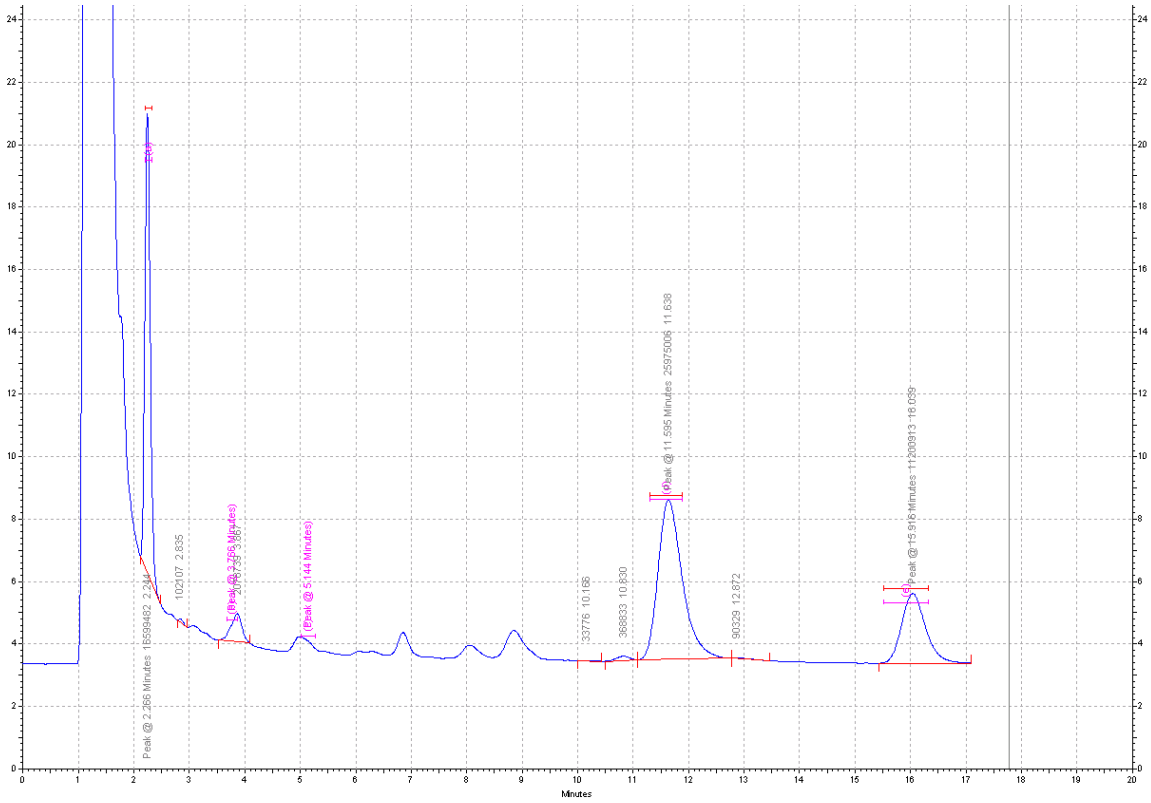


Figure 5: Chromatogram of plasma of TAM treated patients

Appendix C

Calibration curves of TAM, NDMT and END

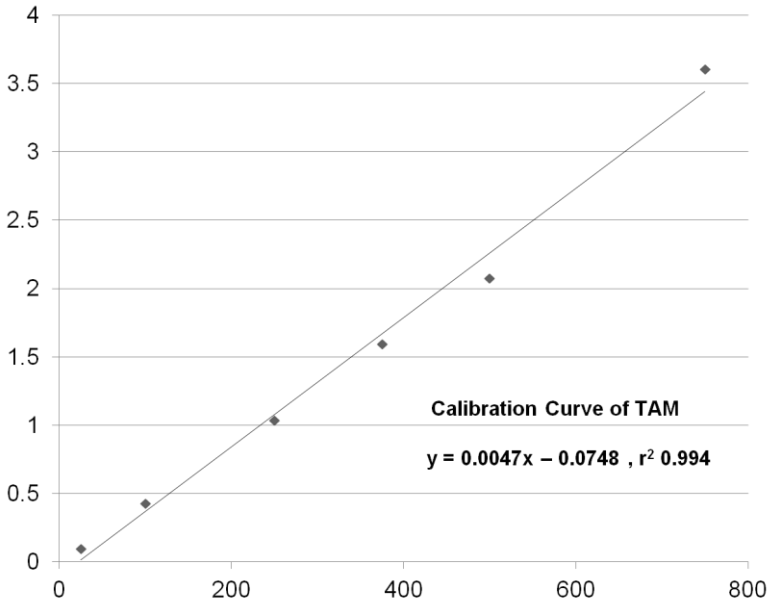


Figure 6 TAM calibration curve

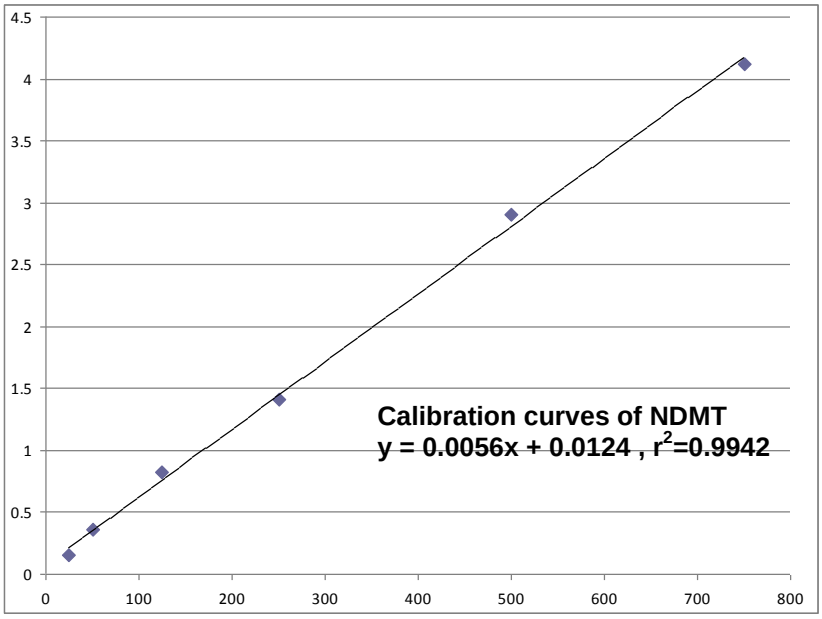


Figure 10 Calibration curve of NDMT

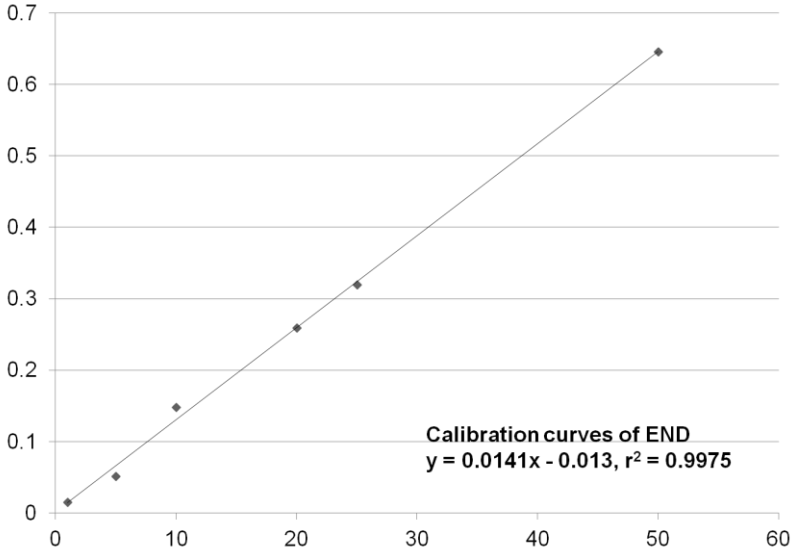
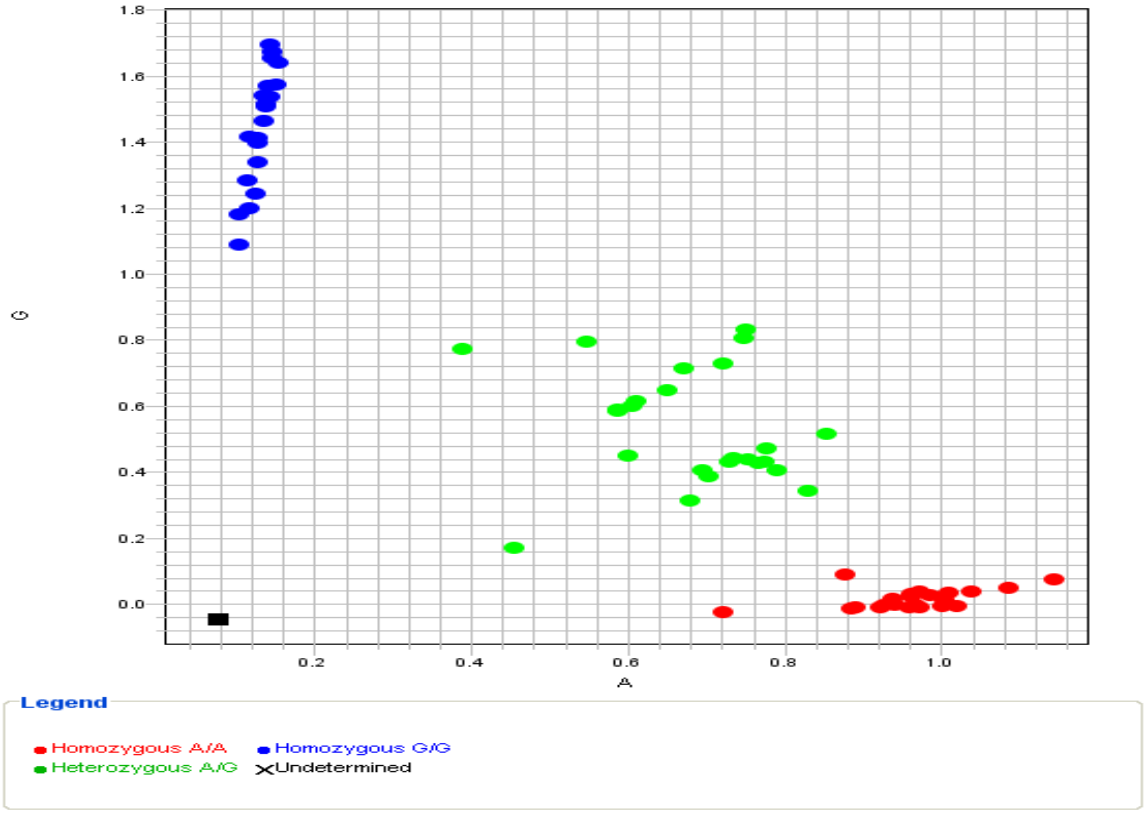


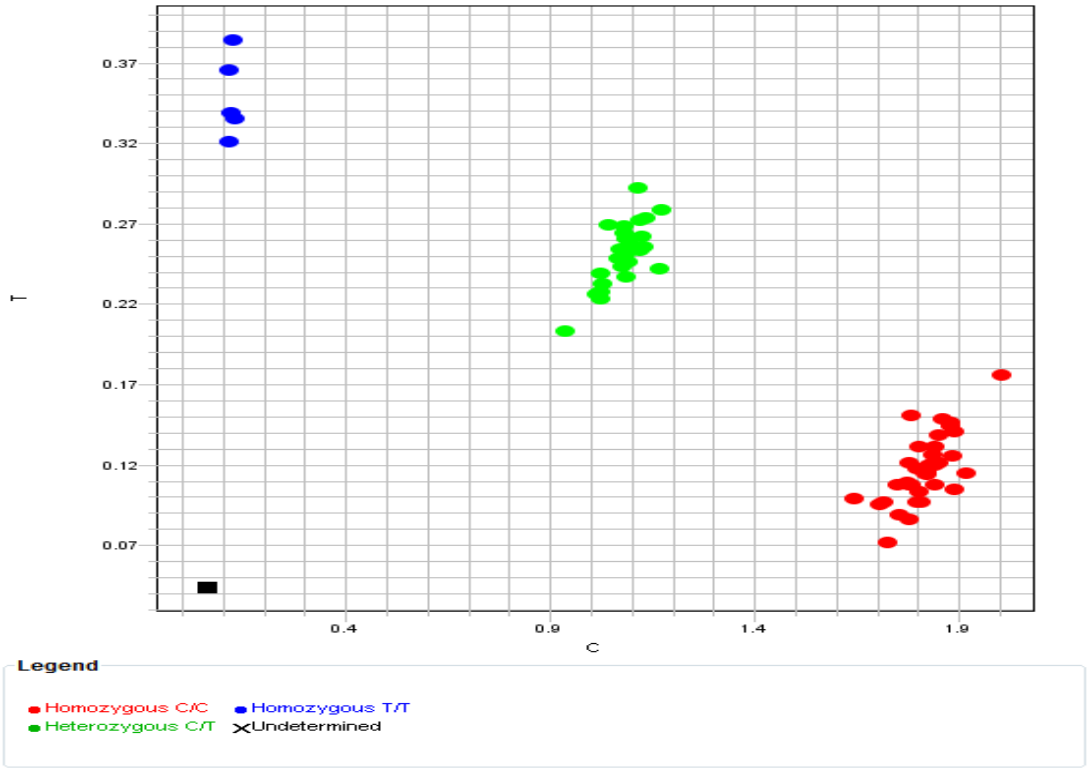
Figure 11 Calibration curve of END

Appendix D

CYP2D6 allele discrimination plot



UGT2B7 allele discrimination plot



Appendix E

CYP2D6 and *UGT2B7* genotypes with TAM and its metabolites of each patient

No	*CYP2D6*	*UGT2B7*	END	NDMT	TAM
1	*1/*10	*1/*2	35.91	87.19	280.26
2	*10/*10	*1/*1	6.95	667.02	487.32
3	*10/*10	*1/*1	14.12	687.86	562.43
4	*1/*10	*1/*1	6.27	441.42	272.75
5	*1/*10	*1/*2	15.67	429.44	47.51
6	*1/*10	*1/*2	11.95	195.31	144.90
7	*10/*10	*2/*2	31.36	306.73	314.21
8	*1/*10	*1/*1	19.76	298.61	340.36
9	*1/*10	*1/*2	42.12	223.31	289.63
10	*10/*10	*1/*1	10.85	973.23	609.48
11	*10/*10	*1/*1	8.20	480.76	251.03
12	*1/*1	*1/*2	6.97	584.37	409.12
13	*1/*1	*1/*1	47.18	557.13	512.21
14	*10/*10	*1/*1	10.02	618.25	454.42
15	*10/*10	*1/*2	11.53	803.14	574.61
16	*1/*10	*1/*1	37.71	477.22	133.58
17	*1/*1	*1/*1	23.20	612.84	384.84
18	*10/*10	*1/*2	66.15	182.70	279.52
19	*1/*10	*1/*1	11.23	532.70	231.92
20	*1/*10	*1/*1	3.47	230.26	149.04
21	*10/*10	*1/*2	7.86	537.00	258.66
22	*1/*1	*2/*2	28.74	372.76	264.18
23	*10/*10	*1/*2	6.52	477.89	315.10
24	*10/*10	*1/*2	1.88	370.95	300.35

No	*CYP2D6*	*UGT2B7*	END	NDMT	TAM
25	*1/*1	*1/*2	20.38	356.70	318.60
26	*10/*10	*1/*2	7.70	622.87	398.08
27	*1/*10	*1/*1	10.70	292.04	300.64
28	*10/*10	*1/*2	9.23	675.94	395.74
29	*1/*1	*1/*1	32.98	406.07	270.68
30	*10/*10	*2/*2	31.38	878.89	564.62
31	*1/*1	*1/*2	20.50	609.75	322.28
32	*10/*10	*1/*1	3.26	1355.71	853.27
33	*1/*10	*1/*2	11.75	779.85	533.94
34	*1/*1	*1/*1	9.64	360.68	209.74
35	*1/*1	*1/*1	40.53	467.02	373.35
36	*1/*10	*1/*1	16.51	556.42	362.96
37	*1/*10	*1/*1	25.34	967.67	659.12
38	*10/*10	*1/*2	22.17	916.03	543.48
39	*1/*10	*1/*2	16.31	583.26	558.11
40	*1/*10	*1/*2	12.13	649.47	542.99
41	*10/*10	*2/*2	18.84	909.22	611.14
42	*1/*1	*1/*1	21.27	491.59	323.59
43	*1/*10	*1/*2	15.33	463.36	360.38
44	*1/*1	*1/*1	28.97	338.88	332.80
45	*1/*10	*1/*1	13.58	724.14	503.51
46	*10/*10	*1/*1	2.33	156.22	228.49
47	*10/*10	*1/*2	7.46	599.74	358.02
48	*10/*10	*1/*1	16.54	780.90	385.74
49	*1/*10	*1/*2	21.90	411.48	399.56
50	*1/*10	*1/*1	9.86	720.43	409.57
51	*1/*10	*1/*1	17.04	597.46	318.14

No	*CYP2D6*	*UGT2B7*	END	NDMT	TAM
52	*1/*1	*1/*1	21.83	271.06	241.73
53	*1/*1	*1/*1	11.82	223.82	197.76
54	*1/*10	*1/*2	25.71	478.88	338.81
55	*1/*10	*1/*1	14.90	302.55	236.09
56	*1/*1	*1/*1	35.73	479.13	336.51
57	*1/*1	*1/*1	2.86	634.66	297.24
58	*1/*10	*1/*2	16.36	634.47	321.65
59	*1/*1	*2/*2	6.35	572.89	382.65

VITA

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