ความสัมพันธ์ระหว่างภาวะพหุสัณฐานของยีนที่นำส่งยาทีโนโฟเวียร์กับระดับยาทีโนโฟเวียร์ในพลาสมาในผู้ป่วยไทยที่ติดเชื้อเอชไอวี

นางสาวศิวพร มิตรรัก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ASSOCIATION BETWEEN POLYMORPHISMS OF TENOFOVIR TRANSPORTERS AND TENOFOVIR PLASMA LEVELS IN THAI HIV-INFECTED PATIENTS.

Miss Siwaporn Mitruk

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Clinical Pharmacy

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ศิวพร มิตรรัก : ความสัมพันธ์ระหว่างภาวะพหุสัณฐานของยีนที่ลำเลียงทีโนโฟเวียร์กับระดับทีโนโฟเวียร์ในพลาสมาในผู้ป่วยไทยที่ติดเชื้อเอชไอวี. (ASSOCIATION BETWEEN POLYMORPHISMS OF TENOFOVIR TRANSPORTERS AND TENOFOVIR PLASMA LEVELS IN THAI HIV-INFECTED PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ดร.บราลี ปัญญาวุธโธ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : พญ.อัญชลี อวิหิงสานนท์, 59 หน้า

มีหลายปัจจัยที่มีผลต่อระดับทีโนโฟเวียร์ในพลาสมา เช่น น้ำหนัก การทำงานของไต และความผันแปรทางพันธุกรรมของยีนที่ทำหน้าที่ลำเลียงยา แต่ในปัจจุบันยังไม่พบการศึกษาความสัมพันธ์ของปัจจัยเหล่านี้กับระดับยาในพลาสมา การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างความผันแปรทางพันธุกรรมของยีน *ABCC2* และ *ABCC4* รวมทั้งปัจจัยอื่น ๆ ได้แก่ น้ำหนัก อัตราการกรองของไต และการทำหน้าที่ผิดปกติของท่อไต กับระดับทีโนโฟเวียร์ในพลาสมา

การศึกษานี้เป็นการศึกษาแบบภาคตัดขวาง ทำการศึกษาในผู้ป่วยทั้งหมด 106 คน ซึ่งได้รับยาทีโนโฟเวียร์ด้วยขนาดคงที่เป็นเวลาอย่างน้อย 2 สัปดาห์ ตรวจวัดระดับทีโนโฟเวียร์ในพลาสมาด้วย HPLC และตรวจรูปแบบทางพันธุกรรมด้วย Taqman allelic discrimination assays ใช้สถิติ Independent t-test เพื่อวิเคราะห์ความแตกต่างของระดับทีโนโฟเวียร์ในพลาสมาระหว่างกลุ่มผู้ป่วยที่มีภาวะพหุสัณฐานของยีนที่แตกต่างกัน และใช้การวิเคราะห์ถดถอยพหุคูณเชิงเส้นเพื่อหาความสัมพันธ์ระหว่างปัจจัยต่าง ๆ ที่มีผลต่อระดับยาในพลาสมา

ผลการศึกษาพบว่าระดับทีโนโฟเวียร์ในพลาสมาของผู้ป่วยที่มีลักษณะยีน *ABCC4* 3463A>G และ 4131T>G แบบปกติมีความแตกต่างจากผู้ป่วยที่มียีนผิดปกติอย่างน้อย 1 อัลลีลอย่างมีนัยสำคัญทางสถิติ จากการวิเคราะห์ถดถอยพหุคูณเชิงเส้นพบว่าน้ำหนักและอัตราการกรองของไต มีความสัมพันธ์กับระดับทีโนโฟเวียร์ในพลาสมา อย่างไรก็ตามเมื่อนำปัจจัยทางพันธุกรรมมาพิจารณาร่วมกับน้ำหนักและอัตราการกรองของไต พบว่าภาวะพหุสัณฐานของยีน *ABCC4* ไม่มีผลต่อระดับยาในพลาสมา

ภาควิชา	เภสัชกรรมปฏิบัติ	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชกรรมคลินิก	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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Several factors including, body weight, kidney function and transporter polymorphisms are postulated that they can influence tenofovir levels. A present, there are no studies investigating the relationship between tenofovir transporter polymorphisms and these factors on drug plasma levels. This study aimed to investigate the influence of the *ABCC2*, *ABCC4* polymorphisms and other factors, including body weight, glomerular filtration rate (GFR) and kidney tubular dysfunction (KTD) on tenofovir plasma levels.

This is a cross sectional study performed in 106 patients who had stable tenofovir dosage regimen for at least 2 weeks. Tenofovir plasma levels were determined using HPLC method. Genotyping of *ABCC2* and *ABCC4* was carried out by Taqman allelic discrimination assays. Independent t-test was used to compare tenofovir plasma levels among groups of different polymorphisms. Multiple regression analysis was performed to investigate an association between factors and tenofovir plasma levels.

The results found that tenofovir plasma levels of patients with wild type of *ABCC4* 3463A>G and 4131T>G were significantly different from patients with at least 1 variant allele. The stepwise regression model showed that weight and GFR influence tenofovir plasma levels. However, after adjusting for weight and GFR, the influence of *ABCC4* polymorphisms was not found.

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

Field of Study : Clinical Pharmacy Advisor's Signature .....

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LIST OF ABBREVIATIONS

AUC	=	Area under the curve
Cmax	=	The maximum concentration
DNA	=	Deoxyribonucleic acid
GFR	=	Glomerular filtration rate
HPLC	=	High-performance liquid chromatography
HWE	=	Hardy Weinberg equilibrium
kg	=	Kilogram
L	=	Litters
µg	=	microgram
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
MLR	=	Multiple linear regression
ng	=	nanogram
OD	=	Optical density
PCR	=	Polymerase chain reaction
rs number	=	Reference single nucleotide polymorphisms number
Scr	=	Serum creatinine
SNPs	=	Single nucleotide polymorphisms
Tmax	=	Time to peak concentrations
VIF	=	Variance inflation factor

CHAPTER I

INTRODUCTION

Background and Rationale

Tenofovir is a nucleotide reverse transcriptase inhibitors (NtRTIs) widely used for the treatment of human immunodeficiency virus (HIV) - infection and chronic hepatitis B virus (HBV) infection.[1-4] For the treatment of HIV infection, tenofovir is selected for using as a first line drug.[3, 5] Tenofovir is highly effective, has low toxicity[6] and can be administered once daily.[1] Although tenofovir has few side effects, there are side effects that seriously affect the kidneys. It is reported that the use of tenofovir is associated with abnormalities of the kidneys with the incidence of 1.4% to 22%[7, 8], which cause the drug unsafe in long-term use.

When tenofovir was given 300 mg once daily, the median decrease in $\log_{10}$ HIV-1 RNA after 35 days of dosing was 1.22 $\log_{10}$ copies/mL. The median peak tenofovir concentration (C_{max}) was 326 ng/mL, and the trough tenofovir concentration was 40 ng/mL. When the dose lower than 300 mg/day was given, tenofovir plasma levels and a reduction of HIV-1 RNA levels was lower.[9]

In addition, studies have found that tenofovir plasma levels are associated with kidney tubular dysfunction.[10] Tenofovir plasma levels in patients with kidney tubular dysfunction were higher than in patients with normal kidney tubular function. Moreover, tenofovir plasma levels more than 160 ng/mL were found to be associated with kidney tubular dysfunction. Patients having tenofovir concentration greater than 160 ng/mL were 4.8 times greater risk of kidney tubular dysfunction compared to patients with tenofovir plasma concentration below 160 ng/mL.[10]

All of the mentioned above showed that tenofovir plasma levels correlate with efficacy and toxicity of the drug. Several factors may contribute to the variation of tenofovir levels, including patient characteristics, body weight, renal function and genetic variation.

Tenofovir disoproxil fumarate is a prodrug that is converted into an active form, tenofovir diphosphate. Tenofovir is primarily excreted by the glomerular filtration and tubular secretion via the proximal tubule. The elimination half-life was 17 hours after oral administration of a single dose of

tenofovir disoproxil fumarate 300 mg.[1, 11] The process of tubular secretion requires transporters, including organic anion transporter 1 (OAT1), organic anion transporter 3 (OAT3), multidrug resistance protein 2 (MRP2) and multidrug resistance protein 4 (MRP4). The organic anion transporters, mainly OAT1 and OAT3 influx transporters encoded by the solute carrier genes *SLC22A6* and *SLC22A8*,[11-13] are responsible for transporting tenofovir into the epithelial cells of kidney tubule through the basolateral membrane, and tenofovir is excreted into the urine via apical surface by MRP2 and MRP4 efflux transporter encoded by the adenosine triphosphate–binding cassette (ABC) genes *ABCC2* and *ABCC4* at the luminal membrane.[14-18] These transporters showed genetic polymorphisms among ethnicities.[19, 20] Therefore, the genetic variation of these transporters may affect the concentrations of drugs in the plasma and within cells, and influence the efficacy and risk of kidney dysfunction of the drug.

The association between genetic variation of drug transporters and plasma drug concentrations has been previously illustrated.[21, 22] Studies have shown a relationship between genetic variation of transporters and methotrexate plasma levels. Methotrexate is excreted via the kidneys by tubular secretion. The studies by Rau T, et al[21] and Hagleitner MM, et al[22] found that genetic variations of *ABCC2* gene are associated with methotrexate plasma levels. The same as methotrexate, tenofovir is excreted into the urine by *ABCC2* gene. Thus, genetic variations of these genes may also affect tenofovir plasma levels.

Even though, several factors i.e., patient characteristics, body weight, renal function and transporter polymorphisms are postulated that they may influence tenofovir levels, at present, there are no studies investigating the relationship of tenofovir transporter polymorphisms and other factors on drug plasma levels. Therefore, this study aimed to investigate the influence of the *ABCC2* and *ABCC4* polymorphisms and other factors, including body weight, glomerular filtration rate (GFR) and kidney tubular dysfunction (KTD) on tenofovir plasma levels. The results will be useful for individualizing dosage regimens of tenofovir that could, achieve the optimal drug levels to ensure efficacy and safety of the drug.

Hypothesis

1. Genetic factors including *ABCC2* and *ABCC4* polymorphisms are associated with tenofovir plasma levels.
2. Non-genetic factors including body weight, glomerular filtration rate (GFR) and kidney tubular dysfunction (KTD) are associated with tenofovir plasma levels.

Objective

1. To determine the prevalence of *ABCC2* and *ABCC4* genotypes.
2. To determine the relationship between genetic factors including *ABCC2* and *ABCC4* polymorphisms and tenofovir plasma levels.
3. To determine the relationship between non-genetic factors including body weight, GFR and KTD and tenofovir plasma levels.

Scope of this study

This study investigated the influence of genetic factors, *ABCC2* and *ABCC4* polymorphisms and other non-genetic factors, including body weight, GFR and KTD on tenofovir plasma levels in patients with HIV-infection at HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT).

Significance of the study

The results from this study can be use as prior information for tenofovir dose adjustment in Thai HIV-infected patients to achieve optimal plasma levels and prevent the possible adverse events.

Conceptual framework

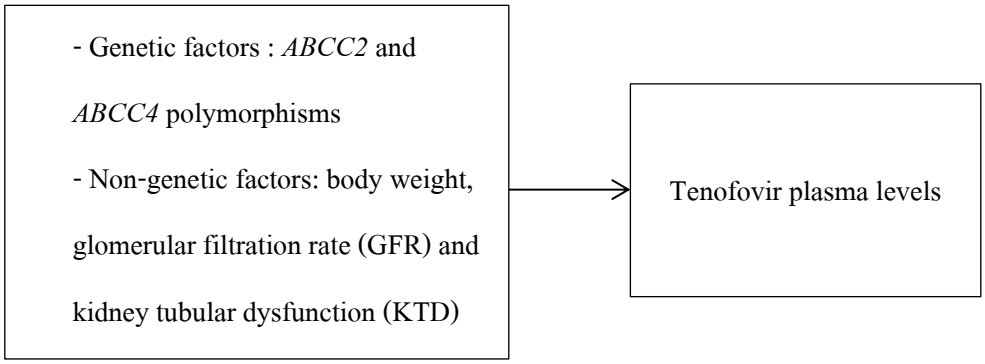

Figure 1 Conceptual framework

Limitation of this study

An application of the results from this study should be limited to specific patients having similar characteristics with the patients in this study.

Operational definition

1. Single Nucleotide Polymorphisms (SNPs) are genetic variations which is most common in the population. Each SNP refers to the change of a single nucleotide.[23]
2. Glomerular filtration rate (GFR) calculated from the Modification of Diet in Renal Disease (MDRD) Equation[24] : GFR (ml/min/1.73 $m.^2$) = 186.3 x $S_{Cr}^{-1.154}$ x Age $^{-0.203}$ x (0.742 for female)
3. Kideny tubular dysfunction (KTD) was defined by the presence of at least 2 of these abnormalities including:
   (1) nondiabetic glycosuria : urine glucose level > 300 mg daily
   (2) Tubular maximum Phosphate Reabsorption per Glomerular Filtration Rate (TmP/GFR) < 2.5
   (3) fractional tubular reabsorption of phosphate (1 - [(urine phosphorus X plasma creatinine)/ (plasma phosphorus X urine creatinine)]) < 0.82
   (4) total excretion of phosphorus (urine phosphorus X urine volume) > 1200 mg daily

(5) fractional excretion of uric acid ([(urine uric acid X plasma creatinine)/(urine creatinine X plasma uric acid)] X 100) > 15%

(6) β2-microglobulinuria : β2-microglobulin level > 1 mg daily

CHAPTER II

LITERATURE REVIEWS

1. Tenofovir

Tenofovir is an antiretroviral drug in a class of nucleotide reverse transcriptase inhibitors (NtRTIs), which inhibits the activity of human immunodeficiency virus 1 (HIV-1) reverse transcriptase and hepatitis B virus (HBV) polymerase by terminating the DNA chain. Tenofovir is administered orally in the form of a prodrug, tenofovir disoproxil fumarate. In plasma, tenofovir disoproxil fumarate requires diester hydrolysis for a conversion to tenofovir , which will be further transformed to tenofovir diphosphate in intracellular, which is an active drug as shown in figure 2 and 3.[26]

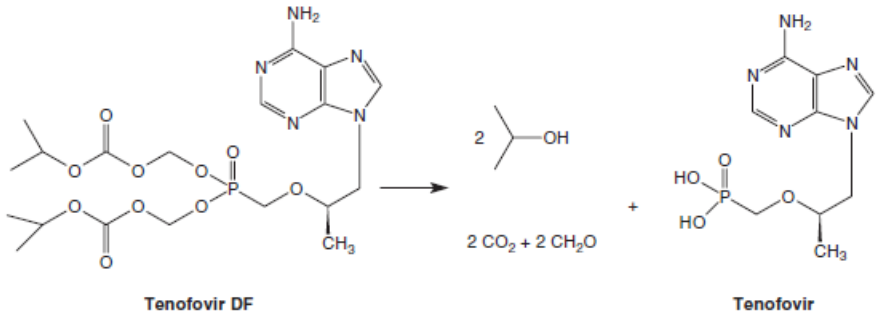

Figure 2 Conversion of tenofovir disoproxil fumarate to tenofovir.[26]

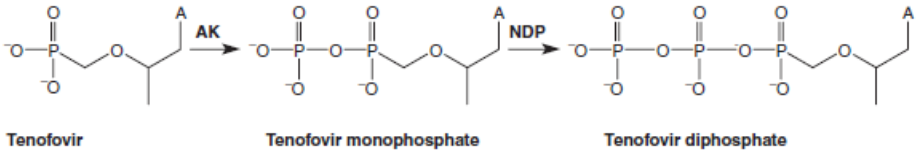

Figure 3 Intracellular anabolism of tenofovir to tenofovir diphosphate. A = adenine; AK = adenylate kinase; NDP = nucleoside diphosphate kinase.[26]

1.1 Dosage and administration

The recommended dose of tenofovir for the treatment of HIV-1 or chronic hepatitis B in patients aged ≥12 years and having weight ≥35 kg is 300 mg once daily taken orally, without regard to food. The recommended dose for a treatment of HIV infection in 2 to younger than 12 years is 8 mg/kg up to the maximum dose of 300 mg orally once daily. The dosing interval of tenofovir should be adjusted in patients with baseline creatinine clearance <50 mL/min, according to Table 1. For patients having creatinine clearance less than 10 mL/min and do not receive hemodialysis, dose recommendation is not available.[1, 25]

Table 1 Dosage Adjustment for Patients with Altered Creatinine Clearance.[1, 25]

	Creatinine Clearance (mL/min)			**Hemodialysis Patients**
	≥ 50	**30-49**	**10-29**	
Recommended 300 mg Dosing interval	Every 24 hours	Every 48 hours	Every 72 to 96 hours	Every 7 days or after a total of approximately 12 hours of dialysis

1.2 Pharmacokinetics

- Absorption[1, 25]
  - Tmax, oral: 1 hr
  - Bioavailability, oral: 25%
  - Effect of food: Tmax delayed by 1 hr; increased bioavailability with high-fat meal
- Distribution[1, 25]
  - Vd: 1.2 L/kg to 1.3 L/kg
  - Protein binding, human plasma: less than 0.7%; serum proteins: 7.2%
- Metabolism[1]
  - Minimal systemic metabolism

- Excretion[1, 25]
    - Renal: 70% to 80% unchanged (IV); 32% unchanged (oral)
    - Renal clearance: 243.5 mL/min
    - Renal clearance: 168.6 mL/min (mild renal impairment); 100.6 mL/min (moderate renal impairment); 43 mL/min (severe renal impairment)
    - Total body clearance: 203 mL/hr/kg
    - Total body clearance, Pregnant women, HIV-infected: 39% greater
- Elimination Half Life[1, 25]
    - Oral: 17 hr

The pharmacokinetic study found that when 300 mg of tenofovir was given, the mean peak plasma level (±SD) of tenofovir was 0.3 ± 0.09 microgram per milliliter (mcg/mL)[9] and mean trough plasma level was 0.064 mcg/mL.[36] Tenofovir is primarily excreted from the kidneys by glomerular filtration and tubular secretion via the proximal tubule.[1, 9, 26] The tubular secretion process requires drug transporters including organic anion transporter 1 (OAT1), organic anion transporter 3 (OAT3), multidrug resistance protein 2 (MRP2) and multidrug resistance protein 4 (MRP4).[27, 28] The organic anion transporters, mainly OAT1 and OAT3 influx transporters encoded by the solute carrier genes *SLC22A6* and *SLC22A8*,[11-13] are responsible for transporting tenofovir into the epithelial cells of kidney tubule through the basolateral membrane, and it is excreted to the urine via apical surface by MRP2 and MRP4 efflux transporter encoded by the adenosine triphosphate–binding cassette (ABC) genes *ABCC2* and *ABCC4* at the luminal membrane.[14-18] Protein transporters involved in tenofovir elimination at the proximal renal tubule are shown in Figure 4.

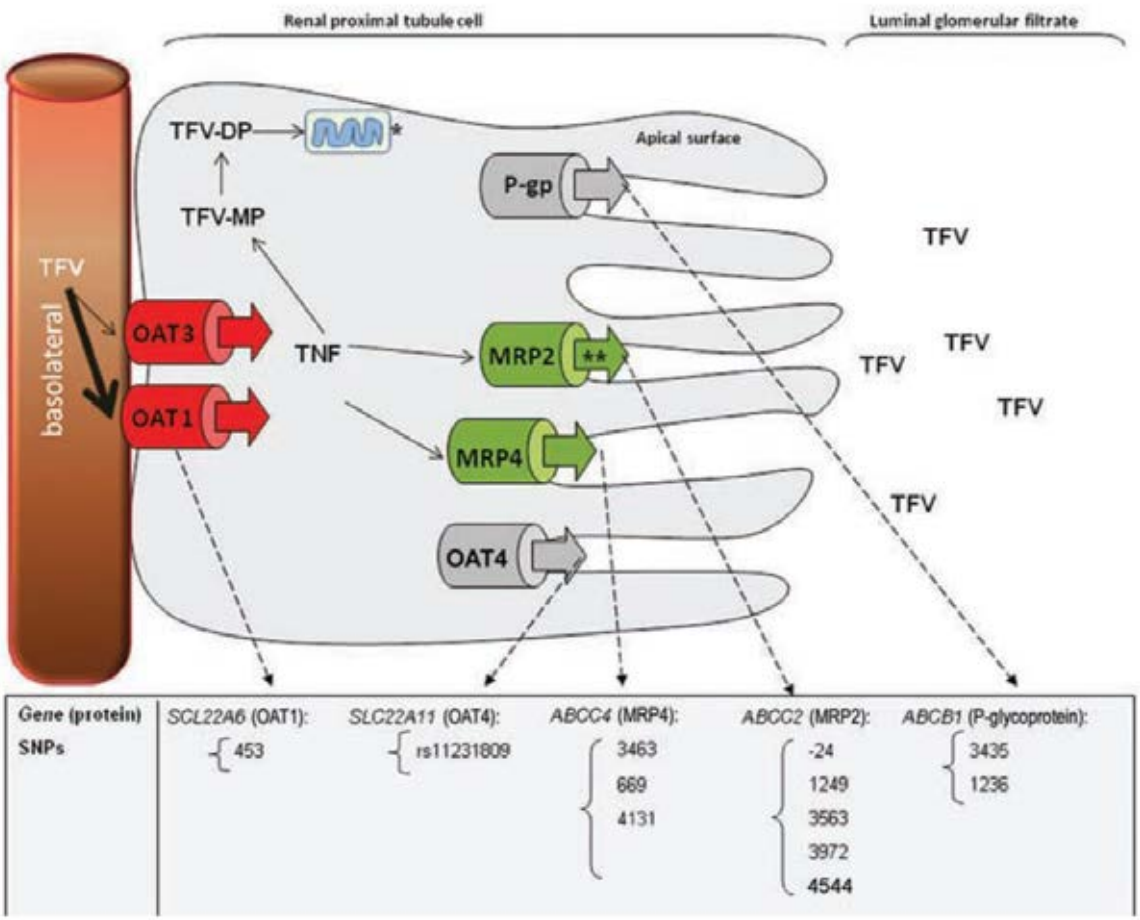

Figure 4 Protein transporters involved in tenofovir elimination at the proximal renal tubule. MRP2, multidrug-resistance protein 2; MRP4, multidrug-resistance protein 4; OAT1, organic anion transporter 1; OAT3, organic anion transporter 3; OAT4, organic anion transporter 4; P-gp, P-glycoprotein; SNP, single-nucleotide polymorphism; TFV-DP, tenofovir diphosphate; TFV-MP, tenofovir monophosphate.[11]

2. The relationship between tenofovir plasma levels and efficacy

Tenofovir plasma levels were shown to be related to its efficacy. Barditch-Crovo P et al[29] investigated the pharmacokinetics of tenofovir in HIV- infected patients. In this study, 49 patients were divided into five groups, each group received tenofovir 75, 150, 300, 600 mg and placebo for 35 days. The results found that the use of tenofovir 300 mg showed the greatest reduction of HIV-1 RNA levels in plasma (1.22 log10 copies/mL). The use of 600 mg/day showed an increase of tenofovir plasma levels, however the reduction of HIV-1 RNA levels in plasma was similar to the

dose of 300 mg/day (0.80 log 10 copies/mL). Moreover, the use of a lower than 300 mg/day of tenofovir could reduce tenofovir plasma levels and also decreases a reduction of HIV-1 RNA levels.

3. The relationship between tenofovir plasma levels and kidney tubular dysfunction

The important adverse reactions of tenofovir was toxicity to the kidney. The reported incidence of renal toxicity among patients receiving tenofovir was 1.4 to 22 %.[7, 8] Previous study found the relationship between tenofovir plasma levels and kidney toxicity. The study of Rodríguez-Nóvoa et al[10] in 18 patients with kidney tubular dysfunction and 74 patients with normal kidney tubular function, showed that tenofovir mean plasma level measured at 10-14 hours after dose administration in patients with kidney tubular dysfunction was significantly higher than those with normal kidney function (P = 0.001). Based on the results from this study, it was suggested that patients having tenofovir plasma levels greater than 160 ng/mL had a 4.8 times higher risk of kidney tubular dysfunction compared with patients having tenofovir plasma level below 160 ng/mL.

The results from these studies supported an evidence of the relationship between tenofovir plasma levels and efficacy and toxicity. Therefore, the study investigating factors influencing tenofovir plasma levels is important. Further study is required in order to identify factors that could affect the pharmacokinetics of tenofovir.

4. Factors contribute to the variation of tenofovir plasma levels.

Several factors, including genetics and non-genetic factors, may contribute to the variation of tenofovir plasma levels. The study of Gagnieu MC et al[30] found that body weight and serum creatinine (Scr) affected tenofovir plasma levels. Moreover, the study of Jullien V et al[39] showed that body weight/serum creatinine ratio was related to apparent clearance of tenofovir. Furthermore, an administration of TDF with high fat meal (1,000 kcal, 50% fat) resulted in a 40% increase of AUC_{∞} and 14% higher of Cmax.[26]

Even though, tenofovir was mainly excreted unchanged into the urine and, therefore drug-drug interaction is unlikely. Studies found some of the co-medications including protease inhibitors, and tacrolimus may altered tenofovir plasma levels. A 24% and 32% increase of tenofovir

AUC when co-administered with atazanavir and ritonavir-boosted lopinavir was reported.[1,25] Additionally, a 14% and 13% increase of tenofovir Cmax was observed when tenofovir was co-administered with indinavir and tacrolimus, respectively.[25] The interactions between tenofovir and co-administered drugs are presented in Table 2.

Table 2 Drug Interactions: Changes in Pharmacokinetic Parameters for Tenofovir[a] in the Presence of Coadministered Drug[1,25]

Coadministered Drug	Dose of Coadministered Drug (mg)	N	% Change of Tenofovir Pharmacokinetic Parameters[b] (90% CI)		
			C_{max}	AUC	C_{min}
Abacavir	300 once	8	-	-	NC
Atazanavir[c]	400 once daily × 14 days	33	↑ 14 (↑ 8 to ↑ 20)	↑ 24 (↑ 21 to ↑ 28)	↑ 22 (↑ 15 to ↑ 30)
Didanosine[d]	250 or 400 once daily × 7 days	14	-	-	-
Emtricitabine	200 once daily × 7 days	17	-	-	-
Entecavir	1 mg once daily x 10 days	28	-	-	-
Indinavir	800 three times daily × 7 days	13	↑ 14 (↓ 3 to ↑ 33)	-	-
Lamivudine	150 twice daily × 7 days	15	-	-	-
Lopinavir/ Ritonavir	400/100 twice daily × 14 days	24	-	↑ 32 (↑ 25 to ↑ 38)	↑ 51 (↑ 37 to ↑ 66)
Saquinavir/ Ritonavir	1000/100 twice daily × 14 days	35	-	-	↑ 23 (↑ 16 to ↑ 30)
Tacrolimus	0.05 mg/kg twice daily x 7 days	21	↑ 13 (↑ 1 to ↑ 27)	-	-

a. Subjects received VIREAD 300 mg once daily.,b. Increase = ↑; Decrease = ↓; No Effect = - ; NC = Not Calculated

c. Reyataz Prescribing Information, d. Subjects received didanosine buffered tablets.

Genetic factor could be one of the important factors contributes to the variability of drug plasma level which may affect drug's efficacy and toxicity. For drugs excreted by renal tubular secretion, such as methotrexate and olmesartan, it was found that genetic variation of renal efflux transporters including *ABCC2* -24C>T and *ABCC2* 3972C>T could affect drug plasma levels. A study by Rau T et al[21] investigating the influence of genetic variation of *ABCC2* genes with methotrexate plasma levels in children with acute lymphoblastic leukemia, found that the mean methotrexate area under the curve from 36 to 48 hours among female patients having *ABCC2* -24 C> T polymorphism was 2-fold higher than all other patients (P<0.001).

Moreover, Hagleitner MM et al[22] studied the relationship between genetic variation of transporters and methotrexate plasma levels in 49 patients with acute lymphoblastic leukemia and 43 patients with osteosarcoma. Of 41 SNPs investigated, the association between *ABCC2* 3972C>T and methotrexate plasma level was observed. The patients carrying *ABCC2* 3972TT had a higher methotrexate plasma level compared with those having *ABCC2* 3972CC.

In addition, a previous study determining the relationship between *ABCC2, SCLO1B1* and *ABCG2* polymorphisms with olmesartan plasma levels in 68 healthy male volunteers found that the peak plasma level and area under the plasma concentration-time curve of *ABCC2* -24CT genotype group were significantly higher than *ABCC2* -24CC genotype group.[31]

For tenofovir, the study investigating the relationship between plasma levels and genetic variation of transporter is not established. However, the relationship between genetic variation of transporters and intracellular tenofovir diphosphate concentrations was observed.[32]

5. The relationship between intracellular tenofovir diphosphate concentrations and genetic variations of tenofovir transporters

Kiser JJ et al[32] investigated the relationship between intracellular tenofovir diphosphate concentrations with the polymorphisms of drug transporter genes, including *ABCC2, ABCC4* and *SLC22A6* in 30 HIV-infected patients. Six single nucleotide polymorphisms (SNPs) including *ABCC2* -24C>T, *ABCC2* 1249G>A, *ABCC4* 3463A>G, *ABCC4* 4131 T>G, *SLC22A6* 728G>A and *SLC22A6* 453G>A were investigated. The study found that intracellular tenofovir diphosphate concentrations of

patients with *ABCC4* 3463AA genotype were significantly lower than those with AG or GG genotype (P = 0.04).

6. The relationship between tenofovir plasma levels and intracellular tenofovir diphosphate concentrations

A relationship between tenofovir plasma levels and and intracellular tenofovir diphosphate (TFV-DP) was observed. Baheti G et al[37] investigated the relationship between plasma tenofovir (TFV) and intracellular tenofovir diphosphate (TFV-DP) concentrations in 55 HIV-infected patients. The result found that an indirect response model can best describe the relationship between tenofovir plasma and intracellular TFV-DP levels, in which an increase in tenofovir plasma levels results in an increase of intracellular concentrations. In consistent with a study by Pruvost et al,[38] this found that when plasma exposure of TFV increased by 50%, the intracellular TFV-DP AUC_{0-4} was increased by 59%.[38]

7. The relationship between genetic variations of tenofovir transporters and kidney tubular dysfunction

Besides an evidence of the association between genetic variation of drug transporters and plasma levels, the association of drug transporters' polymorphisms and toxicity was observed. Several studies found a relationship between genetic variations of tenofovir transporters and kidney tubular dysfunction.

A case-control study by Izzedine H et al[18] investigated a relationship between genetic variations of *ABCC2*, *ABCC4* and *ABCB1* genes (*ABCC2* -24C>T; 1058G>A; 1249 G>A; 3563 T>A; 3972 C>T; 4544 G>A, *ABCC4* 559G>T; 669C>T; 912 G>T; 951G>A; 969G>A; 1497C>T; 3310T>C; 3348A>G; 3609G>A, *ABCB1* 1236C>T; 2677G>T/A; 3435C>T) and renal proximal tubulopathy in 30 HIV-infected patients receiving tenofovir. Of 30 HIV-infected patients (29 Whites and 1 African American), 13 had renal proximal tubulopathy. Eighteen SNPs from three drug transporters including *ABCC2, ABCC4* and *ABCB1* were investigated. The results showed that genetic variation of *ABCC2* 1249G>A significantly associated with renal proximal tubulopathy.

A cross-sectional study by Rodríguez-Nóvoa S et al[11] investigated the relationship between the polymorphisms of *ABCC2, ABCC4, ABCB1, SLC22A6* and *SLC22A11* genes (*ABCC2* -24C>T; 1249G>A; 3563T>A; 3972 C>T; 4544G>A, *ABCC4* 669C>T; 3463 A>G; 4131 T>G, *ABCB1* 3435 C>T; 1236 C>T, *SLC22A6* 453G>A, *SLC22A11* rs11231809) and kidney tubular dysfunction in HIV-infected patients treated with tenofovir. Among 115 patients included in the study, there were 103 White patients, 2 Black patients and 10 other ethnicities. In this study, 19 patients had kidney tubular dysfunction (16.5%) and 96 patients had normal kidney tubular function (83.5%). The results demonstrated that the number of patients with kidney tubular dysfunction was significantly higher in a group of patients having *ABCC2* -24CC genotype, compared with *ABCC2* -24CT or TT genotypes (P = 0.02).

A cohort study by Nishijima T, et al[33] investigated the relationship between polymorphisms of *ABCC2, ABCC4, ABCC10, ABCB1*, and *SLC22A6* genes with the occurrence of kidney tubular dysfunction in 190 Japanese patients with HIV-infection treated with tenofovir. Among 190 patients, 19 patients had kidney tubular dysfunction(10%) and 171 patients had normal kidney tubular function (90%). The polymorphisms of five drug transporters, including *ABCC2, ABCC4, ABCC10, ABCB1* and *SLC22A6* (*ABCC2* -24C>T; 1249G>A; 2366C>T; 2934G>A, *ABCC4* 559G>T; 912G>T; 2269G>A; 3348A>G; 4135T>G; 4976T>C, *ABCC10* 526G>A; 2759T>C, *SLC22A6* 180C>T, and *ABCB1* 2677T>A/G) were analyzed. The results demonstrated a significant association between CC genotype at position -24 and AA genotype at position 1249 of *ABCC2* gene and kidney tubular dysfunction. This study is considered to be the first study investigating the association of drug transporter polymorphisms and tenofovir-associated kidney tubular dysfunction in Asian population.

It should be noted that, none of these studies measured tenofovir plasma levels, even though, a relationship between tenofovir plasma levels and kidney tubular dysfunction was presented.[10] Based on the results from these studies, it is possible that tenofovir-associated renal toxicity could be a result of a high plasma level of tenofovir due to genetic variation. However, the relationship between tenofovir plasma levels, genetic variations and kidney tubular dysfunction requires further investigation.

As there is an evidence of the association between drug transporters and plasma levels, which would be associated with its efficacy and toxicity, the study investigating factors that influence the pharmacokinetics of tenofovir will be useful for dose adjustment. Therefore, this study aimed to investigate the influence of genetic variations of tenofovir transporters and other factors on tenofovir plasma levels. The results of this study will be useful for tenofovir dose adjustment to obtain the optimal plasma levels and reduce adverse events.

CHAPTER III

PATIENTS AND METHODS

1. Study design

This is a cross-sectional study. The data used in the analysis were part of the study entitled "Incidence and predictor of tenofovir diproxilfumarate (TDF) associated nephrotoxicity and pharmacokinetic of TDF in HIV-1 infected Thai patients study", in which study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2. Patients

2.1 Population and samples

2.1.1 The population is HIV-1 infected Thai patients receiving tenofovir as part of the antiretroviral therapy .

2.1.2 The samples are HIV-1 infected Thai patients receiving tenofovir as part of the antiretroviral therapy at the HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT) during 1 January to 1 September 2012.

2.2 Inclusion and exclusion criteria

This study contained two parts. The first study aimed to determine the prevalence of *ABCC2* and *ABCC4* polymorphisms in Thai patients. The inclusion criteria were :

- Patients with HIV-1 infection receiving tenofovir as part of the antiretroviral therapy at HIV- NAT during 1 January to 1 September 2012.
- Patients participating in the study "incidence and predictor of TDF associated nephrotoxicity and pharmacokinetic of TDF in HIV-1 infected Thai patients".

The second study aimed to determine the relationship between genetic factors including *ABCC2, ABCC4* polymorphisms and non-genetic factors including body weight, glomerular filtration rate (GFR) and kidney tubular dysfunction (KTD) with tenofovir plasma

levels. The patients from the first study who met the inclusion and exclusion criteria as shown below were enrolled in the second study.

- Patients aged greater than 18 years.
- Patients who were treated with 300 mg/day of tenofovir for at least 2 weeks.
- Patients who had tenofovir plasma levels measured at 10-14 hours after receiving a dose of tenofovir, and had peripheral blood mononuclear cells (PBMC) samples at HIV-NAT laboratory for genetic testing.
- Patients with asymptomatic kidney tubular dysfunction
- All participants consented to enroll in the study.

The exclusion criteria for the second study were as follows:

- Patients with incomplete record of demographic data including age, sex, body weight and serum creatinine.
- Patients with GFR < 50 ml/min/1.73 m^2
- Patients who were receiving concomitant use of protease inhibitors and/or other co-medication that may have interaction with tenofovir including tacrolimus, acyclovir, valacyclovir and ganciclovir.

2.3 Sample size determination

The sample size was calculated according to the estimated sample size for multiple linear regression equation: $N \geq 15p$ [35]

Where N was the sample size of the patients and p was the number of tested variables including: *ABCC2* -24C>T, *ABCC2*1249G>A, *ABCC4* 3463A>G, *ABCC4* 4131 T>G, body weight, glomerular filtration rate (GFR) and kidney tubular dysfunction (KTD)

Therefore $N \geq 15 \times 7$

$N \geq 105$

The sample size of this study was at least 105 patients.

3. Study protocol

3. 1 Patients were enrolled according to inclusion and exclusion criteria.

3. 2 Patient demographic and tenofovir plasma levels data were extracted from the HIV-NAT database system.

3. 3 DNA were extracted from PBMC samples by QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, German)

3. 4 *ABCC2* and *ABCC4* genotyping were determined by Taqman allelic discrimination assays at Chula Medical Research Center and department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3. 5 The relationship between genetic and non-genetic factors with tenofovir plasma levels was determined by independent t-test and multiple linear regression (MLR).

3. 6 Discussions and conclusion.

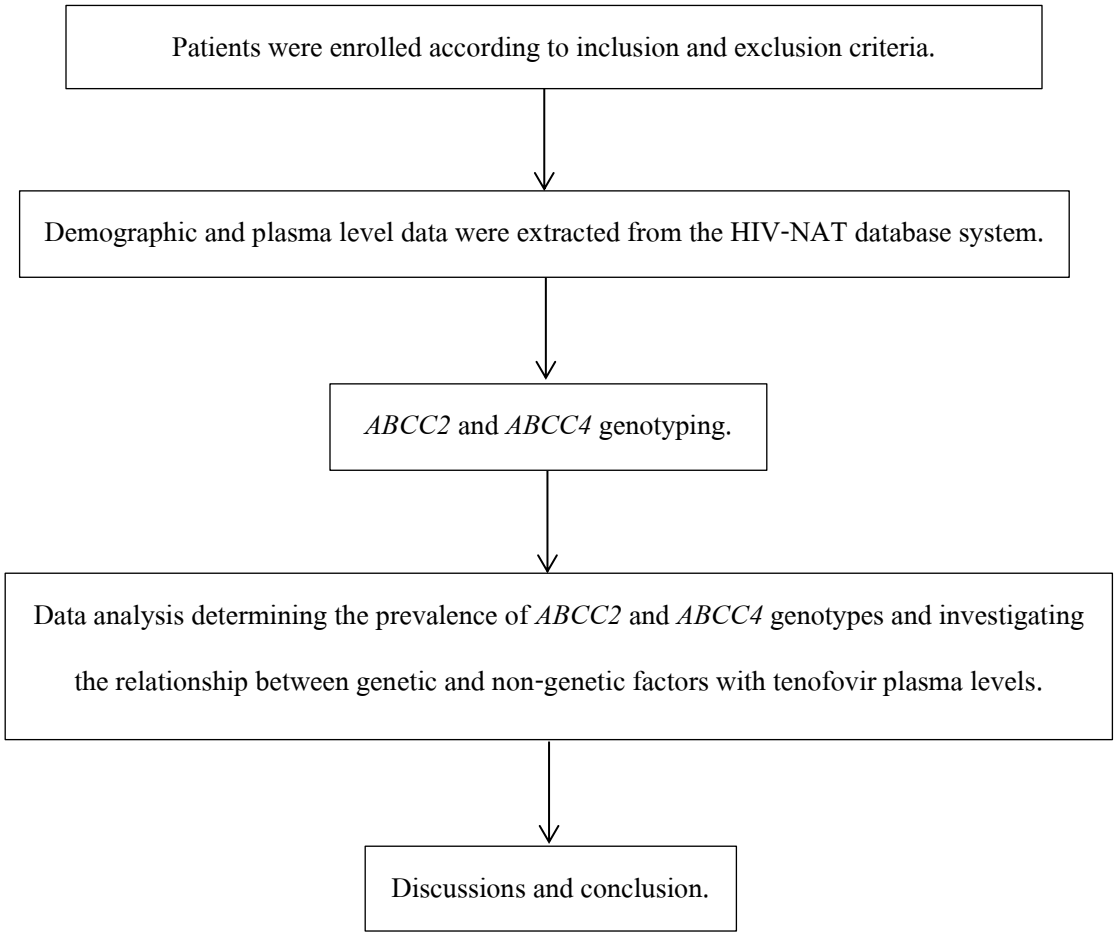

Figure 5 Flow chart of the study protocol

4. Bioanalysis

4.1 Tenofovir plasma levels

Tenofovir levels were determined by using a validated high-performance liquid chromatography assay with a fluorimetric detector by a modified method, previous described by Droste JA et al.[34] The analysis of tenofovir plasma levels was performed in laboratory of HIV-NAT. The detailed procedures and method validation were presented in Appendix B.

4.2 *ABCC2* and *ABCC4* genotyping

4.2.1 DNA extraction

Peripheral blood mononuclear cells (PBMC) were used for DNA extraction by QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, German) using a procedure recommended by the manufacturer. The materials and methods for DNA extraction were presented in Appendix C.

4.2.2 Determination of concentration, yield, and purity

DNA yields are determined from the concentration of DNA in the elution, measured by absorbance at 260 nm. Absorbance readings at 260 nm should be between 0.1 and 1.0. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7–1.9. The measure of DNA quantification and quality was performed by optical density (OD) measurement as follows:

1) Dilute a sample of DNA isolation to 1:5, by using DNA 20 µL and deionized water (dH_2O) 80 µL.
2) Prepare dH_2O 100 µL for control
3) Set spectrophotometer at 260 and 280 nm.
4) Calculate OD 260/280 ratio to determine purity and estimate the concentration of DNA according to the formula:

   DNA concentration (µg/mL or ng/µL) = OD260 x 50 µg/mL x dilution

4.2.3 *ABCC2* and *ABCC4* genotyping

Four SNPs including *ABCC2* -24C>T, *ABCC2* 1249G>A, *ABCC4* 3463 A>G and *ABCC4* 4131 T>G were genotyped. All genotyping was determined by Taqman allelic discrimination assays with specific probe and primer (designed by Applied Biosystems, California, USA). All reactions were analyzed by the Step One Plus® Real-Time polymerase chain reaction (PCR) System. The detailed procedures were presented in Appendix D.

5. Data analysis

The data analysis was determined by the Statistical Package for Social Sciences software (SPSS version 17, SPSS Co., Ltd., Bangkok Thailand). The significance level of 0.05 was used as criteria for justification of statistical significance. The data were analyzed as follows:

1) Demographic data were presented as the mean ± standard deviation (SD) for continuous data or percentage and frequency for categorical data.
2) Prevalence of *ABCC2* and *ABCC4* genotypes were determined as a frequency (%). The deviation from Hardy-Weinberg equilibrium was determined by Chi-square test. Allele frequencies were compared between populations using Chi-square test.
3) The comparisons of tenofovir plasma levels between 2 groups of patients (group 1 is patients with homozygous wild type and group 2 is patients with at least one variant allele) were analyzed by independent t-test.
4) The influence of genetic (*ABCC2* and *ABCC4* polymorphisms) and non-genetic (body weight, glomerular filtration rate and Kidney tubular dysfunction) factors on tenofovir plasma levels was determined by multiple linear regression with stepwise method.

CHAPTER IV

RESULTS

This study investigated the influence of genetic factors including *ABCC2, ABCC4* polymorphisms and non-genetic factors including body weight, glomerular filtration rate (GFR), and kidney tubular dysfunction (KTD) with tenofovir plasma levels in patients with HIV-infection receiving treatment at the HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT) during 1 January to 1 September 2012, which participated in the incidence and predictor of TDF associated nephrotoxicity and pharmacokinetic of TDF in HIV-1 infected Thai patients study.

1. Population allelic frequencies

For the allele frequency analysis, a total of 400 PBMC samples from HIV-infected patients participating in the study "incidence and predictor of TDF associated nephrotoxicity and pharmacokinetic of TDF in HIV-1 infected Thai patients" were genotyped and included in the analysis.

1.1 Demographic data of patients (N = 400)

Of 400 patients enrolled in this study, 180 patients were female (45%) and 220 patients were male (55%). The average age (± standard deviation) of participants was 43.47 ± 7.85 years. The summary of patient characteristics is presented in Table 3.

Table 3 Demographic data of patients enrolled in the study (n=400 patients)

Characteristics	**Frequency (mean ± S.D.)**	**% (range)**
Gender - female - male	180 220	45 55
Age (years)	(43.47 ± 7.85)	(22-71)
Weight (kg)	(60.31 ± 12.12)	(37.6-117.7)
Height (cm.)	(163.37 ± 8.32)	(145-185)
BMI (kg/m^2)	(22.34 ± 3.76)	(15.28-36.73)
GFR ($ml/min/1.73\ m^2$)	(93.66 ± 19.59)	(32.69-135.68)
Patients with KTD	52	13

1.2 The results of SNPs analysis

The results from SNPs analysis (N=400) showed that the frequencies of the homozygous wild-type of *ABCC2* -24C>T; 1249G>A, *ABCC4* 3463A>G and 4131T>G were 5.2%, 1%, 4% and 25.2% respectively. The frequency of the heterozygous variant of *ABCC2* -24C>T; 1249G>A, *ABCC4* 3463A>G and 4131T>G were 33%, 13.5%, 31.5% and 48% respectively.

Therefore, the allele frequencies of *ABCC2* -24C>T; 1249G>A, *ABCC4* 3463A>G and 4131T>G were 21.8%, 7.8%, 19.8% and 49.2% respectively. All polymorphisms were in Hardy-Weinberg equilibrium (P>0.05). The genotype frequencies of *ABCC2* and *ABCC4* genes are shown in Table 4.

Table 4 Genotype frequencies of *ABCC2* and *ABCC4* in Thai patients (n=400)

Gene (Protein)	Polymorphisms (SNP identification)	Genotype	Frequency	%	P value*
ABCC2 **(MRP2)**	1249G>A (rs2273697)	GG	342	85.5	0.264
		AG	54	13.5	
		AA	4	1.0	
	-24C>T (rs717620)	CC	247	61.8	0.542
		CT	132	33.0	
		TT	21	5.2	
ABCC4 **(MRP4)**	3463A>G(rs1751034)	AA	258	64.5	0.900
		AG	126	31.5	
		GG	16	4.0	
	4131T>G (rs3742106)	TT	107	26.8	0.426
		TG	192	48.0	
		GG	101	25.2	

*Test for Hardy-Weinberg Equilibrium, Chi-square test

1.3 Comparison of *ABCC2* and *ABCC4* allele frequencies among different populations.

The comparison of the allele frequencies of *ABCC2* and *ABCC4* between Thai population and those previously reported from other populations are shown in Table 5 and Table 6. The results showed that the allele frequencies of *ABCC2* in this population were similar to those observed in other Asian populations. However, the allele frequency of *ABCC2* 1249G>A in this population was lower than those reported in Caucasian population (7.8% vs23.3%).[20] For *ABCC4*, the allele frequency of *ABCC4* 3463A>G was lower than those reported in Japanese population (19.8% vs 34.3%)[20], whereas the frequency of *ABCC4* 4131T>G was significantly higher than the frequency observed in African population.[20]

Table 5 Comparison of *ABCC2* allele frequencies between Thai and other populations

Gene (Protein)	**Polymorphisms (SNP identification)**	**Ethnicity**	**Sample Size**	**Allele frequency (%)**		**P value***
ABCC2	-24C>T (rs717620)			C	T	
(MRP2)		Thai (this study)	400	78.2	21.8	
		Thai[20]	376	73.1	26.9	0.051
		Chinese[20]	168	78.6	21.4	0.863
		Mexican[20]	154	75.3	24.7	0.617
		European[20]	330	80.3	19.7	0.728
ABCC2	1249G>A			G	A	
(MRP2)	(rs2273697)	Thai (this study)	400	92.2	7.8	
		Thai[20]	376	96.0	4.0	0.248
		Japanese[33]	190	86.0	14.0	0.175
		Chinese[20]	168	89.9	10.1	0.621
		Mexican[20]	154	87.0	13.0	0.249
		European[20]	330	76.7	23.3	0.003

*Chi-square test

Table 6 Comparison of *ABCC4* allele frequencies between Thai and other populations

Gene (Protein)	Polymorphisms (SNP identification)	Ethnicity	Sample Size	Allele frequency (%)		Pvalue*
ABCC4	3463A>G			A	G	
(MRP4)	(rs1751034)	Thai (this study)	400	80.2	19.8	
		Japanese[20]	172	65.7	34.3	0.026
		Chinese[20]	166	81.9	18.1	0.718
		African[20]	166	78.3	21.7	0.728
		European[20]	330	86.7	13.3	0.182
ABCC4	4131T>G (rs3742106)			T	G	
(MRP4)		Thai (this study)	400	50.8	49.2	
		Chinese[20]	168	46.4	53.6	0.479
		African[20]	166	65.1	34.9	0.045
		Caucasian[19]	46	58.0	42.0	0.320

*Chi-square test

2. Effect of genetic factors and non-genetic factors on tenofovir plasma levels

2.1 Demographic data of patients (N=106)

Of 106 patients enrolled in this study, 45 patients were female (42.4%) and 61 patients were male (57.6%). The average age (± standard deviation) of participants was 41.6 ± 7.1 years. The average body weight (± standard deviation) was 59.9 ± 11.1 kilograms. The average of glomerular filtration rate (GFR) was 101.2 ± 18.7 ml/min/1.73 $m.^2$ The summary of antiretroviral drug regimens were:

Lamivudine (3TC) + Efavirenz (EFV) + Tenofovir (TDF) (n=68),

3TC+Nevirapine (NVP) +TDF (n=8),

EFV+FTC+TDF (n=24),

EFV+Zidovudine(ZDV) + TDF (n=6).

The summary of demographic data is presented in Table 7.

Table 7 The summary of demographic data (N=106)

Characteristics	Frequency, (mean±SD)	%, (range)
Age (years)	(41.6± 7.1)	(23.7-61.3)
Gender		
- Female	45	42.4
- Male	61	57.6
Body weight (kg)	(59.9 ± 11.1)	(38-100)
Height (cm.)	(163.7 ± 7.4)	(147-180)
BMI (kg/m^2)	(22.3 ± 3.4)	(15.4-36.7)
GFR($ml/min/1.73\ m^2$)	(101.2 ± 18.7)	(55.1-132.9)
Patients with KTD	11	10.4
Hepatitis B	49	46.2
Hepatitis C	6	5.7
ARV regimen*		
- 3TC+EFV+TDF	68	64.1
- 3TC+NVP+TDF	8	7.6
- EFV+FTC+TDF	24	22.6
- EFV+ZDV+TDF	6	5.7

*EFV (Efavirenz), FTC (emtricitabine), 3TC (Lamivudine), NVP (Nevirapine), TDF (Tenofovir), ZDV (Zidovudine)

2.2 The demographic data in patients with KTD and patients with normal tubular function

From a total of 106 patients, 11 patients had kidney tubular dysfunction (KTD). When tenofovir plasma levels were compared between patients with KTD and patients with normal tubular function, the results showed that tenofovir plasma levels in patients with KTD was not significantly different from the levels in patients with normal tubular function. GFR of patients with KTD were significantly lower than patients with normal tubular function.

The summary of demographic data in patients with KTD and patients with normal tubular function is presented in Table 8.

Table 8 The summary of demographic data in patients with KTD and patients with normal tubular function (N=106)

Demographic data	Frequency or Mean±SD		p-value
	KTD (n=11)	Normal tubular function (n=95)	
Gender (Female/Male)*, n (%)	4(36.4)/7(63.6)	41(43.1)/54(56.9)	0.756
Age (years)**	42.55 ± 3.94	41.52 ± 7.35	0.649
Body weight (kg)**	65.07 ± 12.71	59.33 ± 10.82	0.105
BMI (kg/m^2) **	23.77 ± 4.35	22.11 ± 3.32	0.133
GFR($ml/min/1.73\ m.^2$)**	90.05 ± 17.34	102.54 ± 18.54	0.036
Tenofovir plasma levels (ng/mL)**	86.36 ± 31.67	84.77 ± 26.95	0.856

*Fisher's exact test **Independent t-test

2.3 Comparisons of patient's tenofovir plasma levels among groups of different ARV regimen

When tenofovir plasma levels were compared between groups of patients receiving different ARV regimens, it was found that tenofovir plasma levels was not significantly different between groups as shown in Table 9.

Table 9 Comparisons of patient's tenofovir plasma levels among groups of different ARV regimen (N=106)

ARV regimen	Number of patients	Tenofovir plasma levels Mean±SD (ng/mL)	p-value*
- 3TC+EFV+TDF	68	84.26 ± 27.86	0.511
- 3TC+NVP+TDF	8	79.25 ± 16.53	
- EFV+FTC+TDF	24	91.12 ± 27.93	
- EFV+ZDV+TDF	6	75.50 ± 30.71	

*One-way ANOVA

2.4 The comparison of tenofovir levels between between patients carrying homozygous wild type and those carrying at least 1 variant alleles for each SNPs

When tenofovir plasma levels were compared between patients carrying homozygous wild type and those carrying at least 1 variant alleles for each SNPs, the results found that:

For *ABCC2* -24C>T, tenofovir plasma levels in patients carrying homozygous wild type (C/C) was not significantly different from the levels in patients carrying at least 1 variant allele (C/T or T/T) (Table 10 and Figure 6)

Table 10 Comparison of the demographic data between patients categorized into 2 groups based on *ABCC2* -24C>T (rs717620) genotypes (N=106)

Demographic data	Frequency or Mean±SD		p-value
	C/C (n=64)	C/T (n=38) or T/T (n=4)	
Gender (Female/Male)*, n (%)	24(37.5)/40(62.5)	21(50.0)/21(50.0)	0.203
Age (years)**	41.36 ± 6.89	42.04 ± 7.40	0.627
Body weight (kg)**	61.50 ±11.00	57.54 ±10.95	0.072
BMI (kg/m^2) **	22.62 ± 3.43	21.77 ± 3.47	0.221
GFR(ml/min/1.73 $m.^2$)**	99.34 ± 16.31	104.14 ± 21.84	0.199
Tenofovir plasma levels (ng/mL)**	82.76 ± 23.58	88.26 ± 32.21	0.313

*Chi-square test **Independent t-test

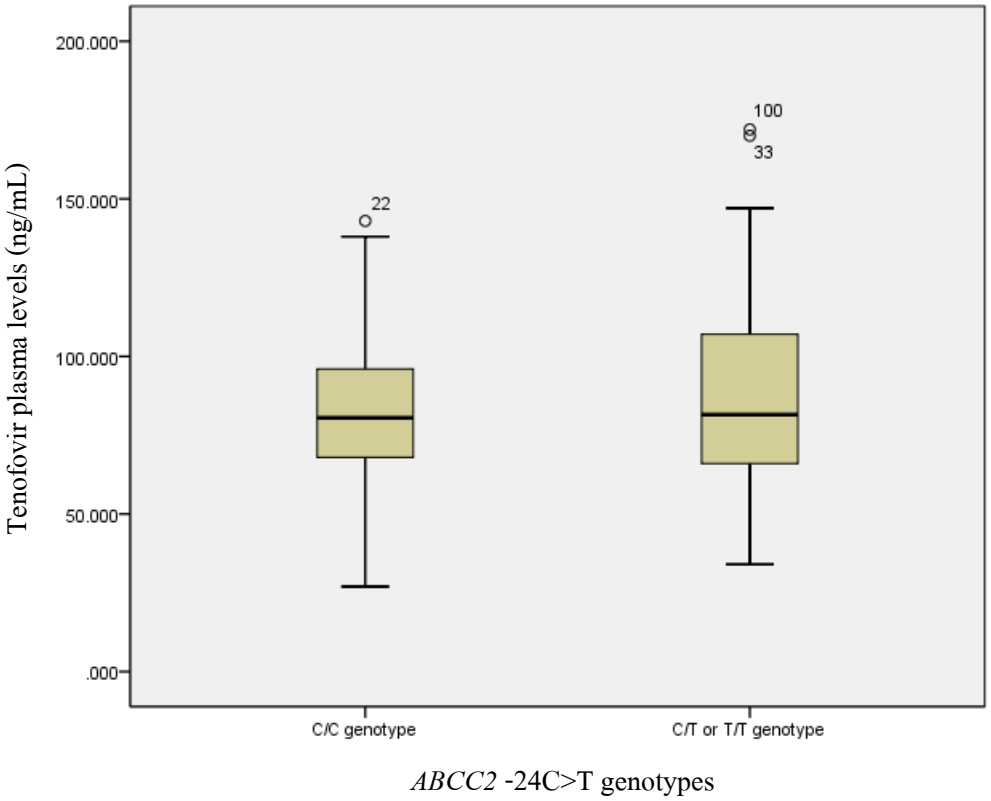

Figure 6 Boxplot of tenofovir plasma levels for the different *ABCC2* -24C>T genotypes

For *ABCC2* 1249 G>A, tenofovir plasma levels between patients having homozygous wild type alleles (G/G) and patients with at least 1 variant allele (A/G or A/A) were not significantly different. (Table 11 and Figure 7)

Table 11 Comparison of the demographic data between patients categorized into 2 groups based on *ABCC2* 1249G>A (rs2273697) genotypes (N=106)

Demographic data	Frequency or Mean±SD		p-value
	G/G (n=90)	A/G (n=14) or A/A (n=2)	
Gender (Female/Male)*, n (%)	40(44.4)/50(55.6)	5(31.2)/11(68.8)	0.325
Age (years)**	42.06 ± 6.79	39.21 ± 8.32	0.137
Body weight (kg)**	59.38 ± 11.26	63.04 ± 9.93	0.226
BMI (kg/m^2) **	22.24 ± 3.60	22.49 ± 2.60	0.795
GFR($ml/min/1.73\ m.^2$)**	101.08 ± 19.43	102.18 ± 14.78	0.830
Tenofovir plasma levels (ng/mL)**	85.70 ± 28.05	80.69 ± 23.05	0.501

*Chi-square test **Independent t-test

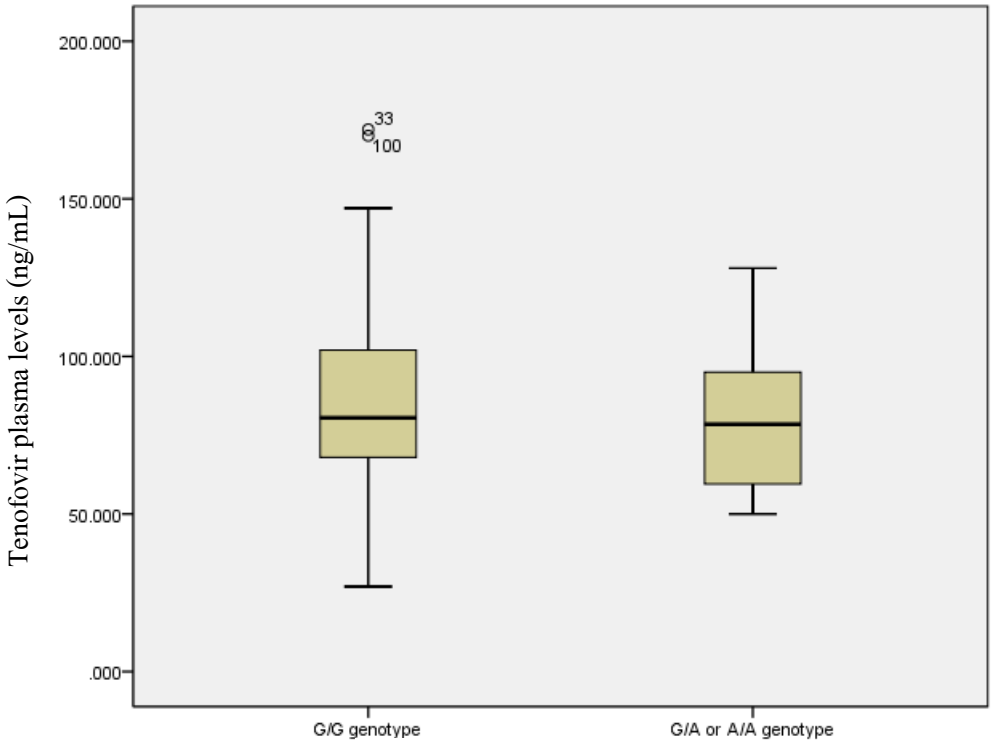

Figure 7 Boxplot of tenofovir plasma levels for the different *ABCC2* 1249G>A genotypes

For *ABCC4* 3463A>G, the results found that tenofovir plasma levels of patients with A/A genotype were significantly higher than among patients with at least 1 variant allele (A/G or G/G). (Table 12 and Figure 8)

Table 12 Comparison of the demographic data between patients categorized into 2 groups based on *ABCC4* 3463A>G (rs1751034) genotypes (N=106)

Demographic data	Frequency or Mean±SD		p-value
	A/A (n=72)	A/G (n=32) or G/G (n=2)	
Gender (Female/Male)*, n (%)	29(40.3)/43(59.7)	16(38.1)/18(61.9)	0.51
Age (years)**	41.59 ± 6.80	41.72 ± 7.72	0.926
Body weight (kg)**	60.25 ± 10.75	59.26 ± 11.96	0.672
BMI (kg/m^2) **	22.28 ± 3.24	22.30 ± 3.92	0.975
GFR($ml/min/1.73\ m^2$)**	98.94 ± 15.85	106.14 ± 23.26	0.064
Tenofovir plasma levels (ng/mL)**	88.87 ± 26.22	76.62 ± 28.09	0.030

*Chi-square test **Independent t-test

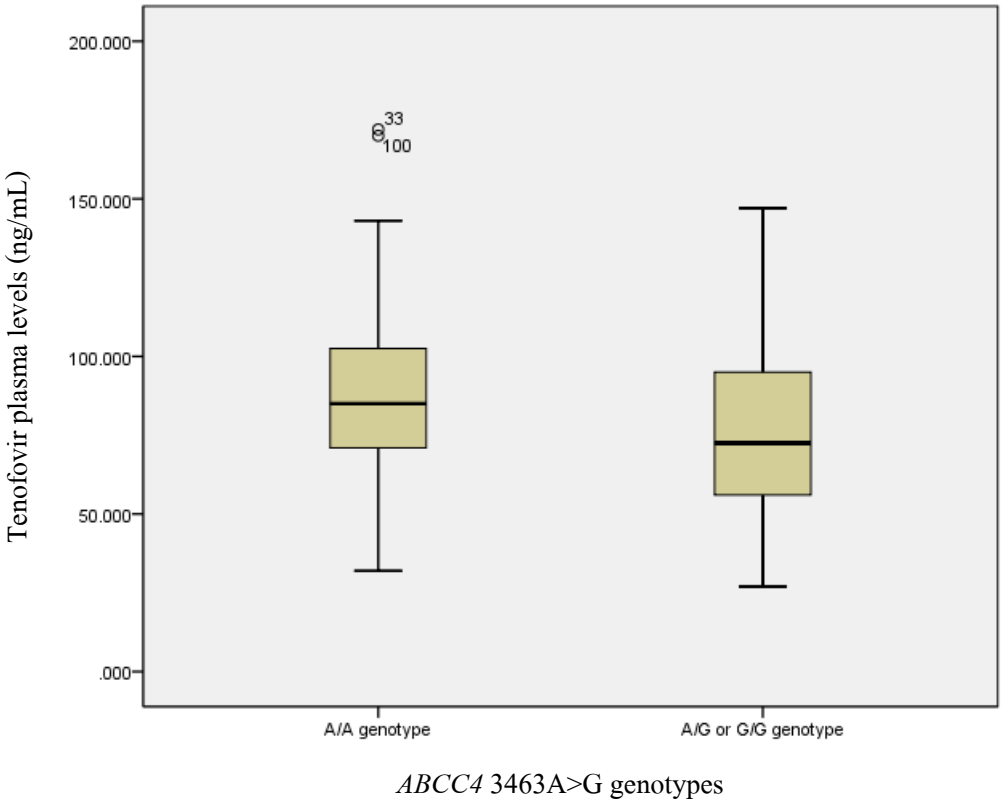

Figure 8 Boxplot of tenofovir plasma levels for the different *ABCC4* 3463A>G genotypes

For *ABCC4* 4131T>G, the tenofovir plasma levels of patients with T/T genotype were significantly lower than patients with at least 1 variant allele (T/G or G/G) (Table 13 and Figure 9)

Table 13 Comparison of the demographic data between patients categorized into 2 groups based on *ABCC4* 4131T>G (rs3742106) genotypes (N=106)

Demographic data	Frequency or Mean±SD		p-value
	T/T (n=22)	T/G (n=57) or G/G (n=27)	
Gender (Female/Male)*	9(40.9)/13(59.1)	36(42.9)/48(57.1)	0.869
Age (years)**	41.99 ± 6.85	41.54 ± 7.16	0.792
Body weight (kg)**	59.34 ± 11.94	60.09 ± 10.95	0.778
BMI (kg/m^2) **	21.47 ± 2.95	22.50 ± 3.56	0.216
GFR($ml/min/1.73\ m^2$)**	105.08 ± 18.41	100.25 ± 18.81	0.284
Tenofovir plasma levels (ng/mL)**	73.91 ± 24.32	87.83 ± 27.45	0.033

*Chi-square test **Independent t-test

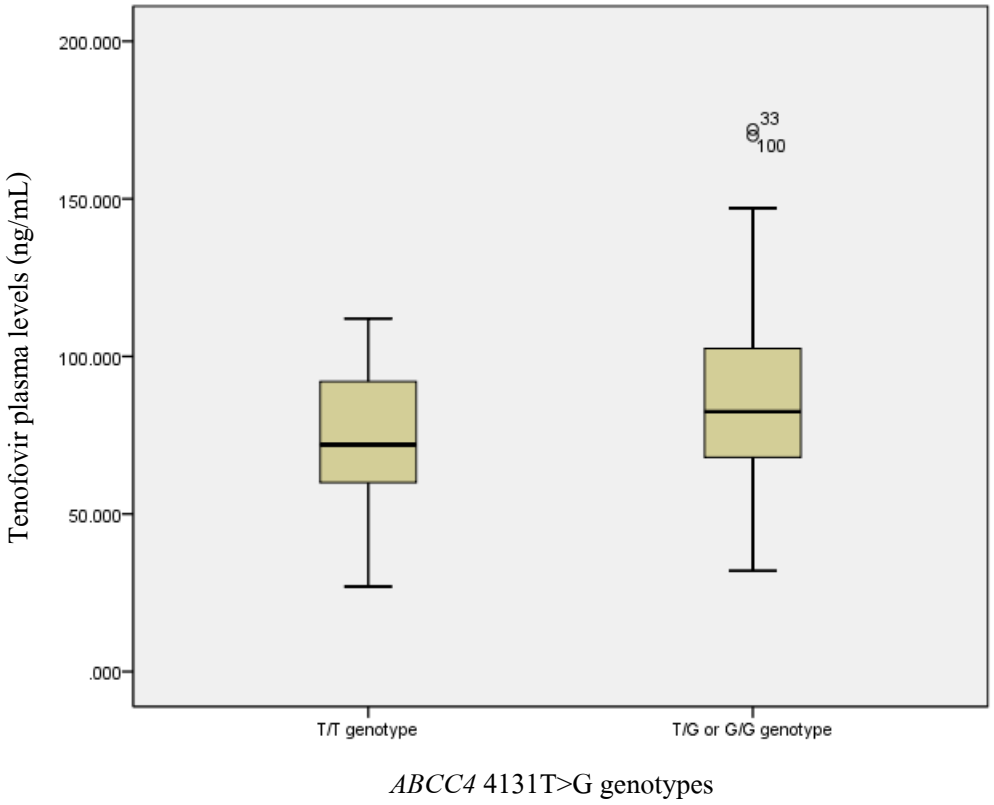

Figure 9 Boxplot of tenofovir plasma levels for the different *ABCC4* 4131T>G genotypes (2 groups)

As there is sufficient number of patients in each genotyping groups for *ABCC4* 4131T>G, the patients were categorized into 3 groups (T/T, T/C, and C/C). Tenofovir plasma levels were compared between groups. Tenofovir plasma levels were not significantly different among groups as shown in Table 14 and Figure 10.

Table 14 Comparison of the demographic data between patients categorized into 3 groups based on *ABCC4* 4131T>G (rs3742106) genotypes (N=106)

Demographic data	Frequency or Mean±SD			p-value
	T/T (n=22)	T/G (n=57)	G/G (n=27)	
Gender (Female/Male)*	9(40.9)/13(59.1)	24(42.1)/33(57.9)	12(44.4)/15(55.6)	0.967
Age (years)**	41.99 ± 6.85	41.75 ± 8.07	41.08 ± 4.84	0.891
Body weight (kg)**	59.34 ± 11.94	60.38 ± 11.88	59.48 ± 8.83	0.906
BMI (kg/m^2) **	21.47 ± 2.95	22.59 ± 3.81	22.30 ± 3.03	0.438
GFR($ml/min/1.73\ m^2$)**	105.08 ± 18.41	101.10 ± 20.42	98.44 ± 15.03	0.470
Tenofovir plasma levels (ng/mL)**	73.91 ± 24.32	87.10 ± 30.04	89.37 ± 21.40	0.097

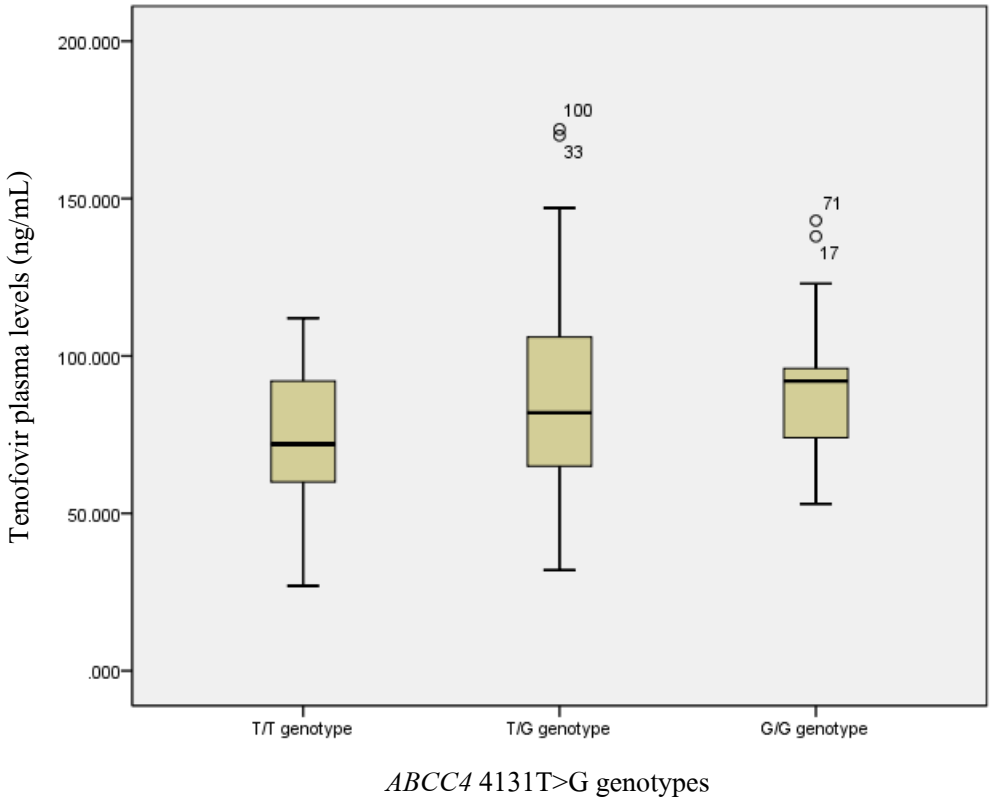

Figure 10 Boxplot of tenofovir plasma levels for the different *ABCC4* 4131T>G genotypes (3groups)

3. Regression equations of tenofovir plasma levels

The influence of genetic (*ABCC2* and *ABCC4* polymorphisms) and non-genetic (body weight, glomerular filtration rate and kidney tubular dysfunction) factors on tenofovir plasma levels was investigated by multiple linear regression with stepwise method. A total of 106 patients meeting the inclusion and exclusion criteria were enrolled for multiple linear regression analysis.

The results of multiple linear regression with stepwise method are shown in Table 15. The results found that model 2 was the best model. This model included two factors: body weight and glomerular filtration rate (GFR). Therefore, body weight and GFR were significant factors influencing tenofovir plasma levels. The adjusted R-Square of the model was 0.194 refers to that body weight and GFR could explain 19.1% of the variability of tenofovir plasma levels.

Table 15 The summary of multiple linear regression with stepwise method for prediction of tenofovir plasma levels

Model	Variable	R	R-Square	Adjusted R-Square	Sig (F change)	Model Sig. (ANOVA)
1	GFR	0.400	0.160	0.152	<0.001	<0.001
2	Body weight GFR	0.454	0.207	0.191	0.016	<0.001

The coefficient of each variable in the selected model and multi-colinearity tests are shown in Table 16. Multi-colinearity testes showed that all factors in the final model are not highly correlated.

Table 16 Coefficients of factors in the regression model for prediction of tenofovir plasma levels and multi-colinearity test.

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Colinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
2	(Constant)	182.272	20.424		8.924	.000		
	GFR	-0.643	0.130	-0.441	-4.937	.000	0.966	1.036
	Body Weight	-0.538	0.220	-0.219	-2.450	.016	0.966	1.036

Based on the results from regression analysis, the final equation can be presented as follows:

Tenofovir plasma levels (ng/mL) = 182.272 + (-0.643)[GFR(ml/min/1.73 m^2)] + (-0.538)[Body weight (kg)]

- Glomerular filtration rate (GFR) calculated from the Modification of Diet in Renal Disease (MDRD) Equation[24] : GFR (ml/min/1.73 m^2) = 186.3 x $S_{Cr}^{-1.154}$ x $Age^{-0.203}$ x (0.742 for female)

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Drug transporters are expressed in many tissues, such as intestine, liver, kidney, and brain. They play an important role in drug absorption, distribution, and excretion. Genetic variants of these transporters can affect the pharmacokinetics of drugs and major pharmacologically active metabolites which may lead to the difference in drug efficacy and safety among patients. An understanding of genetic variants of drug transporter genes could result in a better approach for optimal drug therapy.

This study contained two parts. The first study aimed to determine the prevalence of *ABCC2* and *ABCC4* polymorphisms in Thai patients with HIV-1 infection receiving tenofovir as part of the antiretroviral therapy at HIV- NAT. The second study aimed to determine the relationship between genetic factors including *ABCC2, ABCC4* polymorphisms and non-genetic factors including body weight, glomerular filtration rate (GFR) and kidney tubular dysfunction (KTD) with tenofovir plasma levels.

To determine the prevalence of *ABCC2* and *ABCC4* polymorphisms in Thai patients, four hundred patients were available for genotyping. The prevalence of *ABCC2* -24C>T; 1249G>A, *ABCC4* 3463A>G and 4131T>G were investigated. The prevalence of genetic polymorphisms examined in this study was comparable to those observed in other Asian populations, except *ABCC4* 3463 A>G. Interestingly, the polymorphism of *ABCC4* 3463A>G was found to be different among Asian populations. The allele frequency of *ABCC4* 3463A>G observed in this population is similar to the frequency found in Chinese population (19.8% vs 18.1%)[20], but it was significantly lower than that found in Japanese population (19.8% vs 34.3%).[20] However, this difference could be due to a small sample size of Japanese population in the study.

When comparing with Caucasian population, the allele frequencies of *ABCC2* 1249G>A found in Caucasian population were higher than those found in Thai population. On the other hand, the allele frequencies of *ABCC4* 4131T>G found in African population were lower than those observed in this study.

The difference of the polymorphisms of drug transporters may partly explain the interindividual variability in drug's pharmacokinetics, pharmacodynamics, and toxicity. Previous study in Japanese population found the polymorphisms of *ABCC2* -24C>T and *ABCC2* 1249G>A were associated with kidney tubular dysfunction (KTD) in tenofovir-treated patients.[33] Additionally, a study by Nishijima T et al reported that patients carrying *ABCC2* -24CC and *ABCC2* 1249AA have a higher risk of tenofovir-induced KTD.[33] As the polymorphisms of these genetic variants were presented in Thai population, a close monitoring of renal function is warranted in patients receiving tenofovir, especially those carrying these genetic variations.

Besides their contribution to drug's toxicity, genetic variation is one of the major factors contributing to the variability of drug's pharmacokinetics. A study by Rau T et al[21] found a 2-fold higher of the mean area under the curve of methotrexate from 36 to 48 hours after starting the infusion in female patients carrying at least one mutation allele of *ABCC2* -24T compared with other groups of patients. As the polymorphism of *ABCC2*-24C>T is observed in Thai population, side effects from drugs that are substrates of this polymorphism should be aware in patients carrying this genetic variant possibly due to higher drug level.

Therefore, this is the first study determining the allele frequencies of *ABCC2* and *ABCC4* in Thai population. The results revealed the difference of the genetic polymorphisms between Thai and other ethnicities. The results from this study can be used as prior information for an understanding of genetic variants in drug transporters and guiding the study investigating an association between genetic variations and pharmacokinetics/pharmacodynamics of drugs in the future.

In the second study, a total of 106 patients were enrolled for investigating the relationship between genetic factors and non-genetic factors and tenofovir plasma levels. Tenofovir plasma levels were not different between patients with KTD and normal tubular function. Additionally, when tenofovir plasma levels were compared between groups of different antiretroviral drug, the results found that tenofovir plasma levels were not significantly different among groups. Thus, the results from this study supported the previous findings that efavirenz, emtricitabine, lamivudine, nevirapine and zidovudine do not affect to tenofovir plasma levels.

Tenofovir plasma levels were compared between patients carrying homozygous wild type and those carrying at least 1 variant alleles for each SNPs. The results showed that mean tenofovir plasma level in patients carrying *ABCC2* -24C>T homozygous wild type (C/C) was not significantly different from the mean level in patients carrying at least 1 variant allele (C/T or T/T). However, the mean of tenofovir plasma level in patients with C/C genotype tended to be lower than the level in patients with C/T or T/T genotype.

For *ABCC2* 1249 G>A, tenofovir plasma levels between patients having homozygous wild type alleles (G/G) and patients having at least 1 variant allele (A/G or A/A) were not significantly different. Nevertheless, the mean of tenofovir plasma levels in patients with G/G genotype tended to be higher than in patients with A/G or A/A genotype.

The results from our study found that, for *ABCC4* 3463A>G, tenofovir plasma levels of patients with A/A genotype were significantly higher than the levels of patients with at least 1 variant allele (A/G or G/G). Interestingly, this study found the relationship between *ABCC4* 4131T>G and tenofovir plasma levels. The mean tenofovir plasma levels of patients having T/T genotype were significantly lower than the patients having at least 1 variant allele (T/G or G/G). Therefore, it is possible that patients with *ABCC4* 3463 A/G or G/G genotype may have an ability to eliminate tenofovir greater than patients with *ABCC4* 3463 A/A genotype. On the contrary, patients with *ABCC4* 4131 T/G or G/G genotype may have an ability to eliminate the tenofovir lower than patients with *ABCC4* 4131 T/T genotype. Even though, when patients were categorized into 3 groups according to *ABCC4* 4131T>G genotype (T/T, T/G, and G/G), the tenofovir plasma levels were not significantly different among groups. However, the mean of tenofovir plasma level in patients with G/G genotype tended to be higher than the level in patients with T/G or T/T genotype.

Based on the results from our study, it demonstrated role of *ABCC4* polymorphisms on the pharmacokinetics of tenofovir. Additionally, the results from this study supported the previous findings showing that tenofovir was a substrate of *ABCC4*, but not *ABCC2*.[12] As the association between tenofovir plasma levels and renal tubular dysfunction was presented, renal toxicity should be aware in patient tentatively having high plasma levels of tenofovir.

The results of multiple linear regression with stepwise method found body weight and glomerular filtration rate (GFR) were significant factors influencing tenofovir plasma levels which is consistent with the study of Gagnieu MC et al[30] and Jullien V et al.[39] The adjusted R-Square of the model was 0.194 refers to that body weight and GFR could explain 19.1% of the variability in tenofovir plasma levels. However, the influence of genetic factors was not observed in the linear regression analysis. This could be due to the influence of weight and GFR on the plasma levels of tenofovir. After adjusting for weight and GFR in regression analysis, the influence of genetic polymorphisms was not detected.

In conclusion, the results from this study found that *ABCC4* 3463A>G and 4131T>G polymorphisms influence tenofovir plasma levels. However, when considering the results from multiple linear regression which were adjusted for body weight and GFR, the genetic polymorphisms of drug transporters did not found to be a factor describing the variability of tenofovir plasma levels. Therefore, the association between genetic variations of tenofovir transporter with tenofovir plasma concentration requires further examination. The results from this study can be use as prior information when adjusting tenofovir dosage regimens in Thai and Asian HIV-infected patients to achieve optimal plasma levels and prevent possible adverse events.

Limitation

1. The polymorphisms of *ABCC2* and *ABCC4* were examined in this study, however the polymorphisms of other genes such as *SLC22A6* and *ABCC10* may also influence tenofovir plasma levels.
2. The analysis investigating the influence of genetic polymorphisms on tenofovir plasma levels (part 2) included a small number of patients (N=106). Therefore, the allele frequency of some polymorphisms was low. This may lead to a lack of power for detecting the significant influence of these polymorphisms in this study.
3. An application of the results from this study should be limited to specific patients having similar characteristics with the patients in this study.

4. As tenofovir diphosphate concentrations in intracellular were not measured, a complete explanation of the elimination of tenofovir cannot be made

Further study

An association between drug transporter polymorphisms and tenofovir pharmacokinetics requires further investigation in the study with a larger sample size. The relationship between tenofovir pharmacokinetics and other genetic polymorphisms should be that were not investigated in this study should be evaluated in the future study.

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APPENDICES

APPENDIX A

Certificate of Approval from the Institutional Review Board of the Faculty of Medicine, Chlalongkorn University, Bangkok, Thailand.

หมายเลขโครงการ 375/52

คณะกรรมการจริยธรรมการวิจัยในคน
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
1873 ถ.พระราม 4 เขตปทุมวัน กรุงเทพฯ 10330 โทร. 0-2256-4455 ต่อ 14, 15

หนังสือรับรองเอกสารที่เกี่ยวข้องกับโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในคน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ดำเนินการให้การรับรองเอกสารที่เกี่ยวข้องกับโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ : อุบัติการณ์ การทำนายโรค ของการเกิดโรคไตจากยาทีโนโฟเวียร์ และการศึกษาทางด้านเภสัชจลนศาสตร์และเภสัชพันธุศาสตร์ของยาทีโนโฟเวียร์ในผู้ป่วยติดเชื้อเอชไอวี

เลขที่โครงการวิจัย : HIV-NAT114/TDF renal toxicity

ผู้วิจัยหลัก : แพทย์หญิงอัญชลี อวิหิงสานนท์

สังกัดหน่วยงาน : HIV-NAT

เอกสารที่ได้รับการรับรอง :

1. เอกสารโครงร่างการวิจัย HIV-NAT114/TDF renal toxicity ฉบับภาษาไทย , ฉบับที่ 1.4 ลงวันที่ 28 มิถุนายน 2555

ลงนาม.....
(ศาสตราจารย์กิตติคุณแพทย์หญิงธาดา สืบหลินวงศ์)
ประธาน
คณะกรรมการจริยธรรมการวิจัยในคน

ลงนาม.....
(ผศ.ดร.พญ. ประภาพรรณ รัชตะปีติ)
กรรมการและผู้ช่วยเลขานุการปฏิบัติหน้าที่แทน
เลขานุการคณะกรรมการจริยธรรมการวิจัยในคน

วันที่รับรอง: 7 สิงหาคม 55

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

นักวิจัยทุกท่านที่ผ่านการรับรองจริยธรรมการวิจัยต้องปฏิบัติดังต่อไปนี้

1. ดำเนินการวิจัยตามที่ระบุไว้ในโครงร่างการวิจัยอย่างเคร่งครัด
2. ใช้เอกสารแนะนำอาสาสมัคร ใบยินยอม (และเอกสารเชิญเข้าร่วมวิจัยหรือใบโฆษณาถ้ามี) แบบสัมภาษณ์ และหรือ แบบสอบถาม เฉพาะที่มีตราประทับของคณะกรรมการพิจารณาจริยธรรมเท่านั้น และส่งสำเนาเอกสารดังกล่าวที่ใช้กับผู้เข้าร่วมวิจัยจริงรายแรกมาที่ฝ่ายวิจัย คณะแพทยศาสตร์ เพื่อเก็บไว้เป็นหลักฐาน
3. รายงานเหตุการณ์ไม่พึงประสงค์ร้ายแรงที่เกิดขึ้นหรือการเปลี่ยนแปลงกิจกรรมวิจัยใดๆ ต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ภายใน 5 วันทำการ
4. ส่งรายงานความก้าวหน้าต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ตามเวลาที่กำหนดหรือเมื่อได้รับการร้องขอ
5. หากการวิจัยไม่สามารถดำเนินการเสร็จสิ้นภายในกำหนด ผู้วิจัยต้องยื่นขออนุมัติใหม่ก่อนอย่างน้อย 1 เดือน
6. เอกสารทุกฉบับที่ได้รับการรับรองครั้งนี้ หมดอายุตามอายุของโครงการวิจัยที่ได้รับการรับรองก่อนหน้านี้ (หมายเลขโครงการ 375/52)

* รายชื่อของคณะกรรมการจริยธรรมการวิจัยในคน (ชื่อและตำแหน่ง) ที่อยู่ในที่ประชุมวันที่รับรองโครงการวิจัยได้แนบมาด้วย เอกสารที่รับรองทั้งหมดจะถูกส่งไปยังผู้วิจัยหลัก

Protocol Number 375/52

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University
1873 Rama 4 Road, Patumwan, Bangkok 10330, Thailand, Tel 662-256-4455 ext 14, 15

Approval of Documents related to Study Protocol

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the following study which is to be carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title : Incidence and predictor of TDF associated nephrotoxicity and pharmacokinetic of TDF in HIV-1 infected Thai patients: A sub-study of HIV-NAT 006 long term cohort

Study Code : HIV-NAT114/TDF renal toxicity

Principal Investigator : Anchalee Avihingsanon .MD.

Study Center : HIV-NAT

Document Approval :
1. Thai Protocol for HIV-NAT 114 / TDF renal toxicity, version 1.4, Dated 28 June 2012

Signature ..... Signature .....
(Emeritus Professor Tada Sueblinvong MD) (Assistant Professor Prapapan Rajatapiti, MD.,PhD)
Chairperson Member and Assistant Secretary, Acting Secretary
The Institutional Review Board The Institutional Review Board

Date of Approval : August 7, 2012

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;
2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for the record;
3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;
4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;
5. If the study cannot be finished within the expire date of the approval certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.
6. All the above approved documents are expired on the same date of the previously approved protocol (Protocol Number..375/52.....)

* A list of the Institutional Review Board members (names and positions) present at the meeting of Institutional Review Board on the date of approval of this study has been attached. All approved documents will be forwarded to the principal investigator.

APPENDIX B

Determination of tenofovir plasma levels and method validation

Tenofovir concentrations were determined using a validated high-performance liquid chromatography assay with a fluorimetric detector according to previous literature.[34] The analysis of tenofovir plasma levels was performed in laboratory of HIV-NAT. Analytical method including :

1) Add 200 µl of acetonitrile to 100 µl of human plasma
2) Add 200 µl of 0.34% chloroacetaldehyde in 50 mM acetate buffer (pH 4.5).
3) Kept sample in freezer at -20°C for 5 min
4) 10 µl of sample was injected onto the column.

Table A Chromatographic condition for HPLC

Parameters	**Description**
Mobile phase	Phosphate buffer 50 mM, pH 6.8 and Acetonitrile (96:4; vol/vol)
Analytical column	3.5 µm, 150 x 4.6 mm
Column temperature	30°C under isocratic conditions with extinction and emission wavelengths of 232 and 420 nm, respectively.
Injection volume	10 µL
Flow rate	1 mL/min

Table B Result of Method Validation

Item	Result
Analysis	tenofovir
Internal standard	adefovir
The concentrations of the quality controls (ng/mL)	15, 30, 210, 1200
Intra-assay accuracy (%dev)	1.2% to +5.7%
Intra-assay precision (%CV)	1 to 3%
Inter-assay accuracy (%dev)	0.0% to +1.7%
Inter-assay precision (%CV)	+1.6% to +2.8%

Overall, the intra- and inter-day accuracy and precision for tenofovir were within the acceptable limits.

APPEDDIX C

DNA Extraction

Peripheral blood mononuclear cells (PBMC) were used for DNA extraction by QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, German) using a procedure recommended by the manufacturer.

1. Materials

Chemical and reagents

1)	Ethanol (100%)	Carlo erba	Italy
2)	Buffer AL	Qiagen	Germany
3)	Buffer AW1	Qiagen	Germany
4)	Buffer AW2	Qiagen	Germany
5)	Buffer AE	Qiagen	Germany
6)	QIAGEN® protease	Qiagen	Germany
7)	Protease solvent	Qiagen	Germany

Apparatus

1)	Centrifuge (Universal 320)	Hettick	Germany
2)	Vortex mixer (S0100-220)	Labnet	USA
3)	Heating block (Dri-block DB-2D)	Techne	UK
4)	Microcentrifuge (5415R)	Eppendorf	Germany
5)	Spectrophotometer (Smart spec 3000 Bio-radTM)		USA
6)	Freezer	Sanyo	Japan
7)	Real-Time PCR system (Applied Biosystems 7500)		USA

Supplies

1)	Microcentrifuge tube 1.5 mL	Treff AG.	Switzerland
2)	Pipette tips (Blue and Yellow)	Scientific Plastics	USA
3)	Micropipette 1,000 mcL	Eppendorf	Germany
4)	Micropipette 200 mcL	Eppendorf	Germany
5)	Micropipette 20 mcL	Eppendorf	Germany
6)	QIAamp Mini spin Columns	Qiagen	Germany
7)	Collection tubes 2 mL	Qiagen	Germany
8)	Disposable gloves		

2. DNA Extraction methods

1) Equilibrate samples to room temperature.
2) Heat a heating block to 56°C.
3) Equilibrate Buffer AE to room temperature.
4) If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.
5) Pipet 20 µl QIAGEN Protease into the bottom of a 1.5 mlmicrocentrifuge tube.
6) Add 200 µlsample to the microcentrifuge tube.
7) Add 200 µl Buffer AL to the sample. Mix by vortex mixer for 15 seconds.
8) Incubate at 56°C for 10 min.
9) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the insideof the lid.
10) Add 200 µl ethanol (100%) to the sample, and mix again by vortex mixer for 15 seconds. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to removedrops from the inside of the lid.
11) Carefully apply the mixture to the QIAamp Mini spin column (in a 2 mlcollection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.

12) Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 withoutwetting the rim. Close the cap and centrifuge at 8000 rpm for 1 min.Place the QIAamp Mini spin column in a clean 2 ml collection tube, anddiscard the collection tube containing the filtrate.
13) Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2without wetting the rim. Close the cap and centrifuge at full speed 14,000 rpm for 3 min.
14) Place the QIAamp Mini spin column in a new 2 ml collection tubeand discard the old collection tube with the filtrate. Centrifuge at fullspeed for 1 min.
15) Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully openthe QIAamp Mini spin column and add 200 µl Buffer AE.Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 8000 rpm for 1 min.
16) Storing DNA (eluting in buffer AE) at -20°C until genotyping.

APPENDIX D

ABCC2 and *ABCC4* genotyping

Overview

TaqMan® SNP Genotyping Assays provide optimized assays for genotyping SNPs. The products use the 5′ nuclease assay foramplifying and detecting specific SNP alleles in purified genomicDNA samples. Each assay allows to genotype for a specific SNP.

The SNP Genotyping Assay contains:

• Sequence-specific forward and reverse primers to amplify thepolymorphic sequence of interest.

• Two TaqMan® probes (The probes for all SNPs were presented in Table A) :

– One probe labeled with VIC® dye detects the Allele 1sequence

– One probe labeled with FAM™ dye detects the Allele 2Sequence

Table A Context sequence of ABCC2, ABCC4, ABCC10 and SLC22A6polymorphisms.

SNPs	**Context Sequence [VIC/FAM]**
ABCC2 -24C>T	ACAATCATATTAATAGAAGAGTCTT**[C/T]**GTTCCAGACGCAGTCCAGGAA TCAT
ABCC2 1249G>A	CAACTTGGCCAGGAAGGAGTACACC**[A/G]**TTGGAGAAACAGTGAACCTG ATGTC
SNPs	**Context Sequence [VIC/FAM]**
ABCC4 3463A>G	TGCATACCTGAGGTATGATTGACAT**[G/A]**TTCTTCCTTAAATCGTGAAGTC CAA
ABCC4 4131T>G	GTTTACATAGTCCAAAAACTAGTGG**[T/G]**AAATGCCTTCGGAACGGACTT GACA

Chemical and reagents

1) Taqman® SNP Genotyping Assays — Applied Biosystems — USA
2) Taqman® Universal PCR Master Mix — Applied Biosystems — USA

Apparatus

1) MicroAmp Optical 96-well reaction plate
2) MicroAmp Optical adhesive Film
3) Vortex mixer
4) Real-Time PCR system (Applied BiosystemsStepOnePlus®) — USA

Supplies

1) Disposable gloves
2) Pipette tip 10 mcL (White) — Scientific Plastics — USA
3) Micropipette 10 mcL — Eppendorf — Germany

Procedure

1) Prepare the reaction plate using the following components for one reaction as shown in the table below.

Reaction Components	**Volume/Well (20 mcL volume reaction)**	**Final Concentration**
Taqman® Universal PCR Master Mix (2X)	10 mcL	1X
20X Taqman® SNP Genotyping	0.5 mcL	1X
Genomic DNA (1-20 ng/mcL)	2 mcL	20 ng
dH_2O	7.5 mcL	-
Total	20 mcL	-

2) Run the plate on Real-Time PCR system using the following thermal cycling conditions:

	AmpliTaq Gold Enzyme Activation	PCR	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/Extend
Time	10 min	15 sec	1 min
Temp	95 ^{o}C	92 ^{o}C	60 ^{o}C

3) The clusters of output of the fluorescent data were interpreted as follows:

3. 1 Only FAM signal: Homozygous allele 1

3. 2 Both VIC and FAM signal: Heterozygous

3. 3 Only VIC signal: Homozygous allele 2

VITAE

Miss Siwaporn Mitruk was born on the second of August 1983 in Bangkok. She graduated Bachelor degree of Pharmacy from the Faculty of Pharmacy, Chiang Mai University in 2008. She started to work as hospital pharmacist at Camillian Hospital in March 2008. Then she started to work as hospital pharmacist at Faculty of Medicine Vajira Hospital, Bangkok in November 2008 until now. She has been enrolled in a Master degree program, Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University since June 2011.