Pharmacogenetic markers of CYP2B6 associated with efavirenz plasma concentrations in HIV-1 infected Thai adults

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Interindividual variability in efavirenz plasma concentrations is associated with CYP2B6 genetic polymorphisms.
- Twenty-nine different alleles of the CYP2B6 gene are listed. CYP2B6 *6, *9, *16, *26, *27 and *28 carriers are reported to be associated with slower efavirenz oral clearance.
- The allelic variant 516G>T is associated with diminished activity of CYP2B6 and high efavirenz plasma concentrations are associated with an increase risk of neuropsychological toxicity.

WHAT THIS STUDY ADDS

- This study identified three SNPs in the CYP2B6 gene which could potentially act as additional independent predictors of efavirenz plasma concentrations beyond that provided by the CYP2B6 c.516G>T polymorphism.
- The GAC-CYP2B6 haplotype (G516T/A785G/C21563T) is associated with higher plasma efavirenz concentrations in HIV-infected Thai adults.
- The CYP2B6 g.18492 T>C polymorphism was significantly associated with lower efavirenz concentrations than those with the homozygous wild-type.

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Aims

To investigate the frequency of CYP2B6 polymorphisms and the influence of haplotype structure on plasma efavirenz concentrations in Thai adults with HIV-1 infection.

Methods

Genotyping of nine single nucleotide polymorphisms (SNPs, c.64C>T, c.499C>G, c.516G>T, c.785A>G, c.1375A>G, c.1459C>T, g.3003T>C, g.18492C>T and g.21563C>T) of CYP2B6 were performed using real-time PCR-based allelic discrimination on blood samples from 52 HIV-infected adults who had received an efavirenz-based regimen. Plasma efavirenz concentrations were measured by high performance liquid chromatography.

Results

The minor allele frequencies for c.64C>T, c.516G>T, c.785A>G, g.3003C>T, g.18492T>C and g.21563C>T were 0.087, 0.365, 0.413, 0.308 and 0.356, respectively. However, no variant alleles were identified for three SNPs (c.499 C>G, c.1375 A>G and c.1459 C>T). Efavirenz plasma concentrations were significantly associated with c.516G>T ($P = 0.0095$), c.785A>G ($P = 0.0017$), g.21563C>T ($P = 0.0036$) and g.18492C>T ($P = 0.0011$). The composite CYP2B6 of three SNPs (c.516G $\geq$ T, c.785A $\geq$ G and g.21563C $\geq$ T) genotypes were significantly associated with higher efavirenz concentrations.

Conclusions

Our data indicate that the GAC-CYP2B6 haplotype is associated with higher plasma efavirenz concentrations in HIV-infected Thai adults.

Introduction

Efavirenz is potent and effective non-nucleoside reverse transcriptase inhibitor (NNRTI) that is commonly used as part of the first line antiretroviral regimen for the treatment of HIV/AIDS. Efavirenz-containing highly active antiretroviral therapy (HAART) is also preferred in patients with tuberculosis co-infection requiring rifampicin-containing therapy [1]. Although its safety profile is considered satisfactory, central nervous system (CNS) side effects are commonly reported. The CNS side effects range from headaches and dizziness to insomnia, hallucinations, acute mania and psychosis [2]. The exact mechanism responsible for CNS toxicity associated with efavirenz remains unknown [2, 3], but high plasma concentrations have been reported to be a predictor of early neuropsychological disturbances in patients initiating an efavirenz containing antiretroviral regimen [2, 4–6]. Significant inter-individual variability in efavirenz plasma concentrations in adults and children has been observed [2, 3, 7, 8]. Mid-dose or trough efavirenz plasma concentrations below 1000 ng ml⁻¹ have been associated with an increased risk of virological failure [2, 8, 9], whereas concentrations above 4000 ng ml⁻¹ have been associated with a higher risk of CNS side effects [2, 8].

Efavirenz is metabolized primarily by the hepatic CYP2B6 enzyme to form 8-hydroxy and 7-hydroxy efavirenz metabolites, with minor contributions from CYP3A4/5 and CYP2A6 [10, 11]. The CYP2B6 gene is highly polymorphic and has been mapped to chromosome 19, which is 28 kb long and consists of nine exons [12]. Previous studies have shown that inter-individual variability in efavirenz plasma concentrations is associated with the CYP2B6 genetic polymorphisms [5, 8, 13, 14]. Currently, 29 different alleles of the CYP2B6 gene are listed. CYP2B6 *6, *9, *16, *26, *27 and *28 carriers have been reported to be associated with slower efavirenz oral clearance [14–18]. The most commonly studied allele is *6 (c.516G ≥ T and c.785A ≥ G). The *6 allele is associated with higher efavirenz plasma concentrations and an increased risk of CNS toxicity [15, 16]. The allelic variant c.516G>T is associated with diminished activity of CYP2B6, increased efavirenz plasma concentrations and neuropsychological toxicity [2, 5, 7, 15].

Minimizing drug associated toxicity is a major challenge. It is thought that pharmacogenetic information may be able to help identify patients at an increased risk of drug toxicity. Recently, it has been reported that CYP2B6-516G>T significantly affected the drug metabolism of efavirenz in HIV-infected Thai children and high concentrations were associated with psychiatric side effects [7]. The objective of this study was to determine the frequency of nine CYP2B6 polymorphisms in HIV-infected Thai adults and identify SNPs and/or haplotypes in this population that are associated with efavirenz plasma concentrations.

Methods

Sample collection

This retrospective analysis used stored plasma and cell pellets that were collected from HIV infected Thai adults followed in a prospective observational cohort study (<http://www.clinicaltrials.gov>, NCT00433030). HIV-infected patients receiving two NRTIs plus efavirenz for at least 4 weeks with plasma and cell pellet samples available were included. The first plasma sample available after 4 weeks of treatment was selected for analysis. Patients receiving concomitant treatments that could potentially affect efavirenz pharmacokinetics were excluded. All subjects provided written informed consent within the cohort study, which included the use of stored samples for future research following specific ethical clearance. This specific retrospective analysis, performed on anonymized DNA and plasma samples, was approved by the Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital (ID 06–51–31) and the Faculty of Associated Medical Sciences, Chiang Mai University (Sor Thor 6393(4)/Vor Jor 202).

Sample preparation and DNA extraction

Peripheral blood samples were obtained and the plasma was aliquoted and frozen within 1 h of collection at –20°C. For each blood draw the remaining EDTA cell pellets were stored at –20°C. DNA was isolated from the stored EDTA cell pellets using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was quantified by a u.v. spectrophotometer ND-1000 at 260 nm (NanoDrop Technologies, Wilmington, DE).

CYP2B6 genotyping

A total of nine SNPs within CYP2B6 were genotyped (GeneBank accession number NG_000008.7). SNPs c.516G ≥ T (rs3745274) and c.785A ≥ G (rs2279343) have previously been reported to influence enzyme activity and efavirenz concentrations [14] and three CYP2B6 tagSNPs, g.3003T ≥ C (rs8100458), g.18492C ≥ T (rs2279345) and g.21563C ≥ T (rs8192719), identified using HapMap (<http://www.hapmap.org>) data on Japanese and Han Chinese populations with an $r^2 \geq 0.8$ were also included [19]. The c.499C ≥ G (rs3826711) SNP, part of the *26 allele, was associated with high efavirenz plasma concentrations in Japanese subjects, and three other SNPs also assessed in this study, c.64C ≥ T (rs8192709), c.1375A ≥ G (rs3211369) and c.1459C ≥ T (rs3211371) were also selected for assessment [15].

Pre-designed TaqMan assays (Applied Biosystems, Foster City, CA) were used to genotype the CYP2B6 g.3003T>C (assay ID C__2818167_10), c.516G>T (assay ID C__7817765_60), g.18492C>T (assay ID C__26823975_10), g.21563C>T (assay ID C__22275631_10), c.64C>T (assay ID C__2818162_20), c.499C>G (assay ID C__2752377_10), c.1375A>G (assay ID C__2741754_10) and c.1459C>T

(assay ID C__30634242_40). The CYP2B6 c.785A>G were performed by custom TaqMan assays (Applied Biosystems). The sequences of primers and probes were: CYP2B6 c.785A>G, TGGAGAAGCACCGTGAAC (forward), TGGAG-CAGGTAGGTGTCGAT (reverse), VIC-CCCCAAGGACCTC-MGB (wild-type), FAM-CCCCAGGGACCTC-MGB (mutant).

For SNP analysis, these nine SNPs were genotyped using an allele-specific fluorogenic 5' nuclease chain reaction assay with predesigned primers and TaqMan MGB probes (TaqMan SNP Genotyping Assay; Applied Biosystems, Foster City, CA). Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest used two TaqMan MGB probes. One probe was labelled with VIC dye and detected the Allele 1 sequence and the second probe was labelled with FAM dye and detected the Allele 2 sequence.

Measurement of efavirenz plasma concentrations

Blood samples were centrifuged and the plasma was aliquoted and frozen within 4 h of collection at -20°C . Efavirenz plasma drug concentrations were measured at the Faculty of Associated Medical Sciences, Chiang Mai University using an isocratic reversed-phase high performance liquid chromatography (HPLC) method with ultra-violet detection at 245 nm [7]. Briefly, patient plasma samples (300 μl) and all calibration and control samples were heat inactivated in a water bath at 56°C for 30 min prior to assay. Sample pretreatment involved protein precipitation with acetonitrile (360 μl) and following centrifugation the sample supernatant was injected into the HPLC machine. Chromatography was performed using an Agilent 1100 HPLC machine with an Omnispher C18 (150 $\times$ 4.6 mm i.d./particle size 5 μm) analytical column (Varian, CA, USA), a Chromquard RP guard column and a mobile phase consisting of 10 mM KH_2PO_4 pH 3.1: acetonitrile (50 : 50, v/v). This method was validated using the AIDS Clinical Trials Group (ACTG) method validation guidelines [6] over the concentration range of 78–10 000 ng ml^{-1} . The average accuracy was 102–105% and precision (both inter- and intra-assay) was $\leq 5\%$ of the coefficient of variation (CV). Overall extraction recovery was 106% and efavirenz was stable under various storage conditions. Plasma samples with efavirenz concentrations $\geq 10\,000$ ng ml^{-1} were diluted and re-assayed. This laboratory participates in the international external quality Pharmacology Quality Control (Precision Testing) programme of the AIDS Clinical Trial Group, USA.

Statistical analysis

All genotype distributions were tested for Hardy–Weinberg equilibrium using exact tests. SNPs with a call rate $< 95\%$ were omitted. Tagging of SNPs was analyzed using Haploview 4.2 software. A Kruskal–Wallis test was used to test for significant difference in efavirenz concentrations between genotypes. Mann–Whitney U-tests were

used to compare efavirenz concentrations between two genotypes. The QTLHAPLO program was used to identify the association between the CYP2B6 haplotypes and the efavirenz plasma concentration. Statistical significance was defined as $P < 0.05$.

Results

Study population

Samples from 68 subjects were selected for assessment. Fifty-two subjects had detectable efavirenz concentrations and were genotypes for nine CYP2B6 SNPs. All individuals were >18 years of age and taking 600 mg of efavirenz once daily for at least 4 weeks. Efavirenz plasma concentrations were measured at an average of 13 h after last drug intake. Patient characteristics are summarized in Table 1.

Frequencies of CYP2B6 genetic polymorphisms

The genotype of the nine CYP2B6 SNPs could be determined for all 52 subjects and the frequencies of each SNP are summarized in Table 2. All CYP2B6 polymorphisms were found to be in Hardy–Weinberg equilibrium ($P > 0.05$). The minor allele frequencies (MAFs) for c.64C>T, c.516G>T, c.785A>G, g.3003C>T, g.18492T>C and g.21563C>T were 0.087, 0.365, 0.413, 0.308 and 0.356, respectively. No variant alleles were identified for three SNPs (c.499 C>G, c.1375 A>G and c.1459 C>T) in these subjects.

Assessment of individual CYP2B6 polymorphisms with efavirenz plasma concentrations

In the entire cohort, the median (range) efavirenz plasma concentration was 2622 ng ml^{-1} (IQR 1727–3983). Efavirenz plasma concentrations in patients with either the 516GT or TT genotype were significantly higher than in

Table 1

Patient characteristics

Characteristic*	Total (n = 52)
Gender Male : Female (%)	24 : 28
Age (years)	33 (18–53)
Weight (kg)	54 (33–72)
HAART regimen, n (%)	
TDF + FTC + EFV	21 (40)
ZDV+3TC + EFV	17 (33)
D4T+3TC + EFV	6 (11)
ZDV + DDI + EFV	4 (8)
D4T + DDI + EFV	2 (4)
3TC + DDI + EFV	2 (4)
Duration of HAART (weeks)	26 (4–269)
Duration between blood sampling and last intake (h)	13 (10–18)

*Median (range), unless otherwise stated. TDF, tenofovir; FTC, emtricitabine; EFV, efavirenz; ZDV, zidovudine; 3TC, lamivudine; D4T, stavudine; DDI, didanosine.

Table 2

Relationship between *CYP2B6* polymorphisms and efavirenz plasma concentrations

Genetic polymorphism	n (%) n = 52	Minor allele frequency	Efavirenz plasma concentration (ng ml ⁻¹), Median (IQR)
CYP2B6 c.64C>T(rs8192709)			
CC	43 (0.827)	T = 0.087	2694 (1704–4192)
CT	9 (0.173)		2049 (1621–2880)
TT	0 (0.0)		–
P value			0.371
CYP2B6 c.499C>G(rs3826711)			
CC	52 (1.0)	G = 0.000	2622 (1721–3983)
CG	0 (0.0)		–
GG	0 (0.0)		–
P value			–
CYP2B6 c.516G>T(rs3745274)			
GG	22 (0.423)	T = 0.365	1902 (1257–3051)
GT	22 (0.423)		2691 (1818–3500)
TT	8 (0.153)		8422 (4068–10 265)
P value			0.0095
CYP2B6 c.785A>G(rs2279343)			
AA	19 (0.365)	G = 0.413	1902 (1304–3157)
AG	23 (0.442)		2274 (1704–3268)
GG	10 (0.192)		7402 (3011–9965)
P value			0.0017*
CYP2B6 c.1375A>G(rs3211369)			
AA	52 (1.0)	G = 0.000	2622 (1721–3983)
AG	0 (0.0)		–
GG	0 (0.0)		–
P value			–
CYP2B6 c.1459C>T(rs3211371)			
CC	52 (1.0)	T = 0.000	2622 (1721–3983)
CT	0 (0.0)		–
TT	0 (0.0)		–
P value			–
CYP2B6 g.3003C>T(rs8100458)			
CC	25 (0.48)	T = 0.308	2806 (1992–5190)
CT	22 (0.423)		2113 (1482–4160)
TT	5 (0.096)		2366 (889–4547)
P value			0.1454
CYP2B6 g.18492T>C(rs2279345)			
TT	30 (0.577)	C = 0.231	2986 (1845–6097)
TC	20 (0.385)		2012 (1589–2964)
CC	2 (0.038)		1668 (–)†
P value			0.0011
CYP2B6 g.21563C>T(rs8192719)			
CC	22 (0.423)	T = 0.356	1902 (1257–3051)
CT	23 (0.442)		2694 (1856–4149)
TT	7 (0.135)		7908 (3126–10 415)
P value			0.0036

*P < 0.05 when comparing 785GG with 785AA and 785AG. †Unable to calculate for IQR, statistical significance was indicated by a Kruskal–Wallis test.

those patients with the homozygous wild-type genotype ($P = 0.0095$, Figure 1A). Subjects with a nucleotide substitution of c.785A>G were observed to have higher efavirenz plasma concentrations than the recommended therapeutic range (1000–4000 ng ml⁻¹) [2] whereas, patients who carried the homozygous variant form (785GG) demonstrated higher efavirenz concentrations than other genotypes (785AA or AG) ($P = 0.0017$, Figure 1B). For SNPs at positions c.64 C ≥ T and g.3003C ≥ T, the efavirenz concentrations were not significantly different ($P = 0.371$ and

0.1454, respectively). The homozygous mutant polymorphisms of g.18492 T ≥ C had a reverse effect to the other SNPs. Efavirenz plasma concentrations for patients with the g.18492 TC (2012 ng ml⁻¹, $n = 20$) or CC (1668 ng ml⁻¹, $n = 2$) genotype were significantly lower than those with the homozygous wild-type (TT; 2986 ng ml⁻¹, $n = 30$) ($P = 0.0011$, Figure 1C). Patients carrying at least one defect allele for the *CYP 2B6* g.21563C>T polymorphism showed higher efavirenz exposure compared with the homozygous wild-type ($P = 0.0095$, Figure 1D).

Figure 1

Impact of CYP2B6 polymorphisms; 516G>T (A), 785A>G (B), 18492T>C (C) and 21563C>T (D) on efavirenz (EFV) plasma concentrations. Middle bar indicates the median and grey bar indicates the interquartile range. Mann–Whitney tests were used to compare efavirenz concentrations between two genotypes

Assessment of CYP2B6 haplotypes and efavirenz plasma concentrations

Significant linkage disequilibrium (LD) in each variant position (CYP 2B6 c.64C>T, c.516G>T, c.785A>G, g.3003 C>T, g.18492T>C and g.21563C>T) exists. From a pairwise tagging analysis (Haploview, $r^2 > 0.8$), CYP 2B6 c.516G>T can be representative of c.785A>G and 21563C>T due to strong LD. While, other SNPs (c.64C>T, g.3003 C>T and g.18492T>C) could not capture any SNPs (four SNPs in four tests captured six of six alleles at $r^2 \geq 0.8$; mean maximum r^2 is 0.963, Figure 2).

From single marker association analysis, three SNPs (c.516G>T, c.785A>G and g.21563C>T) were significantly associated with efavirenz plasma concentrations that

exceeded the therapeutic range ($P = 0.0012$, 0.0006 and 0.0036 , respectively; use Bonferroni correction, $P < 0.0083$). These three SNPs were therefore used to construct a haplotype block. The frequencies of HIV-infected patients with the CYP2B6-GAC (516G $\geq$ T, 785A $\geq$ G and 21563C $\geq$ T) were 36.5% ($n = 19$), 40.4% ($n = 21$) and 23.1% ($n = 12$) for non-GAC, GAC heterozygous and GAC homozygous genotypes, respectively. Median (range) efavirenz plasma concentration were 1934 ng ml⁻¹ (1313–3520 ng ml⁻¹), 2688 ng ml⁻¹ (1780–3276 ng ml⁻¹) and 5544 ng ml⁻¹ (2372–9595 ng ml⁻¹) for patients with non-GAC, GAC heterozygous and GAC homozygous genotypes, respectively. This haplotype was significantly associated with higher efavirenz plasma concentrations ($P = 0.0179$, Figure 3).

Figure 2

Linkage disequilibrium of *CYP2B6* for each polymorphism, data represents six SNPs in Haploview 4.2 software. Red quadrate without D' worth display mean $D' = 1$, $\text{LOD} \geq 2$ while pale blue square mean $D' = 1$, $\text{LOD} < 2$; white quadrate, $D' < 1$ and $\text{LOD} < 2$; pale red square, $D' < 1$ and $\text{LOD} \geq 2$

Figure 3

CYP2B6 GAC haplotype. Middle bar indicates the median and grey box indicates the interquartile range. Asterisks represent an individual subject

Discussion

SNPs within the *CYP2B6* gene contribute towards the high inter-individual variability in efavirenz plasma concentrations [5, 8, 13, 14]. This retrospective study was aimed at assessing the frequency of nine *CYP2B6* SNPs in HIV-infected Thai adults to determine which could act as pre-

dictors of efavirenz plasma concentrations, in addition to that provided by the well-studied 516G>T polymorphism [7, 20–22].

Overall, the detected frequencies of the nine SNPs were in good agreement with published data [15, 19]. The most common SNP identified in this study was the c.785A>G (MAF = 0.413), while c.516G>T was the second most frequent (MAF = 0.365). Both these polymorphisms were more common than in Japanese and Chinese HIV-infected patients [15, 20]. The nucleotide substitutions c.499C ≥ G, c.1375A ≥ G and c.1459C ≥ T were absent in this cohort, whereas a previous study showed a low frequency among the Japanese group [15]. The allelic frequency of the *CYP2B6* SNPs in this study was similar to that reported in Thai HIV-infected children and adults [7, 19].

In agreement with previous studies, we observed a significant gene dose effect between c.516G>T and efavirenz [7, 23–25]. In addition, c.785A>G and g.21563C>T genotypes were also associated with efavirenz plasma concentrations. Conversely, the variant allele of 18492T>C was associated with lower plasma concentrations of efavirenz. However, the plasma efavirenz concentrations in all variants of those SNPs were within the therapeutic range. The clinical significance of 18492T>C polymorphisms with respect to lower efavirenz concentrations is unknown [22] and further studies with a larger sample are required.

To our knowledge, the association of c.516G ≥ T with efavirenz pharmacokinetics is well established [26–28] but the impact of *CYP2B6* haplotype, GAC (516G, 785A and g.21563C) on steady-state efavirenz plasma concentrations is not well-defined. It is unclear why the g.21563C>T polymorphisms, which are unlikely to impact on the functional effect of *CYP2B6*, were associated. However, it has been proposed that g.21563C>T is linked to 785A>G, which is correlated with reduced *CYP2B6* activity [14–16]. Due to the complexity of the *CYP2B6* polymorphisms, it is likely that haplotypes rather than a single polymorphism would be a better predictor of efavirenz plasma concentrations.

There are several limitations to our study. Firstly, the study population is small and the SNPs and haplotypes identified in this study need to be confirmed in a larger population. Also, the retrospective analysis creates several possible biases in the selection of subjects. Specifically; subjects who did not tolerate efavirenz may have been excluded due to switching prior to the samples being obtained. Finally, the sampling time varied from 10 to 18 h after efavirenz administration, which might impact on the interpretation of the efavirenz plasma concentration.

In conclusion, the results of the present study suggest that efavirenz plasma concentrations are significantly higher in HIV-infected Thai adults with *CYP2B6*-c.516G>T, -c.785A>G and -g.21563C>T. Also, multiple SNPs (*CYP2B6* haplotype, GAC) may have potential value in predicting efavirenz concentrations and should be investigated to see

if they can help identify patients who are at an increased risk of adverse drug reactions.

Competing Interests

There are no competing interests to declare.

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