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ARTICLE

Pharmacogenotyping of *CYP3A5* in predicting dose-adjusted trough levels of tacrolimus among Malaysian kidney-transplant patients

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Abstract: Tacrolimus (FK506) is a calcineurin inhibitor with a narrow therapeutic index that exhibits large interindividual variation. Seventy-eight kidney transplant patients treated with tacrolimus were recruited to study the correlation of dose adjusted trough level (level/dose; L/D) of tacrolimus with *CYP3A5* and *ABCB1* genotypes, as well as the mRNA copy number of *ABCB1* in blood. Patients were genotyped for *ABCB1* (*C1236T*, *G2677T*/*A*, and *C3435T*) and *CYP3A5* (*G6986A*), while *ABCB1* mRNA transcript copy number was determined by absolute quantification (real-time PCR) in 46 patients. *CYP3A5*3* genotypes were found to be a good predictor of tacrolimus L/D in kidney-transplant patients. Significantly higher L/D was observed among non-expressors (2.85, 95%: 2.05–3.70 (ng·mL^{-1})/(mg·kg^{-1})) as compared with the expressors (1.15, 95%: 0.95–1.80 (ng·mL^{-1})/(mg·kg^{-1})) of CYP3A5 (Mann–Whitney *U* test; $P < 0.001$). No correlation was observed between L/D and the *ABCB1* genotypes. A significant inverse correlation of blood *ABCB1* mRNA level with L/D was demonstrated (Spearman's Rank Order correlation; $P = 0.016$, $r_s = -0.348$). However, in multiple regression analysis, only *CYP3A5*3* genotype groups were found to be significantly correlated with tacrolimus L/D ($P < 0.001$). These findings highlight the importance of *CYP3A5*3* pharmacogenotyping among kidney-transplant patients treated with tacrolimus, and confirm the role of blood cell P-glycoprotein in influencing the L/D for tacrolimus.

Key words: kidney transpant patients, tacrolimus, *CYP3A5* genotype, *ABCB1* genotype, mRNA copy number of *ABCB1*.

Résumé : Le tacrolimus (FK506) est un inhibiteur de calcineurine dont l'indice thérapeutique est étroit et dont la variabilité de réponse inter-individus est importante. Soixante-dix-huit patients ayant subi une transplantation rénale et traités au tracrolimus ont été recrutés afin d'étudier la corrélation de la dose ajustée par le niveau de tacrolimus (L/D) et les génotypes de *CYP3A5, ABCB1* et du nombre de copies d'ARNm de *ABCB1* dans le sang. Le génotype de *ABCB1* (*C1236T*, *G2677T*/*A* et *C3435T*) et de *CYP3A5* (*G698A*) des patients a été établi et le nombre de copies de l'ARNm de *ABCB1* a été déterminé par quantification absolue par RT-PCR chez 46 patients. Les génotypes *CYP3A5*3* se sont révélés de bons prédicteurs du L/D du patient pour le tacrolimus. Un L/D significativement plus élevé a été observé parmi ceux n'exprimant pas CYP3A5 (2,85, 95 % : 2,05–3,70 (ng·mL^{-1})/(mg·kg^{-1})) comparativement à ceux qui l'expriment (1,15, 95 % : 0,95–1,80 (ng·mL^{-1})/(mg·kg^{-1})) (test Mann–Whitney *U*, $P < 0,001$). Aucune corrélation n'a été observée entre le L/D et les génotypes de *ABCB1*. Une corrélation inverse significative entre le niveau d'ARNm d'*ABCB1* du sang et le L/D a été démontrée (corrélation des rangs de Spearman, $P = 0,016$, $r_s = -0,348$). Cependant, en utilisant une analyse de régression multiple, seuls les génotypes *CYP3A5*3* se sont révélés en corrélation significative avec le L/D du tacrolimus ($P < 0,001$). Ces résultats soulignent l'importance de réaliser un pharmaco-génotypage chez les patients ayant subi une transplantation rénale à qui l'on a prescrit du tacrolimus, et confirment le rôle de la P-glycoprotéine des cellules sanguines sur le L/D du tacrolimus. [Traduit par la Rédaction]

Mots-clés : patients transplantés rénaux, tacrolimus, génotype du *CYP3A5*, génotype du *ABCB1*, nombre de copies d'ARNm de *ABCB1*.

Introduction

Tacrolimus (FK506) has been used as an immunosuppressant to reduce the risk of organ rejection in transplant patients. However, this calcineurin inhibitor has a narrow therapeutic index (NTI) and wide interpatient variation, which has been observed among the Asians and Caucasians (Venkataramanan et al. 1995; Sam et al. 2006). One of the challenges for the safe use of tacrolimus was related to polymorphic CYP3A5 enzyme, which is the predominant enzyme for the metabolism of tacrolimus, with a higher affinity compared with CYP3A4 (Kamdem et al. 2005). The most frequent and functionally significant single nucleotide polymorphism (SNP) of the CYP3A5 enzyme is *CYP3A5*3*. This SNP carries a substitution of A to G at position 6986 within intron 3, which leads to alternative splicing and truncated protein (Hustert et al. 2001). Based on previous studies, patients were classified into 2 categories: (*i*) expressors (*CYP3A5*1*/*CYP3A5*1* or *CYP3A5*1*/*CYP3A5*3*), and (*ii*) CYP3A5 non-expressors (*CYP3A5*3*/*CYP3A5*3*) (Barry and Levine 2010). A previous study has shown that the dose-adjusted ratio of tacrolimus was significantly lower among CYP3A5 expressors compared with CYP3A5 non-expressors (Fukudo et al. 2008).

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P-glycoprotein (P-gp), which pumps tacrolimus into the intestinal lumen, reduces the intracellular concentration of tacrolimus (Benet 1998; Tuteja et al. 2001). A synonymous SNP known as *C3435T* for the *ABCB1* gene (Marchetti et al. 2007) has shown significant correlation with intestinal P-gp levels, and it also influences the uptake of some orally administered P-gp substrates (Hoffmeyer et al. 2000). Linkage analysis has confirmed that this SNP is associated with *G2677T/A* and *C1236T* (Kroetz et al. 2003; Sai et al. 2003; Tang et al. 2004; Leschziner et al. 2006). The variant of *G2677T/A* is a tri-allelic nonsynonymous mutation, which leads to one of 2 possible amino-acid changes with dissimilar prevalence, where *G2677T* (A893S) is far more common than *G2677A* (A893T) (Fung and Gottesman 2009). The synonymous *C1236T* polymorphism, on the other hand, is located on exon 12, and causes the substitution of cytosine with thymine, which encodes for the same amino acid, glycine, at position 412 of the protein (Kimchi-Sarfaty et al. 2007). The lack of association between these 3 genetic variants and *ABCB1* expression led to the hypothesis that there might be other nonsynonymous polymorphisms linked to this SNP. A more user-friendly tool is therefore desired for clinical use (Sim and Ingelman-Sundberg 2012), as genotyping all of the 105 allelic variants of *ABCB1* is too tedious. We hypothesized that quantification of *ABCB1* mRNA levels in peripheral blood mononuclear cells (PBMCs) could be a reliable pharmacological marker to aid in the effort to personalise tacrolimus therapy for transplant patients. Furthermore, *ABCB1* mRNA levels in PBMCs have also been reported to be significantly correlated with the occurrence of acute cellular rejection (ACR) among liver-transplant patients treated

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primers (1236C: 5′-TCGTCCTGGTAGATCTTGAAGGTC-3′ or 1236T: 5′-TCGTCCTGGTAGATCTTGAAGGTT-3′), 100 ng of genomic DNA and 1× GoTaq Green Master Mix (Promega, Madison, Wisconsin, USA) in a total volume of 25 μL. Amplification was carried out with 5 cycles of denaturation at 94 °C for 45 s, touch-down step at 62–66 °C (decrement of 1 °C per cycle) for 30 s and extension at 72 °C for 30 s and followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. To detect *2677G, 2677T*, and *2677A* alleles, 3 separate reactions were carried out in parallel for each individual sample. Each amplification mix contains 0.1 μmol/L of forward common primer (2677 FWC: 5′-CTATAGGTTCCAGGCTTGCTG-3′), 0.2 μmol/L of reverse common primer (2677RVC: 5′-GGAAGGAAGAACAGTGTGAAGAC-3′), and 0.3 μmol/L of an allele-specific primer (2677GFW: 5′TGAAAGATAAGAAAGAACTAGAAGGCG3′; 2677TFW: 5′-TGAAAGATAAGAAAGAACTAGAAGGCT-3′; or 2677AFW: 5′-TGAAAGATAAGAAAGAACTAGAAGGCA-3′), 100 ng of genomic DNA, 1× GoTaq Green Master Mix in a total volume of 25 μL. Amplification was carried out with 25 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. A single reaction was carried out for detection of *6986G* and *6986A* alleles. Each amplification mix contains 0.4 μmol/L of common primers (CYP3A5*3FWC: 5′-ATGACACAGCTCTAGATGTCC-3′ and CYP3A5*3RVC: 5′-ATTTAAATAATGATTGATCCACTG-3′) together with 0.05 and 0.7 μmol/L of allele-specific primers (6986G RV: 5′-ATATCTCTTCCCTGTTTGGAC-3′ and 6986A FW: 5′-TAAAGAGCTCTTTTGTCTTTCAG-3′ respectively), 100 ng of genomic DNA, and 1× GoTaq Green Master Mix in a total volume of 25 μL. Amplification were carried out with 35 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s.

Validation of genotyping

Ten DNA samples from anonymous individuals, collected from the DNA bank, were randomly genotyped and sent for direct sequencing. Sequencing results confirmed the validity of the method.

ABCB1 mRNA expression

Primers and probe were designed using Beacon Designer 7.91 (Premier Biosoft International, California, USA). 6-Carboxylfluorescein (FAM) was used as the reporter dye for the housekeeping gene probe (*B2M*), while HEX reporter dye was used for the probe of the gene of interest (*ABCB1*). The extraction of RNA from patients' blood was carried out using the RiboPure Blood Kit (Ambion; Applied Biosystems) according to manufacturer's guide. RNA integrity and quantity were analyzed using Bioanalyzer (Agilent, Karlsruhe, Germany). It was previously shown that the efficiency of reverse-transcription PCR for a small amplicon length of 70–250 bp is less dependent on RNA quality (Fleige and Pfaffl, 2006). Therefore RNA samples with a RNA integrity number (RIN) of more than 2.0 were used for conversion to cDNA using Qiagen Reverse Transcription kit (Qiagen, Valencia, Calif.). Quantification of *ABCB1* mRNA was achieved by absolute real-time PCR using the IQ5 thermal cycler (Bio-Rad, Munich, Germany). The assay was done in duplex where both the gene of interest, *ABCB1* (NCBI Reference Sequence: NM_000927.3), and the housekeeping gene, *B2M* (NCBI Reference Sequence: NM_003889.3), were quantified in the same reaction well using specific probes. For *ABCB1* the primers and probes were as follows: forward primer, 5′-ATGAATCTGGAGGAAGACATGACC-3′; reverse primer, 5′-GCCAGGCACCAAAATGAAACC-3′; and the probe (HEX), 5′-TAAGCAGCAACCAGCACCCCAGCA-3′ were used; and for B2M we used, forward primer, 5′-AAAGTGGAGCATTCAGACTTGTC-3′; reverse primer, 5′-CCACTTAACTATCTTGGGCTGTG-3′; and the probe (FAM), 5′-TCACATGGTTCACACGGCAGGCAT-3′. To generate the standard curve, the gene of interest and housekeeping genes were cloned into plasmids (pDrive cloning vector; Qiagen). A standard curve for the cloned *ABCB1* and β-microglobulin (B2M) cDNA were calibrated over a range of 50 to 5 000 000 copies at 10-fold increments. Reactions were carried out in final volume of 25 μL with 0.4 μmol/L each of forward and reverse primers, and 0.2 μmol/L of each probe, using the NoROX Multiplex Master mix (Qiagen). Each reaction was done in triplicate. Amplification was carried out for 35 cycles with a denaturation temperature of 95 °C for 60 s and an annealing and extension temperature of 60 °C for 90 s.

Tacrolimus dosage and measurement

Patients were treated with tacrolimus (Prograf; Fujisawa Healthcare, Inc., Deerfield, Illinois, USA) twice daily; doses were weight-adjusted. The trough concentration of tacrolimus was measured using the Microparticle Enzyme Immunoassay (MEIA) with an IM_X analyzer (Abbott Laboratories, Chicago, Ill.) according to the manufacturer's instructions. The coefficient of variation (CV) of the assay for intraday and interday variation was 4.1%–8.7% and 1.7%–2.8% respectively. The tacrolimus trough levels ($ng \cdot mL^{-1}$) per dose ($mg \cdot kg^{-1} \cdot d^{-1}$) were calculated by dividing the trough level ($ng \cdot mL^{-1}$) and patients' tacrolimus dose ($mg \cdot kg^{-1} \cdot d^{-1}$) at the particular time point, and this is expressed as L/D in the unit of $[(ng \cdot mL^{-1})/(mg \cdot kg^{-1})]$.

Statistical analyses

Data were analyzed using PASW 18.0 for Windows (SPSS, Inc., USA). Spearman's Rank Order correlation was used to find association between dose-adjusted level of tacrolimus and *ABCB1* mRNA copy number as well as biochemical data. The Kruskal–Wallis or the Mann–Whitney *U* tests were used to determine the association between tacrolimus L/D and race, as well as the *CYP3A5* and *ABCB1* genotypes. Correlation between *ABCB1* genotypes and *ABCB1* mRNA copy number was tested using 1-way ANOVA if the variances across the groups were equal, with Welch's test being used when the variances across the groups were not equal. Correlation, with $P < 0.05$ was included in the multi-regression analysis. For *CYP3A5* genotypes, patients were classified in 2 functional categories: (*i*) CYP3A5 expressors (*CYP3A5*1/*1* and *CYP3A5*1/*3*) and (*ii*) non-expressors (*CYP3A5*3/*3*) (Barry and Levine 2010). The *CYP3A5* functional categories were then coded using 2 dummy variables (1 coded for expressor and 0 coded for non-expressor). Values for $P < 0.05$ were considered statistically significant, and the confidence intervals (CI) are at 95%.

Results

Correlation of patients' clinical characteristics and dose-adjusted trough level of tacrolimus

The patients' characteristics are shown in Table 1. There were no significant correlations between the dose adjusted levels (L/D) of tacrolimus and age or biochemical parameters, except for levels of sodium and chloride (Spearman's correlation; $P = 0.025$, $r^2 = -0.327$; and $P = 0.005$, $r^2 = -0.409$ respectively). This correlation is corroborated by a study (Hoorn et al. 2011) showing that calcineurin inhibitors are capable of activating the sodium chloride cotransporter, which then leads to hypertension. There is significant correlation between L/D and different ethnic groups (Kruskal–Wallis test, $P = 0.013$). Further analysis showed that the L/D was different between the Malay and Indian patients, and between the Chinese and Indian patients (Mann–Whitney *U* test, $P = 0.042$ and 0.006, respectively), but similar for the Malay and Chinese patients (Mann–Whitney *U* test, $P = 0.102$) with the Indian patients exhibiting the lowest L/D and the Chinese exhibiting the highest L/D (Table 1).

ABCB1 single nucleotide variations, haplotypes, and mRNA expression

A total of 3 single nucleotide polymorphisms (SNPs; *C1236T*, *G2677T/A*, and *C3435T*) on the *ABCB1* gene, and one SNP on the *CYP3A5* gene (*G6986A*) were analyzed in 78 patients. The distribution of CYP3A5 status was similar among the Malay and Indian patients, with a higher frequency of expressors in both groups (χ^2,

Table 2. Allele frequency of *C1236 T*, *G2677 T/A*, *C3435 T*, *A6986 G*, and percentage of expressor and non-expressor of CYP3A5 among kidney-transplant patients of 3 ethnic groups (Malay, Chinese, and Indian).

Race (*N*)	Allele frequency, % (95% CI)		
C1236 T	**C**	**T**	
Malay (33)	40.9 (31.8–50.7)	59.1 (49.3–68.2)	
Chinese (38)	40.8 (31.7–50.6)	59.2 (49.4–68.3)	
Indian (7)	28.6 (20.7–38.1)	71.4 (61.9–79.3)	
G2677 T/A	**G**	**T**	**A**
Malay (33)	57.6 (47.8–66.8)	33.3 (24.8–43.0)	9.1 (4.9–16.4)
Chinese (38)	47.4 (37.9–57.1)	38.2 (29.3–48.0)	14.4 (8.8–22.6)
Indian (7)	28.6 (20.7–38.1)	71.4 (61.9–79.3)	ND
C3435 T	**C**	**T**	
Malay (33)	68.2 (58.5–76.5)	31.8 (23.5–41.5)	
Chinese (38)	63.2 (53.4–72.0)	36.8 (28.0–46.6)	
Indian (7)	28.6 (20.7–38.1)	71.4 (61.9–79.3)	
A6986G	***CYP3A5*1***	***CYP3A5*3***	
Malay (33)	40.9 (31.8–50.7)	59.1 (49.3–68.2)	
Chinese (38)	27.6 (19.8–37.1)	72.4 (62.9–80.2)	
Indian (7)	50.0 (40.4–59.6)	50.0 (40.4–59.6)	
CYP3A5 Status			
	Expressor (*CYP3A5*1/*3* and **1/*1*)	Non-expressor (*CYP3A5*3/*3*)	
Malay (33)	69.7 (60.1–77.8)	30.3 (22.2–39.9)	
Chinese (38)	44.7 (35.3–54.5)	55.3 (45.5–64.7)	
Indian (7)	85.7 (77.5–91.2)	14.3 (8.8–22.5)	

Note: C, T, G, A, single nucleotide polymorphisms; ND, not detected.

$P = 0.389$). However, among the Chinese patients, the frequency of non-expressor was higher, and this distribution was found to be significantly different from the distribution among the Malay patients (χ^2, $P = 0.044$). Similarly, the distribution of CYP3A5 status was also different among the Chinese and Indian patients, but it was not statistically significant (χ^2, $P = 0.054$). The allele frequencies for patients are shown in Table 2. The allele and genotype frequencies of the SNPs were in Hardy–Weinberg equilibrium for all 3 ethnic groups of patients.

In this study, we found that the mRNA expression level of *ABCB1* is different between carriers of homozygous wild-type and homozygous mutant genotypes for *C1236T* (Games–Howell post-hoc test, $P = 0.039$). However, the mRNA expression level for carriers of the heterozygous genotype does not differ from those with homozygous wild-type and mutant genotypes (Games–Howell post-hoc test, $P = 0.330$ and 0.837 respectively). Carriers of the homozygous wild-type of *C1236T* exhibit the lowest mRNA level of *ABCB1*, followed by heterozygous carriers, and finally, homozygous mutant carriers with the highest mRNA copy number of *ABCB1* (Fig. 1*a*). No significant correlation was found between *G2677T/A* genotypes and mRNA copy number of *ABCB1* (1-way ANOVA, $P = 0.921$). A similar trend of mRNA expression was observed across the different genotype groups of *G2677T/A*, with homozygous mutants having the highest mRNA expression level (Fig. 1*b*). Comparison of *ABCB1* mRNA copy number across the different genotypes of *C3435T* reveal no significant association (1-way ANOVA, $P = 0.604$), although a trend of mRNA expression similar to those observed in *C1236T* and *G2677T/A* was seen across different *C3435T* genotypes (Fig. 1*c*).

Subsequently, only 5 haplotype groups with the highest frequency were included in the next analysis. No significant association was observed between mRNA copy number and the different haplotype groups (Levene's *F* statistic, $P = 0.034$ and the Welch test, $P = 0.327$), although a pattern of distribution for mRNA copy number was observed across the 5 different haplotype groups when comparing the median value. Homozygous wild-type haplotype (CGC/CGC) carriers show the lowest mRNA level, while the highest level was observed among the homozygous mutant (TTT/TTT) carriers. The other 3 heterozygous haplotypes (CGC/TGC, CGC/TTT, and TGC/TTT) show an intermediate value for mRNA copy number (Fig. 1*d*).

Association of *CYP3A5* and *ABCB1* genotypes with tacrolimus L/D

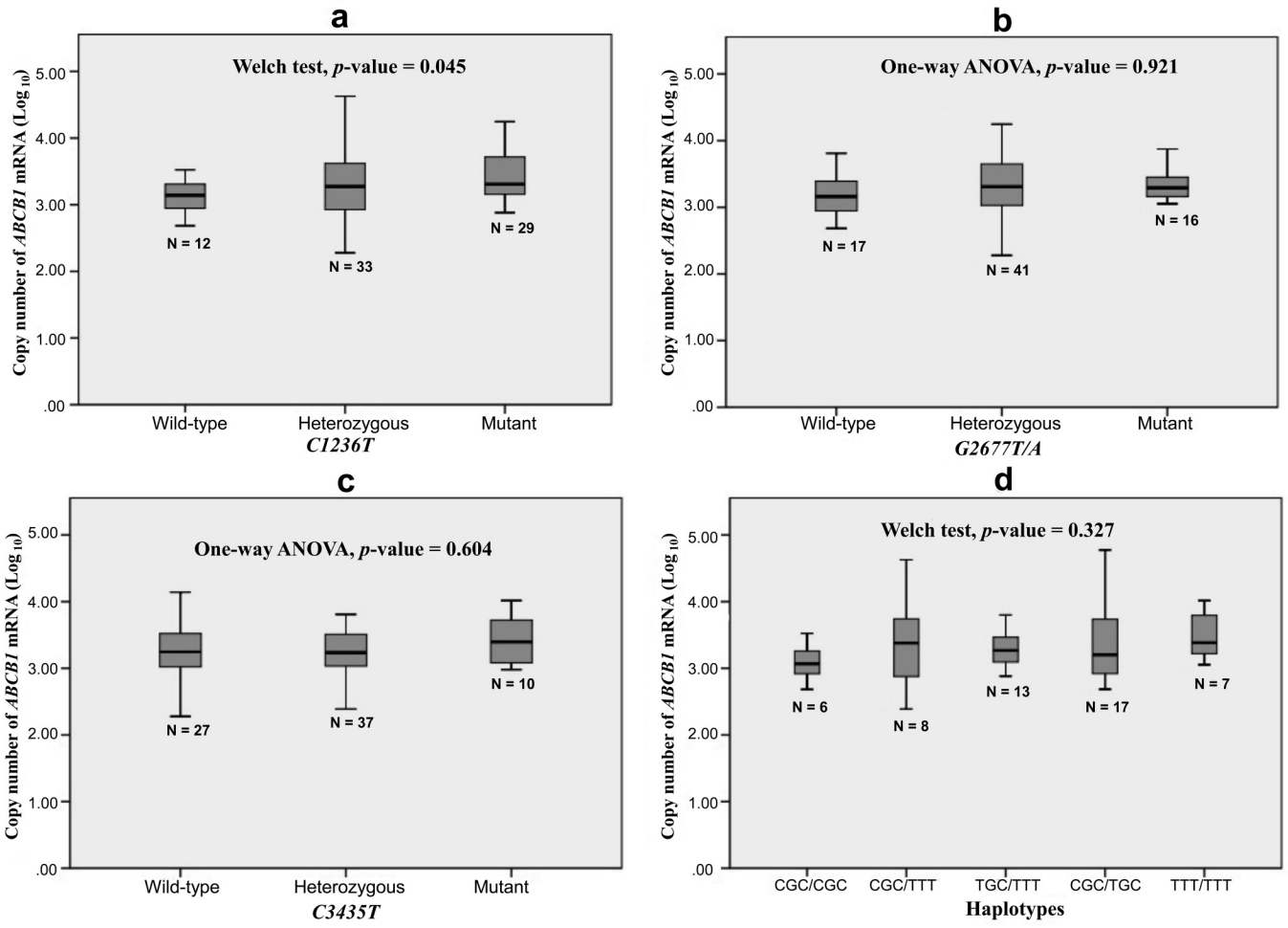
A statistically significant correlation was observed between L/D and *CYP3A5* genotype groups (expressor vs. non-expressor), where the higher L/D was observed among the non-expressors (Mann–Whitney *U* test, $P < 0.001$; L/D = 1.15, 95%; 0.95–1.80 [(ng·mL^{-1})/(mg·kg^{-1})] vs. 2.85, 95%; 2.05–3.70 [(ng·mL^{-1})/(mg·kg^{-1})] for expressors and non-expressors, respectively), as shown in Fig. 2*a*.

The effect of *ABCB1 C1236T*, *G2677T/A* and *C3435T* as well as the 3 major haplotypes (CGC/CGC, CGC/TTT, and TTT/TTT) on tacrolimus L/D was also investigated. None of the polymorphisms or the common haplotypes showed any significant correlation with tacrolimus L/D (Kruskal–Wallis test, $P > 0.05$). Nevertheless, L/D was found to be higher among the carriers of the wild-type genotypes of *C1236T* compared with carriers of the mutant genotypes, while heterozygous carriers were found to have the lowest tacrolimus L/D (Fig. 2*b*). Similarly, carriers of the homozygous wild-type genotype of *G2677T/A* exhibited the highest level of tacrolimus L/D, followed by carriers of the heterozygotes genotype, and the lowest level was found among the carriers of the homozygous mutant genotype (Fig. 2*c*). No distinct patterns of tacrolimus L/D were observed among the different genotypes of *C3435T* and the 3 common haplotypes investigated (Figs. 2*d* and 2*e*).

mRNA expression of *ABCB1* and correlation with tacrolimus L/D

Analysis of mRNA transcript copy numbers of *ABCB1* among kidney-transplant patients treated with tacrolimus was carried out on 46 patients' samples. To test whether the mRNA copy numbers of ABCB1 influenced the trough level of tacrolimus, the copy number was compared with tacrolimus dose-adjusted trough level [L/D (ng·mL^{-1})/(mg·kg^{-1})]. The blood samples used for mRNA quantification and the measurement of tacrolimus trough level were taken on the same day and time, to avoid spurious results. A Spearman's Rank Order correlation was run to determine the relationship between mRNA copy numbers and tacrolimus dose-adjusted trough levels. There was a significant inverse correlation observed between the 2 variables (Spearman's Rank Order correlation, $P = 0.016$, $r_s = -0.348$). From this analysis, it can be deduced that the higher the mRNA copy numbers, the lower the tacrolimus [L/D] levels in the patients.

Fig. 1. (*a*) Relationship between *C1236T* polymorphisms and *ABCB1* mRNA copy number. (*b*) Relationship between *G2677T*/A and *ABCB1* mRNA copy number. (*c*) Relationship between *C3435T* polymorphism and *ABCB1* mRNA copy number. (*d*) Relationship between CGC and TTT haplotypes and *ABCB1* mRNA copy number.



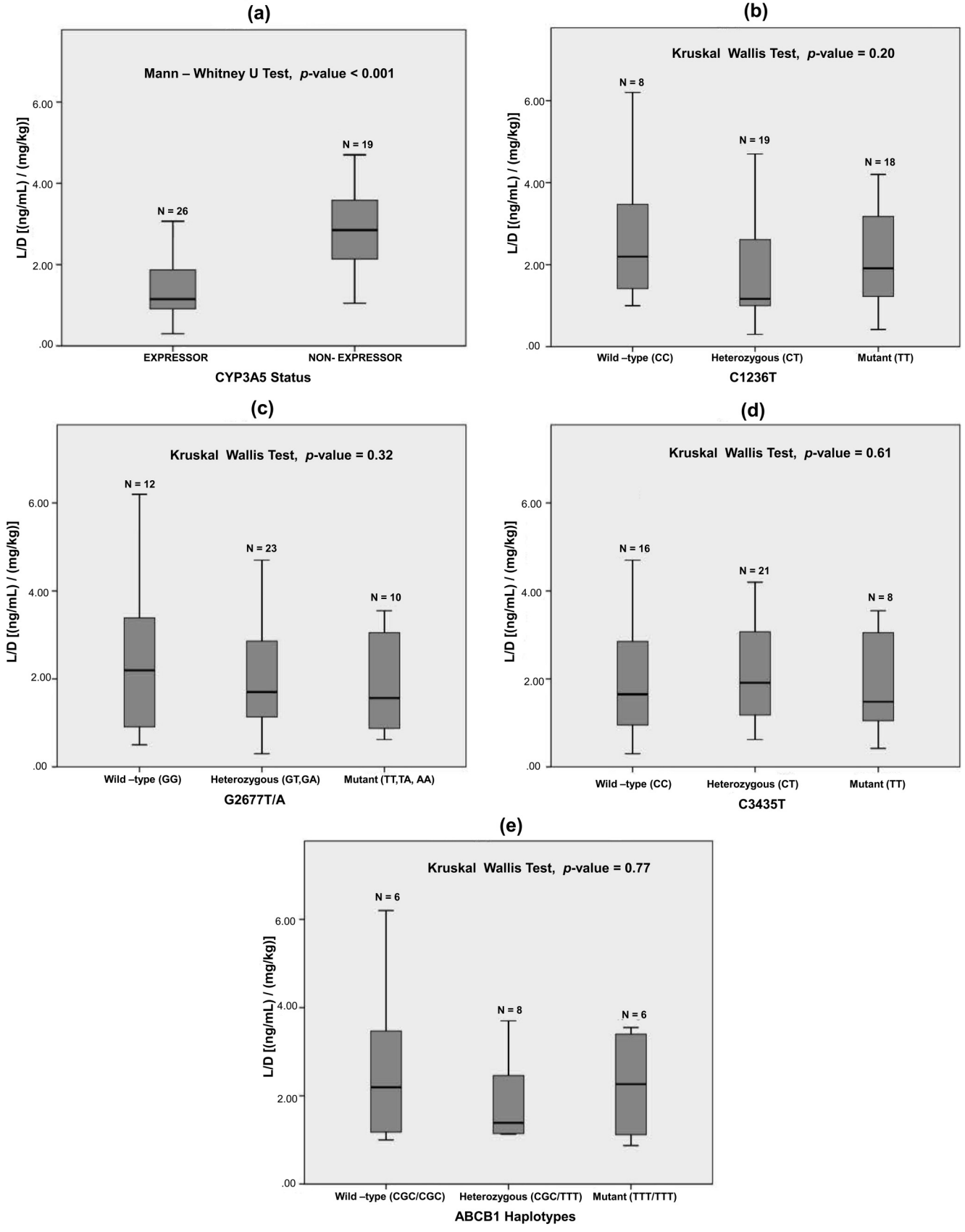
In a multiple regression analysis, L/D was only significantly associated with the *CYP3A5* genotype groups (multiple regression analysis, $P < 0.001$) while association with the mRNA copy number of *ABCB1* was not significant (multiple regression analysis, $P = 0.063$).

Discussion

A significant association was found between tacrolimus L/D and *CYP3A5* genotypes. Such finding is in accordance with previous findings that showed higher tacrolimus trough levels among liver transplant and kidney transplant patients who were non-expressors of CYP3A5 (*CYP3A5*3*/**3*) (Fukudo et al. 2006; Hesselink et al. 2008). This can be explained by an earlier study that reported the formation rate of primary metabolites of tacrolimus, i.e., 13-*O*-desmethyl tacrolimus (13-DMT), 31-*O*-desmethyl tacrolimus (31- DMT), and 12-hydroxy tacrolimus (12-HT), were 1.7-fold higher in human liver microsome among those with *CYP3A5*1*/**3* genotype as compared with the *CYP3A5*3*/**3* genotype (Dai et al. 2006). However no association was observed between tacrolimus L/D and genotypes and haplotypes of the *ABCB1* gene. One of the possible explanations for such findings is the effect of other unknown causal variants that are linked to these polymorphisms.

The rationale for analyzing the mRNA of *ABCB1* in blood is based on the fact that P-gp is shown to be expressed on the surface of lymphocytes (Suzuki et al. 2010). The statistically significant association between *C1236T* and the expression level of *ABCB1* demonstrated in this study is in accordance with the findings of Illmer et al. (2002) who had reported lower *ABCB1* expression in the acute myeloid leukemia blast cells of variant genotype carriers. However, this finding is in contrast with the analysis of *ABCB1* expression level in tumor tissues of breast cancer patients (Vaclavikova et al. 2008). A possible explanation for such discrepancies could be divergence of gene regulation in different human tissues (Vaclavikova et al. 2008). On the other hand, Wang and Sadée (2006) reported that *C1236T* is not the functional polymorphism that leads to imbalanced allelic expression and therefore it will not influence the mRNA level. Lack of association of *G2677T*/*A* and *C3435T* with mRNA copy number demonstrated in this study is similar to earlier studies (Kurata et al. 2002; Hitzl et al. 2004; Owen et al. 2005; Cascorbi 2006; Song et al. 2006; Ansermot et al. 2008; Mosyagin et al. 2008; Vaclavikova et al. 2008). The *G2677T*/*A* polymorphism demonstrates an inconclusive effect because it is located at a region that forms an intracellular loop, and where the function of this domain is unclear (Fung and Gottesman 2009). Since the *ABCB1* SNPs investigated in this study did not explain variations in the mRNA level (Fig. 2), this finding further strengthens the idea that they only act as markers for other unknown causative variants that have a more pronounced effect on the pharmacokinetics of tacrolimus. This also highlights the need to search for the causal variants that can be used to predict L/D of tacrolimus.

Fig. 2. (*a*) Relationship between CYP3A5 status and tacrolimus trough level (L/D). (*b*) Relationship between *C1236T* polymorphisms and tacrolimus L/D. (*c*) Relationship between *G2677T/A* and tacrolimus L/D. (*d*) Relationship between *C3435T* polymorphism and tacrolimus L/D. (*e*) Relationship between CGC and TTT haplotypes and tacrolimus L/D.



In this study, we found a significant negative correlation between *ABCB1* mRNA copy numbers in PBMCs and dose-adjusted trough levels of tacrolimus. Such a correlation is in contrast with the study by Goto et al. (2008), which reported higher whole blood tacrolimus concentrations among patients with higher *ABCB1* mRNA in their PBMCs. However this observation is complicated, owing to the fact that the tacrolimus L/D obtained was primarily from the erythrocyte concentrations, while the *ABCB1* mRNA copy number represents the expression level of *ABCB1* in leukocytes. Therefore it is crucial to establish the correlation between the leukocytic concentration of tacrolimus and *ABCB1* mRNA in PBMCs at multiple time-points before using *ABCB1* mRNA in PBMCs as a pharmacological marker to achieve individually targeted concentrations of tacrolimus.

The significant association of *CYP3A5* genotypes and the L/D from the multiple regression analyses is in agreement with a study on Japanese kidney-transplant patients. It was demonstrated in the aforementioned study that the *CYP3A5* genotype was a determinant for interindividual variation in tacrolimus pharmacokinetics at 1-year post-transplant (Miura et al. 2011). The non-significant association between mRNA copy number of *ABCB1* and tacrolimus L/D in the multiregression analyses could be due to the small sample size in this study. Therefore, it is worth replicating this study with a larger sample size and measuring the leukocyte concentration of tacrolimus before ruling out the potential association of mRNA copy number of *ABCB1* and tacrolimus L/D.

Conclusions

The data clearly demonstrate the significant association of *CYP3A5* genotypes and tacrolimus L/D among patients. This also highlights the need to determine patients' *CYP3A5* genotypes as part of clinical efforts to optimize tacrolimus dose and to avoid possible incidence of toxicity, since it is being clearly shown that *CYP3A5*3*/*CYP3A5*3* individuals had higher tacrolimus L/D. Since the *ABCB1* polymorphisms investigated in this study cannot predict the *ABCB1* mRNA level and tacrolimus L/D, it is suggested that novel SNPs on the *ABCB1* gene influence the phenotype of the *ABCB1* gene product, P-gp. We also recommend a comprehensive study with a larger sample size and a different approach for measuring tacrolimus levels (leukocytic concentration) to identify the true potential of mRNA copy numbers of *ABCB1* in predicting tacrolimus L/D.

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