Chapter 7

Influence of different allelic variants of the cytochrome 3A and adenosine triphosphate-binding cassette B1 gene on the tacrolimus pharmacokinetic profile of Chinese renal transplant recipients

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Abstract

Background and aim

Tacrolimus has a narrow therapeutic window and a wide interindividual variation in its pharmacokinetics. Cytochrome P450 3A (CYP3A) and the adenosine triphosphate-binding cassette B1 (ABCB1) gene encoding protein, multidrug resistance-1 (MDR1) or P-glycoprotein play an important role in the tacrolimus disposition. Therefore this study evaluates whether CYP3A and ABCB1 polymorphisms are associated with the area under the time tacrolimus concentration curve (AUC_{0-12}) calculated using a two time point sample strategy.

Materials and methods

The CYP3A and ABCB1 genotypes are determined by real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays in 103 Chinese renal transplant recipients and consequently related to their dose-normalized AUC_{0-12} ($dnAUC_{0-12}$).

Results

A significant allele-dependent effect (Kruskal Wallis; $P < 0.001$) is observed between the CYP3A5*3 polymorphism and the $dnAUC_{0-12}$. Multiple regression analysis showed that the CYP3A5*3 polymorphism is the most significant independent variable and explained 35% of the dose requirement variability in relation to tacrolimus use. Regarding the ABCB1 G2677T/A and C3435T polymorphisms, a trend is observed between the different genotypes and the $dnAUC_{0-12}$.

Conclusion

The CYP3A5*3 polymorphism may be an important factor in determining the dose requirement for tacrolimus and genotyping can help determine the initial daily dose needed by individual patients for adequate immunosuppression.

Introduction

The calcineurin inhibitor tacrolimus that is used worldwide for primary immunosuppression following renal transplantation has a narrow therapeutic index, which makes close therapeutic drug monitoring necessary to prevent both sub-therapeutic blood levels as well as toxic blood levels. Subtherapeutic tacrolimus blood levels increase the risk of transplant rejection[1-3], while toxic tacrolimus blood levels may lead to severe side effects such as nephrotoxicity and neurotoxicity[4-6]. The high interindividual variability in tacrolimus pharmacokinetics complicates the realisation of this narrow therapeutic index[7,8]. Although tacrolimus trough (C_0) levels have been shown to provide a reliable indication of the total drug exposure[9], their usefulness in differentiating graft rejection episodes from nephrotoxicity has been questioned[2,10]. A more accurate estimation of the tacrolimus exposure by making 12 hour pharmacokinetic profiles may lead to a lower incidence of acute rejection like in the case of cyclosporine[11,12]. Since obtaining complete 12 hour pharmacokinetic profiles for every renal transplant patient is not feasible in clinical practice, parameters have been examined that strongly correlate with the 12 hour pharmacokinetic profile. Recent studies showed higher correlations with the 12 hour pharmacokinetic profiles of tacrolimus when a limited sampling point sample strategy is used instead of C_0 levels alone[13-17]. Additionally, the two time point sample strategy used in the present study is able to calculate reliable the area under the time tacrolimus concentration curve or $AUC_{0\text{-}12}$ in renal transplant patient groups[15,17]. The cytochrome P450 (CYP) 3A iso-enzymes mainly represented by CYP3A4 and CYP3A5 have been identified as the major enzymes responsible for the disposition of tacrolimus[18]. Although a number of CYP3A4 polymorphisms have been identified (website Human Cytochrome P450 (CYP) Allele Nomenclature Committee[101]), these are rare and therefore do not contribute significantly to CYP3A4 expression variability. The single nucleotide polymorphism (SNP) CYP3A5 6986G or *3 displays a sequence variability in intron 3 that creates a cryptic splice site and encodes an aberrantly spliced mRNA with a premature stop codon, leading to the absence of protein expression. This CYP3A5*3 polymorphism occurred homozygously in 90% of the Caucasians, 73% of the Chinese and in 30% of the African American population[19-21]. Although several studies[22-31] have described the association between the CYP3A5*3 polymorphism and tacrolimus C_0 levels, the present study examines the associations with the CYP3A polymorphisms using a more accurate approach to determine the tacrolimus exposure in renal transplant patients. Tacrolimus is also a substrate for the adenosine triphosphate-binding cassette B1 (ABCB1) gene encoding protein multidrug resistance-1 (MDR1) or P-glycoprotein. ABCB1 is an ATP-dependent efflux pump that contributes to the protection of the body from environmental toxins and drugs like tacrolimus by limiting their absorption from the gut lumen or increasing their biliary and urinary excretion[32]. Three partly linked polymorphisms in the ABCB1 gene located on exons 12, 21 and 26 have been studied widely and these polymorphisms account for the major haplotypes

encountered in Caucasians. Whereas two of these ABCB1 polymorphisms, C1236T and C3435T, result in silent mutations, the ABCB1 G2677T/A polymorphism in exon 21 is non-synonymous and results in an amino acid exchange (Ala893Ser/Thr). A number of studies already have reported that there seems to be no association between the ABCB1 polymorphisms and tacrolimus dose-normalised trough (dnC_0) levels[25-27,30,31,33] or the dose-normalised area under the time tacrolimus concentration curve ($dnAUC_{0-12}$)[34,35]. However, some studies found a correlation between individual ABCB1 polymorphisms[23,24,36] or the ABCB1 1236C-2677G-3435C haplotype[37] and a higher tacrolimus dose. The present study evaluates in Chinese renal transplant recipients the impact of CYP3A and ABCB1 polymorphisms on the $dnAUC_{0-12}$ which is calculated according to a two time point sampling strategy. Furthermore, we described two new real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays to determine the genotype for the CYP3A5 A6986G and ABCB1 C1236T polymorphism.

Patients and methods

Study population

A total of 103 Chinese renal transplant recipients who received tacrolimus as part of the immunosuppressive therapy and had regular follow up in Queen Elizabeth Hospital or Tuen Mun Hospital in Hong Kong were included in this retrospective study. There was no change in the daily tacrolimus dose for at least two weeks. Patients were advised to avoid St. John's wort and grapefruit juice which may affect the tacrolimus concentration. None of the patients included were taking medication known to have interaction with tacrolimus, such as calcium channel blockers, anti-epileptics, anti-mycotics and macrolide antibiotics. Additionally, patients who suffered from gastrointestinal disease, liver disease or other disorders that may alter the absorption of tacrolimus were excluded. Apart from tacrolimus and steroids, these patients were normally put on azathioprine, however some patients preferred to use mycophenolic acid on advice of their private physician. The dosage of azathioprine was 1.5 mg/kg/day while the dosage of mycophenolic acid was 0.5 gram twice daily. The initial tacrolimus dosage, administrated twice daily, was 0.3 mg/kg/day for all patients. The daily tacrolimus dose was then adjusted according to the AUC_{0-12} value, which was kept at around 100-150 ng $\times$ hr/ml in the first three months. After three months the target AUC_{0-12} value was decreased to around 80-100 ng $\times$ hr/ml for long term maintenance. These AUC_{0-12} values were based on our previous pilot study[15] and the experiences obtained in our transplantation centers. The steroid regimen for the first month was 30 mg/day of oral prednisolone, progressively tapered by 2.5 mg every two weeks until a daily maintenance dose of 5 mg. Demographic as well as clinical data were collected at the time of the tacrolimus blood sample collection.

Tacrolimus concentration determination

Tacrolimus blood concentrations were determined 2 (C2) and 4 (C4) hours after the morning tacrolimus administration in ethylene diamine tetra-acetic acid (EDTA) whole blood using a semi-automated microparticle enzyme immunoassay (MEIA) on an IMx II clinical analyser (Abbott Laboratories, Abbott Park, IL, USA). The IMx II does not cross-react enormously with the most important tacrolimus metabolite produced by CYP3A5, 13-O-demethyltacrolimus, which has a minor immunosuppressive effect. Furthermore, a few metabolites show significant cross reactivity with the antibody but were present at low concentrations compared to tacrolimus[27]. The two tacrolimus blood concentrations determined were used to calculate the $AUC_{0\text{-}12}$ according to the equation based strategy as described by our group[15]: $AUC_{0\text{-}12} = 16.2 + 2.4 \times C2 + 5.9 \times C4$. The $dnAUC_{0\text{-}12}$ was calculated by dividing the $AUC_{0\text{-}12}$ by the corresponding 24 hour dose on a milligrams per kilogram basis.

Ethics

The study was performed in accordance to the Declaration of Helsinki and its amendments. The protocol was approved by the Medical Ethics Committee of the Queen Elizabeth Hospital in Hong Kong and written informed consent for participation in this study was obtained from all patients.

DNA isolation

Genomic DNA was extracted from 103 Chinese renal transplant recipients by using 200 μl EDTA anticoagulated blood for isolation with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions.

Genotyping of CYP3A and ABCB1 gene polymorphisms

Real-time PCR FRET assays were used for genotyping the CYP3A4 A-392G, CYP3AP1 G-44A, ABCB1 G2677T/A and C3435T polymorphisms with the same primers and probes as described in the original publications[38-41]. The real-time PCR FRET assays for the CYP3A5 A6986G and ABCB1 C1236T polymorphisms were designed, optimised and validated in our laboratory. The presence of the expected polymorphisms was confirmed by sequencing the different allelic variants of CYP3A5 and ABCB1 according to a direct sequence procedure on a capillary sequencer ABI Prism 3100® using the Bridge version 1.1 sequence kit (both products from Applied Biosystems, Fostercity, USA). Genotyping for the CYP3A5 A6986G polymorphism was performed using the primers F16: 5'-TTT gCC TCT TTg TAC TTC TTC ATC-'3 (sense; 172835-858) and R16: 5'- Tag TTg TAC gAC ACA Cag CAA CC-'3 (anti-sense; 173335-313) to amplify a 501 bp part from the CYP3A5 gene which covers the A6986G polymorphism in intron 3 (Genbank acces no: AF280107). Detection was carried out using the anchor probe CYP316R640: 5'-CCC TgT TTg gAC CAC ATT ACC CTT-'3 (sense; 173214-237) which

was labelled at the 5‘ end with LCRed 640 and phosphorylated at the 3‘- end to block extension and the sensor probe CYP3A5A16: 5’-gAg CTC TTT TgT CTT TCA ATA TCT CT-‘3 (sense; 173187-212) which is complementary to the CYP3A5 6986A or *1 allele with the polymorphic nucleotide indicated underlined. This 3‘-fluorescein labelled anchor probe binds with a distance of one base 5’ to the detection probe. The PCR mixture contained 3.3 μl sterile water; 2.0 mmol/l $MgCl_2$; 1.0 μl LC Faststart DNA Master Hybridisation Probes (Roche Diagnostics GmbH, Mannheim, Germany), 0.60 μmol/l of each primer, and 0.20 μmol/l of both anchor and sensor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 μl containing 50-100 ng genomic DNA to the PCR mixture, the total volume is 10 μl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 45 cycles at 95°C for ten seconds; 55°C for ten seconds and 72°C for 20 seconds (all 20°C/second). After amplification was completed, a melting curve was recorded by cooling to 45°C (20°C/second) holding at 45°C for two minutes and then heating slowly to 75°C at 0.1°C/second. Moreover, genotyping for the ABCB1 C1236T polymorphism is performed using the primers F35: 5’-gTT CCT ATA TCC TgT gTC TgT gAA T-‘3 (sense; 208-32) and R35: 5’-AgT CTA gCT CgC ATg ggT CAT C-‘3 (anti-sense; 448-27) to amplify a 241 bp part from the ABCB1 gene which covers the C1236T polymorphism in exon 12 (Genbank access no: M29432). Detection of the ABCB1 C1236T polymorphism was carried out using the anchor probe A35: 5'-gCC ACC gTC TgC CCA CTC TgC AC-3 (anti-sense; 343-321) which is labelled at the 3’-end with fluorescence and binds with a distance of one base 5’ to the sensor probe. This 5’ LCRed640 labelled sensor probe P35: TTC Agg TTC AgA CCC TTC AAg (anti-sense; 319-299) which is phosphorylated at the 3’ end to block extension from the anchor probe and is complementary to the ABCB1 1236T polymorphism which is indicated underlined. The PCR mixture for the ABCB1 C1236T polymorphism contained: 2.95 μl sterile water; 2.0 mmol/l $MgCl_2$; 1.0 μl LC Faststart DNA Master Hybridization Probes; (Roche Diagnostics GmbH, Mannheim, Germany) 0.5 μM of each primer and 0.225 μmol/l of both sensor and anchor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 μl containing 50-100 ng genomic DNA to the PCR mixture, the total volume was 10 μl. The PCR protocol included the following steps: denaturation for 10 minutes at 95°C; 45 cycles at 95°C for 5 seconds, 55°C for 10 seconds and 72°C for 20 seconds (all 20°C/second). After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second) holding at 95°C for 20 seconds; subsequently cooling to 40°C (20°C/second) holding at 40°C for 20 seconds and then heating slowly to 85°C at 0.2°C/second. All PCR and melting curve analysis were performed on the LightCycler (Roche Diagnostics, Almere, the Netherlands).

Statistical analysis

Statistical analysis of the data was performed with use of SPSS 12.0 software for windows (Chicago, IL, USA). To examine the population homogeneity of the patients, the genotype frequencies of the CYP3A and ABCB1 polymorphisms were tested against

Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test. For analysis of the $dnAUC_{0\text{-}12}$ (ng × hr/ml per mg/kg) and the daily tacrolimus dose (mg/kg/day), two groups were compared using the non parametric Mann-Whitney test while the non parameteric Kruskal Wallis test was used to compare several groups. Stepwise multiple regression analyses was used to calculate the relative contribution of several genetic and non-genetic factors on the variability in the daily tacrolimus dose requirement. The genetic factors examined are the CYP3A5*3 polymorphism and three ABCB1 polymorphisms while the non-genetic factors examined are gender, age, serum creatinine concentration, time since transplantation and steroid dosing. *P* values less than 0.05 were considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

Demographic characteristics of patients

Table 7.1 illustrates the characteristics of the 103 Chinese renal transplant patients that fulfilled the inclusion criteria and were enrolled in our study.

Real-time PCR FRET assays for CYP3A5 A6986G and ABCB1 C1236T genotyping

The melting point of the sensor probe covering the CYP3A5 A6986G polymorphism was 57°C when hybridised to the CYP3A5 6986G/G or *3/*3 genotype and 62°C when hybridised to the CYP3A5 6986A/A or *1/*1 genotype. A heterozygous sample contained both type of targets and thus generated both peaks (Figure 7.1A). In addition, for the ABCB1 C1236T polymorphism, the melting point of the sensor probe was 56.5°C when hybridised to the ABCB1 1236CC genotype and 62°C when hybridised to the ABCB1 1236TT genotype. A heterozygote sample for the ABCB1 C1236T polymorphism showed both melting peaks (Figure 7.1B). The presence of the expected polymorphisms was confirmed by sequencing these different allelic variants of CYP3A5 and ABCB1 gene. The resulting characterised heterozygote samples were used as controls in each run performed.

Table 7.1 Demographic characteristics of the renal transplant recipients

Demographic characteristics	
Gender (male/female)	58/45
Age (years, mean ± SD, (range))	43.1 ± 11.1 (21-77)
Weight (kg, mean ± SD, (range))	62.5 ± 11.4 (37.4-87.8)
Primary kidney disease	
Glomerulonephritis	57
Chronic pyelonephritis	1
Diabetic nephropathy	10
Hypertensive nephropathy	9
Polycystic kidney disease	2
Obstructive Uropathy	3
Unknown	18
Other	3
Transplantation number	
First	97
Second	6
Tacrolimus dose (mg/kg body weight/day, mean ± SD, (range))	0.067 ± 0.032 (0.01-0.18)
Tacrolimus conc. 2 hour post dose (ng/ml, mean ± SD, (range))	13.5 ± 4.30 (5.7-29.4)
Tacrolimus conc. 4 hour post dose (ng/ml, mean ± SD, (range))	8.7 ± 2.60 (2.3-16.4)
$AUC_{0\text{-}12}$ (ng × hr/ml, mean, (range))	100.3 (47.8-183.5)
$DnAUC_{0\text{-}12}$ (ng × hr/ml per mg/kg body weight, mean, (range))	1940 (300-11125)
Use of azathiopurine/mycophenolate mofetil	78/25
Current steroid dose (mg, mean ± SD, (range))	6.98 ± 2.37 (4-25)
Time since transplantation (days, mean, (range))	977 (133-4982)
Haemoglobin (mmol/l, mean ± SD, (range))	12.8 ± 2.0 (8.5-19)
Haematocrit fraction (mean ± SD, (range))	0.38 ± 0.058 (0.25-0.57)
ALAT (U/l, mean ± SD, (range))	22 ± 18 (8-147)
Serum albumin (g/l, mean ± SD, (range))	43 ± 3.2 (34-50)
Serum creatinine (µmol/l, mean ± SD, (range))	156 ± 76 (62-462)

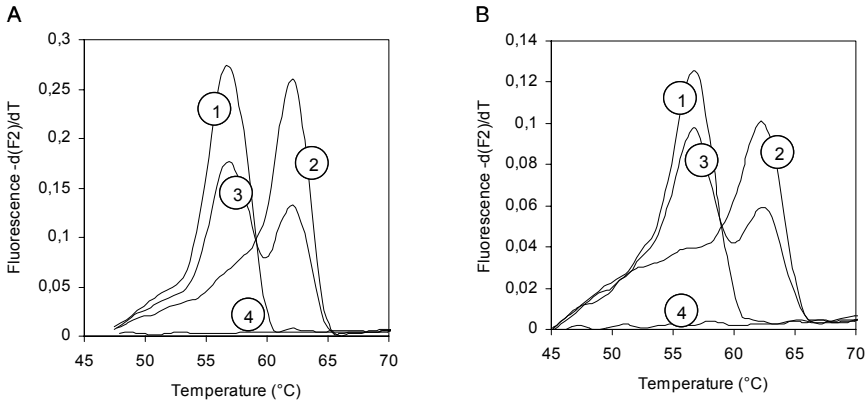

Figure 7.1 Genotyping of CYP3A5 A6986G and ABCB1 C1236T with allele specific fluorescent probes by derivative melting curve plots. The derivative melting curves are read in channel 2 for the detection of the CYP3A5 and the ABCB1 fragments.
A. The derivative melting curve is plotted for a sample homozygous for the CYP3A5 6986G or *3 allele (1; Tm 57°C); a sample homozygous for the CYP3A5 6986A or *1 allele (2; Tm 62°C) and a heterozygous sample (3; Tm 57°C and 62°C). B. The derivative melting curve is plotted for a sample homozygous for the ABCB1 1236C allele (1; Tm 56.5°C); a sample homozygous for the ABCB1 1236T allele (2; Tm 62°C) and a heterozygous sample (3; Tm 56.5°C and 62°C). Melting analysis of a no template control was also performed for both assays (4).

The allele distribution of the different CYP3A and ABCB1 polymorphisms

Table 7.2 shows both the different genotypes and the allele frequencies of the CYP3A and ABCB1 polymorphisms. In our population, we found no homozygous or heterozygous variant allele for the CYP3A4*1B polymorphism, which confirmed its very low frequency in the Chinese population. The allele frequencies found for the CYP3A5, CYP3AP1 and ABCB1 polymorphisms are in line with the literature[20,22,25]. The distribution of all polymorphisms examined is not significantly different from that predicted with the Hardy-Weinberg equilibrium (each $P > 0.05$). The genetic linkage of CYP3AP1 G-44A with CYP3A5 A6986G as already described by previous studies[42,43], was also confirmed in our study.

Table 7.2 Allelic distribution of CYP3A, CYP3AP1 and ABCB1 variants in 103 Chinese renal transplant recipients

SNP	wt/wt	wt/m	m/m	Allele frequency (%)		
CYP3A4				A	G	
A-392G	103 (100%)	0 (0%)	0 (0%)	100	0	
CYP3A5				A	G	
A6986G	10 (10%)	38 (37%)	55 (53%)	28	72	
CYP3AP1				G	A	
G-44A	9 (9%)	40 (39%)	54 (52%)	28	72	
ABCB1, exon 12				C	T	
C1236T	8 (8%)	52 (50%)	43 (42%)	33	67	
ABCB1, exon 21		GT: 32 (31%)	TT: 19 (19%)	G	T	A
G2677T/A	26 (25%)	GA: 16 (15%)	TA: 10 (10%)	48	39	13
ABCB1, exon 26				C	T	
C3435T	46 (45%)	43 (42%)	14 (13%)	66	34	

Effect of CYP3A and ABCB1 polymorphisms on tacrolimus dose requirements and pharmacokinetic profiles

Table 7.3 shows a significant decrease in the $dnAUC_{0\text{-}12}$ which strongly depends on whether the patients were carrier of none, one or two CYP3A5*1 alleles (2143, 1228 and 920 ng × hr/ml per mg/kg; Kruskal Wallis, $P < 0.001$), respectively. Consequently, the daily tacrolimus dose was 80% higher in homozygous carriers of a CYP3A5*1 allele compared to homozygous carriers of the CYP3A5*3 allele (0.09 mg/kg/day *versus* 0.05 mg/kg/day; Kruskal Wallis, $P < 0.001$). This allele-dependent effect of the CYP3A5*3 polymorphism is also illustrated in Figure 7.2. Additionally, a significant correlation is found between the allelic variants of the CYP3AP1 G-44A polymorphism and both the $dnAUC_{0\text{-}12}$ and the daily tacrolimus dose. Likely, this is caused by a very high linkage (> 98%) between CYP3A5*3 and CYP3AP1 -44A (only two patients differed in genotype) in our study.

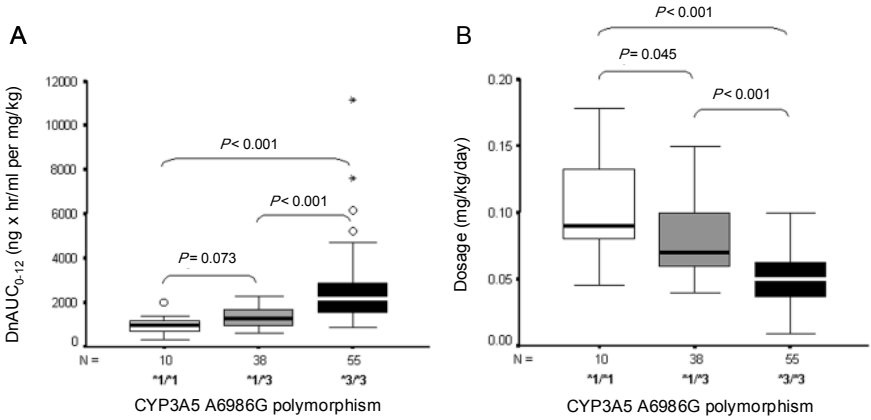

Figure 7.2 Influence of the CYP3A5 A6986G genotype on the pharmacological parameters recorded in 103 renal transplant recipients. A. The boxplot of the dose-normalized (dn)$AUC_{0\text{-}12}$ (ng × hr/ml per mg/kg body weight) clustered according to CYP3A5 A6986G genotype. B. The boxplot of the distribution of the daily tacrolimus dose (mg/kg body weight) clustered according to CYP3A5 A6986G genotype. *P* values for the pairwise comparisons of each genotype are given. Open circles indicate an outlier value at more than 1.5 boxlengths above the box, while asterics indicate an extreme value at more than 3 box lengths above the box.

Regarding the ABCB1 polymorphisms, we examined both the individual ABCB1 polymorphisms and the ABCB1 haplotypes present in the renal transplant patients. Table 7.3 shows the genetic effect of the three individual ABCB1 polymorphisms on both the dn$AUC_{0\text{-}12}$ and the daily tacrolimus dose. Individuals carrying the 2677TT or 3435TT genotype showed no significant difference in the dn$AUC_{0\text{-}12}$ compared to individuals carrying the 2677GG or 3435CC genotype. However, as is shown in Table 7.3, individuals carrying the 2677TT or 3435TT genotype required a significantly higher daily tacrolimus dose than individuals carrying the 2677GG or 3435CC genotype (0.066 mg/kg/day *versus* 0.053 mg/kg/day and 0.073 mg/kg/day *versus* 0.051 mg/kg/day; Mann-Whitney, $P = 0.033$ and $P = 0.044$), respectively. In contrast to ABCB1 G2677T/A and C3435T, the allelic variants of the ABCB1 C1236T polymorphism showed no significant difference on the dn$AUC_{0\text{-}12}$ and the daily tacrolimus dose. Additionally, we analysed the correlation between the ABCB1 haplotypes and the dn$AUC_{0\text{-}12}$ as well as the daily tacrolimus dose.

Table 7.3 Influence of CYP3A, CYP3AP1 and ABCB1 genotypes on the daily tacrolimus dose and the dose-normalized (Dn)AUC_{0-12}.

Genotype	Allelic status (n)	Dose (mg/kg/day)	$DnAUC_{0-12}$ (ng×hr/ml per mg/kg)
CYP3A5	*1/*1 (10)	0.090 (0.05-0.18)[a]	920 (300-1981)[a]
A6986G	*1/*3 (38)	0.070 (0.04-0.15)[a]	1228 (589-2266)[a]
	*3/*3 (55)	0.050 (0.01–0.10)[a]	2143 (827-11125)[a]
CYP3AP1	G/G (9)	0.090 (0.05-0.18)[a]	929 (300-1981)[a]
G-44A	A/G (40)	0.070 (0.04-0.15)[a]	1298 (589-2265)[a]
	A/A (54)	0.050 (0.01–0.10)[a]	2156 (827-11125)[a]
ABCB1	C/C (8)	0.060 (0.03-0.15)	1484 (875-5166)
C1236T	C/T (52)	0.060 (0.01-0.18)	1494 (300-11125)
	T/T (43)	0.063 (0.01-0.15)	1651 (589-7579)
ABCB1	G/G (26)	0.053 (0.03-0.09)[b]	1887 (891-6166)
G2677T/A	G/T (32)	0.070 (0.01-0.18)	1365 (300-7579)
	G/A (16)	0.050 (0.01-0.15)	1864 (676-11125)
	T/A (10)	0.056 (0.05-0.13)	1765 (805-2870)
	T/T (19)	0.066 (0.02-0.15)[b]	1473 (589-4532)
ABCB1	C/C (46)	0.052 (0.01-0.15)[b]	1882 (676-11125)
C3435T	C/T (43)	0.063 (0.03-0.18)	1466 (300-6166)
	T/T (14)	0.073 (0.02-0.15)[b]	1332 (589-4532)
CYP3A5 – ABCB1	*3/*3 – GG (17)	0.048 (0.03-0.08)	2097 (1056-6166)
A6986G – G2677T/A	*3/*3 – GA/GT/TA (26)	0.050 (0.01-0.10)	2369 (827-11125)
	*3/*3 – TT (12)	0.063 (0.02-0.09)	1694 (1237-4532)
	*1/*3 – GG (8)	0.068 (0.04-0.09)	1346 (913-2168)
	*1/*3 – GA/GT/TA (24)	0.073 (0.05-0.15)[c]	1271 (691-1973)[c]
	*1/*3 – TT (6)	0.104 (0.05-0.15)[c]	788 (589-2266)[c]
	*1/*1 – GG (1)	0.0787	891
	*1/*1 – GA/GT/TA (8)	0.110 (0.05-0.18)[c]	878 (300-1981)[c]
	*1/*1 – TT (1)	0.0800	1106[*]
CYP3A5 – ABCB1	*3/*3 – CC (28)	0.046 (0.01-0.08)	2120 (1056-11125)
A6986G – C3435T	*3/*3 – CT (21)	0.059 (0.03-0.10)	2179 (827-6166)
	*3/*3 – TT (6)	0.055 (0.02-0.09)	1845 (1322-4532)
	*1/*3 – CC (15)	0.069 (0.04-0.15)[c]	1106 (870-2168)[c]
	*1/*3 – CT (16)	0.073 (0.05-0.13)[c]	1271 (691-1882)[c]
	*1/*3 – TT (7)	0.098 (0.05-0.15)[c]	841 (589-2266)[c]
	*1/*1 – CC (3)	0.090 (0.08-0.13)[c]	891 (676-950)[c]
	*1/*1 – CT (6)	0.11 (0.05-0.18)[c]	977 (300-1981)[c]
	*1/*1 – TT (1)	0.800	1106

Values are indicated as median (range), [a] $P < 0.001$ (Kruskal Wallis); [b] $P < 0.05$ (Mann-Whitney), [c] $P < 0.05$ (Bonferroni), when the designated CYP3A5 - ABCB1 genotype combination is compared to the reference genotype combination, which is CYP3A5 - ABCB1 G2677T/A *3/*3 – GG and CYP3A5-ABCB1 C3435T *3/*3 – CC, respectively.

Table 7.4 illustrates that in contrast to the two individual ABCB1 polymorphisms, no significant difference or trend was observed between the ABCB1 haplotypes and the $dnAUC_{0-12}$ as well as the daily tacrolimus dose. Moreover, using stepwise multiple regression analysis to examine the relative contribution of the non-genetic and genetic factors to the daily tacrolimus dose variability, the CYP3A5*3 polymorphism appears to be the most significant independent variable when considering the daily tacrolimus dose

as a dependent variable. The CYP3A5*3 polymorphism can explain 35.3% ($P < 0.001$) of the variation in the daily tacrolimus dose observed in the renal transplant recipient population. Furthermore, gender (9.9%; $P < 0.001$) and the ABCB1 C3435T polymorphism (3.7%; $P < 0.009$) have a significant contribution in the variation of the daily tacrolimus dose. The two ABCB1 polymorphisms C1236T and G2677T/A, ABCB1 haplotypes and the non-genetic factors: age, serum creatinine concentration, time since transplantation and steroid dosing have no significant contribution on the variation of the daily tacrolimus dose.

Table 7.4 ABCB1 haplotypes in correlation to tacrolimus dose and dose-normalised (Dn)AUC_{0-12}

C1236T	G2677T/A	C3435T	n	Frequency (%)	Dose (mg/kg/day)	$DnAUC_{0-12}$ (ng × hr/ml per mg/kg)
CC	GG	CC	4	3.9	0.064 (0.03-0.08)	1484 (1301-5166)
CC	GA	CC	4	3.9	0.054 (0.03-0.15)	1862 (875-3948)
CT	GG	CC	11	10.7	0.070 (0.03–0.09)	1073 (891-4002)
CT	GG	CT	2	1.9	0.034	4519
CT	GT	CC	2	1.9	0.067	1650
CT	GT	CT	15	14.6	0.070 (0.03–0.18)	1227 (300-4702)
CT	GT	TT	2	1.9	0.056	2156
CT	GA	CC	11	10.7	0.050 (0.01-0.13)	1846 (676-11125)
CT	TA	CC	1	1.0	0.050	1918
CT	TA	CT	8	7.8	0.056 (0.05-0.13)	1765 (805-2870)
TT	GG	CC	9	8.7	0.050 (0.03-0.08)	1919 (1438-3717)
TT	GT	CC	2	1.9	0.021	4814
TT	GT	CT	11	10.7	0.080 (0.05-0.10)	1325 (837-2309)
TT	GA	CC	1	1.0	0.039	2933
TT	TA	CC	1	1.0	0.072	1106
TT	TT	CT	7	6.7	0.063 (0.05-0.08)	1685 (1237-2626)
TT	TT	TT	12	11.7	0.075 (0.02-0.15)	1277 (589-4532)

Figure 7.3 suggests that a combined analysis of the CYP3A5*3 and ABCB1 G2677T/A or ABCB1 C3435T polymorphism may be helpful to identify those individuals that require a strong aberrant daily tacrolimus dose, although the contribution of the individual ABCB1 polymorphisms on the daily tacrolimus dose variation is not significant or minor compared to the CYP3A5*3 polymorphism.

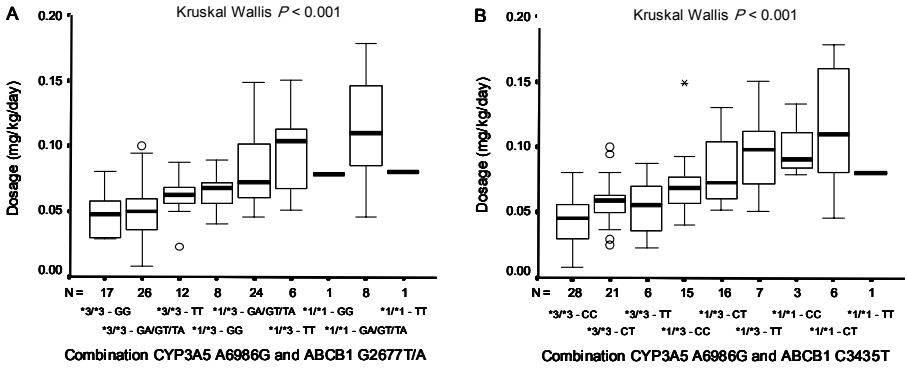

Figure 7.3 The boxplot of the distribution of the daily tacrolimus dose (mg/kg) clustered according to the combination of CYP3A5 A6986G and ABCB1 G2677T/A genotypes. B. The boxplot of the distribution of the daily tacrolimus dose (mg/kg body weight) clustered according to the combination of CYP3A5 A6986G and ABCB1 C3435T genotypes. A *P* value is given for comparison of several groups of both CYP3A5 and ABCB1 genotype combinations. Open circles indicate an outlier value at more than 1.5 boxlengths below/above the box, while asterics indicate an extreme value at more than 3 box lengths above the box.

Discussion

Although most centres still use C_0 levels for monitoring tacrolimus there are conflicting data about the correlation with the systemic tacrolimus exposure. Several studies reported a reasonable squared correlation between C_0 levels and $AUC_{0\text{-}12}$ of tacrolimus[44-46], while other studies found very poor correlations[13-16,47,48]. Recently, Scholten *et al.*[17] reported a reduction of the 95% prediction interval by 50%, when a two point sampling strategy was used consisting of a C_0 level and a second sample obtained between two and four hour post dose. Moreover, Scholten *et al.*[17] evaluated the predictive performance of the two time point sample strategy used in the present study and confirmed its suitability. It is known that several non-genetic factors can modulate the postoperative CYP3A phenotype, especially in the early period after transplantation[49]. Therefore, it is important to demonstrate with close drug monitoring of tacrolimus that steady state blood concentrations have been achieved in order to examine the genotype-phenotype associations more accurately. Therapeutic drug monitoring of tacrolimus can be performed accurately using complete $AUC_{0\text{-}12}$ or an $AUC_{0\text{-}12}$ calculated by a limited sampling strategy. In the present study a limited sampling strategy is used to calculate the $AUC_{0\text{-}12}$ in 103 Chinese renal transplant recipients. To the best of our knowledge, two recent studies[34,35] previously examined, in Japanese renal transplant recipients, the association between the CYP3A5*3 and the ABCB1 C3435T polymorphism and the pharmacokinetic parameters; $(dn)C_0$,

(dn)$AUC_{0\text{-}12}$, (dn)C_{max}, T_{max} and $t_{1/2}$. Although several studies found a significant effect of CYP3A5*3 polymorphism[22-24,26-31,35] on tacrolimus levels or dosages, the number homozygous carriers of the CYP3A5*1 polymorphism was limited in these studies. Recently, Zhang *et al.*[25] correlated the different genotypes of the CYP3A5*3 and ABCB1 C3435T polymorphism of 118 Chinese renal transplant recipients with their corresponding C_0 levels and daily tacrolimus dosages respectively, 1 week, 1 month and 3 months after transplantation. Our findings showed in line with the results of Zhang *et al.*[25] a strong significant genetic effect between the CYP3A5*3 polymorphism and both the dn$AUC_{0\text{-}12}$ and the daily tacrolimus dose. After a mean time of 2.5 years post transplantation, carriers of the CYP3A5*3 polymorphism still require a remarkable lower daily tacrolimus dose compared to carriers of the CYP3A5*1 polymorphism. An estimation of the contribution of the different CYP3A and ABCB1 polymorphisms in the variation of the daily tacrolimus dose is performed by Haufroid *et al.*[27]. They found that the CYP3A5*3 polymorphism is with a relative contribution of 38% the most significant independent variable when considering the daily tacrolimus dose as dependent variable, which is in line with the findings observed in the present study. The significant contribution of the gender in the variation of the daily tacrolimus dose is also found by Kuypers *et al.*[14] although they examined the influence of several non-genetic variables within the first year after transplantation. However, no significant contribution is observed for the time since transplantation in the variation of the daily tacrolimus dose in contrast to the findings observed by Haufroid *et al.*[27]. Despite the ABCB1 G2677T/A and C3435T polymorphisms showed a significant genetic effect after correlating these ABCB1 polymorphisms with the daily tacrolimus dose, no significant contribution is found using multiple regression analysis for the two individual ABCB1 polymorphisms or ABCB1 haplotype. Our findings seem therefore in contrast to several other studies which reported that carriers of the 3435CC allele[23,24,36] and 1236C-2677G-3435C or 2677GG-3435CC haplotype required a higher daily tacrolimus dose[37]. However, the significant genetic effect for ABCB1 polymorphisms or haplotypes found in these studies is like in our study not as pronounced as it is for the CYP3A5*3 polymorphism. The minor influence of the ABCB1 polymorphisms compared to the CYP3A5*3 polymorphism on the tacrolimus pharmacokinetics combined with the differences regarding pharmacokinetic parameter used (C_0 or $AUC_{0\text{-}12}$), the genetic (single SNP *versus* haplotype) analyses that were performed, the number of transplant patients included, and the difference in inclusion criteria for the transplant recipients may explain the differences in genotype-phenotype associations found by these studies. Also after combining the CYP3A5*3 polymorphism with the ABCB1 G2677T/A or C3435T polymorphism, the extra part of the variation in dn$AUC_{0\text{-}12}$ and daily tacrolimus dose that can be clarified above the CYP3A5*3 polymorphism is marginal.

Highlights

- The calcineurin inhibitor tacrolimus, an immunosuppressant used in transplantation therapy, has unpredictable drug levels and therefore requires close therapeutic drug monitoring in order to minimize the risk of under- or overimmunosuppression.
- Tacrolimus is metabolised by the cytochrome P450 3A (CYP3A) iso-enzymes, which is quantitatively the most important metabolic route of tacrolimus. Additionally, tacrolimus is also a substrate for the adenosine triphosphate-binding cassette B1 (ABCB1) gene encoding protein multidrug resistance-1 (MDR1) or P-glycoprotein.
- The present study used a two time point sampling strategy to calculate the area under the time tacrolimus concentration curve (AUC_{0-12}) and correlated these dose-normalised AUC_{0-12} with the corresponding CYP3A and ABCB1 genotypes in 103 Chinese renal transplant recipients.
- Several single nucleotide polymorphisms (SNPs) recently identified in CYP3A and ABCB1 gene may elucidate the inter-individual differences in the tacrolimus pharmacokinetics.
- The CYP3A5*3 allele, which results in the absence of the functional CYP3A5 protein, has been strongly associated with a higher $dnAUC_{0-12}$ and consequently a lower daily tacrolimus dose. The different genotypes of the ABCB1 G2677T/A and C3435T SNPs showed a weaker association with the $dnAUC_{0-12}$ as well as the daily tacrolimus dose.
- Multiple regression analysis demonstrates that the CYP3A5*3 allele is the most important independent variable among several genetic and non-genetic variables examined in the renal transplant recipients.
- Knowing the impact of the CYP3A5*3 allele on the tacrolimus disposition, genotyping prior transplantation may help to assess the initial daily dose needed by individual patients for adequate immunosuppression.

Outlook and conclusion

Tacrolimus has a narrow therapeutic index which requires close monitoring of the drug concentration in order to achieve an optimal efficiency and minimizing the risks of acute rejection and drug overdose. The polymorphisms present in CYP3A and ABCB1 can explain their variability in expression and activity and thus clarify the inter-individual differences in the tacrolimus pharmacokinetics. The majority of studies that examined the CYP3A and ABCB1 genotype-phenotype association were based on tacrolimus C_0 levels, despite the fact that an AUC_{0-12} is the best way to determine the tacrolimus exposure. To the best of our knowledge only two studies reported an association between the CYP3A5*3 polymorphism and both the different pharmacokinetic parameters of tacrolimus and the daily tacrolimus dose. Furthermore, several studies

reported an association between CYP3A5*3 polymorphism and the tacrolimus C_0 levels obtained within the early period after transplantation. However, non-genetic parameters have then also a large impact on the tacrolimus concentration in this period. The present study calculated an AUC_{0-12} according to a two time point sampling strategy for 103 Chinese renal transplant patients with a wide time interval starting on at least three months posttransplantation. Our results clearly point out that the CYP3A5*3 polymorphism is strongly allele-dependent associated with both the $dnAUC_{0-12}$ and the daily tacrolimus dose in renal transplant recipients. A weaker significant association is found for the ABCB1 G2677T/A and ABCB1 C3435T polymorphisms. Multiple regression analysis demonstrates that the CYP3A5*3 polymorphism is the most important independent variable among several genetic and non-genetic variables examined in the renal transplant recipients. Therefore, CYP3A5 genotyping prior transplantation may contribute to determine the initial tacrolimus dose required for individual patients.

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