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Original Article

Associations of *ABCB1*, *NFKB1*, *CYP3A*, and *NR1I2* polymorphisms with cyclosporine trough concentrations in Chinese renal transplant recipients

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Aim: Cyclosporine requires close therapeutic drug monitoring because of its narrow therapeutic index and marked inter-individual pharmacokinetic variation. In this study, we investigated the associations of *CYP3A4*, *CYP3A5*, *ABCB1*, *NFKB1*, and *NR1I2* polymorphisms with cyclosporine concentrations in Chinese renal transplant recipients in the early period after renal transplantation.
Methods: A total of 101 renal transplant recipients receiving cyclosporine were genotyped for *CYP3A4*1G*, *CYP3A5*3*, *ABCB1* C1236T, G2677T/A, C3435T, *NFKB1* -94 ins/del ATTG, and *NR1I2* polymorphisms. Cyclosporine whole blood levels were measured by a fluorescence polarization immunoassay. Trough concentrations of cyclosporine were determined for days 7-18 following transplantation.
Results: The dose-adjusted trough concentration (C_0) of cyclosporine in *ABCB1* 2677 TT carriers was significantly higher than that in GG carriers together with GT carriers [90.4±24.5 vs 67.8±26.8 (ng/mL)/(mg/kg), P=0.001]. *ABCB1* 3435 TT carriers had a significantly higher dose-adjusted C_0 of cyclosporine than CC carriers together with CT carriers [92.0±24.0 vs 68.4±26.5 (ng/mL)/(mg/kg), P=0.002]. Carriers of the *ABCB1* 1236TT-2677TT-3435TT haplotype had a considerably higher CsA C_0/D than carriers of other genotypes [97.2±21.8 vs 68.7±26.9 (ng/mL)/(mg/kg), P=0.001]. Among non-carriers of the *ABCB1* 2677 TT and 3435 TT genotypes, patients with the *NFKB1* -94 ATTG ins/ins genotype had a significantly higher dose-adjusted C_0 than those with the -94 ATTG del/del genotype [75.9±32.9 vs 55.1±15.1 (ng/mL)/(mg/kg), P=0.026].
Conclusion: These results illustrate that the *ABCB1* and *NFKB1* genotypes are closely correlated with cyclosporine trough concentrations, suggesting that these SNPs are useful for determining the appropriate dose of cyclosporine.

Introduction

As a member of the calcineurin inhibitor (CNI) family, cyclosporine (CsA) is a first-line immunosuppressant widely used to prevent allograft rejection after solid organ transplantation. However, the bioavailability of cyclosporine ranges from less than 5% to 89% in transplant patients[1]. Owing to its narrow therapeutic index and marked inter-individual pharmacokinetic variation, therapeutic drug monitoring (TDM) is necessary to adjust the dosage and reduce toxicity[2]. Never-

theless, TDM is hysteretic for optimizing efficacy and limiting toxicity of cyclosporine. Therefore, identifying factors that affect the pharmacokinetics of cyclosporine has great potential for improving the safety and efficacy profile.

Cyclosporine is a substrate of cytochrome 3A4 and 3A5 (CYP3A4 and CYP3A5) and P-glycoprotein (P-gp/MDR1, encoded by *ABCB1*). Most previous studies have focused on the influence of genetic variants in the genes encoding CYP3A4, CYP3A5, and P-gp on CsA pharmacokinetics but have yielded conflicting results[3]. In addition to the differences in study populations, particularly in sample size and ethnicities, there might be other, as-yet undiscovered, genetic factors that influence the expression or function of CYP3A4, CYP3A5 or P-gp to be discovered.

The pregnane X receptor (PXR, encoded by *NR1I2*) is

reported to be the key nuclear receptor regulating the expression of *CYP3A4*, *CYP3A5*, and *ABCB1*[4]. Factors affecting the expression or function of PXR, such as single nucleotide polymorphisms (SNPs), may influence the expression of downstream target genes. T25385C, G24113A, C6994T, C4356T, and G7635A have been reported to be associated with CYP3A4 phenotype, activity and content, whereas A11156C has been associated with variable P-gp levels[5-7]. A24622T and C24446A in exon1 have been found to be associated with PXR levels in previous studies from our laboratory[8], suggesting that these two SNPs may also be correlated with the expression of *CYP3A4*, *CYP3A5*, or *ABCB1*.

NF-κB, a protein complex found in almost all animal cell types, is a transcription factor critical for inflammatory responses. It has long been observed that inflammatory responses and infections decrease drug metabolism capacity in humans[9, 10]. The possible effects of NF-κB on enzymes and transporters related to drug metabolism have been studied because NF-κB is a key regulator of inflammation. Gu *et al* revealed that NF-κB competitively binds to the retinoid X receptor (RXR), thus preventing the PXR-RXR complex from binding to consensus DNA sequences in the regulatory regions of downstream genes, including *CYP3A4* and *ABCB1*[11]. The *NFKB1* gene encodes the p50 subunit of NF-κB, which complexes with p65 to produce the major form of NF-κB, and possesses a functional common insertion/deletion (-94 ins/del ATTG) mutation in its promoter region. Deletion of the ATTG alleles may lead to the loss of binding to nuclear proteins and reduced promoter activity and hence decreased production of the NF-κB p50 isoform[12].

The aim of this study was to comprehensively evaluate the influence of SNPs in *CYP3A4*, *CYP3A5*, *ABCB1*, *NR1I2*, and *NFKB1* on cyclosporine concentration in a group of Chinese renal transplant recipients during the early stage after transplantation.

Materials and methods
Ethics statement
The study was performed in accordance with the Declaration of Helsinki, and ethical approval for this study was obtained from the ethics committee of the First Affiliated Hospital of Sun Yat-Sen University. Written informed consent was obtained from all subjects.

Patients and therapy
A total of 101 renal transplant recipients (70 men and 31 women) who received a renal transplant between 2005 and 2011 at the Department of Organ Transplant, the First Affiliated Hospital, Sun Yat-sen University were enrolled in this study. The average age was 44.5±13.0 years (range 18–75 years), and the average body weight was 60.4±10.6 kg.

All patients were maintained on a triple immunosuppressive regimen consisting of cyclosporine (Neoral, Novartis Pharma, Basel, Switzerland) as a calcineurin inhibitor, mycophenolate mofetil (Cellcept; Roche, Basel, Switzerland) as a purine inhibitor and steroids (prednisolone, Guangdong Huanan

Pharmacy Ltd, Dongguan, China). CsA was administered at a starting dose of 4–6 mg/kg per day following the surgery and administered twice a day in equal amounts. The daily dosage was then adjusted according to trough concentration (C_0), to achieve a target concentration of 200 ng/mL. The steroid regimen was 1 g/d intravenously (IV) administered methylprednisolone at the time of surgery followed by 30 mg/d of oral prednisolone, which was progressively tapered to 15–20 mg/d by the end of the first month. Mycophenolate mofetil was given at a dose of 1 g twice daily (bid) during the first month following surgery. Furthermore, other medications known to affect CsA pharmacokinetics, except for prednisolone, were not administered; these included CYP3A or P-gp inducers and inhibitors, such as calcium channel blockers (diltiazem, nicardipine and verapamil), antiepileptics (phenytoin and carbamazepine), antimycotics (fluconazole and ketoconazole), macrolide antibiotics (erythromycin and clarithromycin) and St John's wort. Patients with abnormal hepatic function and combined organ transplants were also excluded.

Data collection
Body weight, CsA dosages and whole blood CsA concentrations were recorded on days 7–18 after transplantation, when CsA concentrations were stable or dosages had been unchanged for a period of time. Two-milliliter blood samples for the CsA assay were collected before 8:00 AM, immediately before the morning dose. The samples were assayed using the commercially available CsA whole blood monoclonal antibody fluorescence polarization assay[13]. The weight-adjusted CsA dosage (mg/kg per d) and the dose-adjusted trough concentration [C_0/D, (ng/mL)/(mg/kg) per d] were calculated.

DNA extraction and genotyping
Total genomic DNA was extracted from peripheral leukocytes according to a previously described method[14]. *CYP3A5*3* (A6986G), *CYP3A4*1G* (G20230A), *ABCB1* C1236T, G2677T/A, and C3435T polymorphisms were detected by using previously reported polymerase chain reaction restriction-fragment length polymorphism (PCR-RFLP) methods[15-17]. The *NFKB1* -94ins/del ATTG polymorphism was detected by TaqMan® SNP Genotyping Assay. Polymorphisms of *NR1I2*, including T25385C, A24622T, C24446A, G24113A, C4356T, A601G, G7635A, A11156C, and C6994T, were genotyped by direct sequencing[5-8].

Statistical analysis
The pair-wise linkage disequilibrium (LD) for SNPs was estimated by SHEsis (http://analysis2.bio-x.cn/myAnalysis.php). *ABCB1* 1236-2677-3435 haplotype analysis was performed by PHASE 2.1. Groups were compared using nonparametric tests. For the analysis of continuous pharmacologic variables, we used patient genotypes as categorical independent variables. The Hardy-Weinberg equilibrium test was performed using an appropriate χ^2 test. The Mann-Whitney *U* test was used for comparisons between two groups, and the Kruskal-Wallis *H* test was used for comparisons among several groups.

Statistical analysis was performed using SPSS version 17.0 for Windows (SPSS Inc, Chicago, IL, USA). The results are expressed as the mean±standard deviation (SD). A P-value less than 0.05 was considered statistically significant.

Results

Genotypes frequencies

A total of 101 renal transplant recipients were genotyped for polymorphisms in *CYP3A5*, *CYP3A4*, *ABCB1*, *NFKB1*, and *NR1I2*. All mutant allele and genotype frequencies were in agreement with previous reports in Han Chinese populations. No differences were observed between allograft recipients and the healthy population, and no deviations from the Hardy-Weinberg equilibrium were observed.

*CYP3A4*1G* was in moderate linkage disequilibrium (LD) with CYP3A5*3 (D'=0.63). A moderately high degree of LD between *ABCB1* C1236T, G2677T/A, and C3435T was also observed. For C1236T-G2677T/A, G2677T/A-C3435T, and C3435T-C1236T, D' was 0.56, 0.79 and 0.72, respectively, which agreed with previous reports. *NR1I2* T25385C and G24113A were in complete linkage disequilibrium (D'=1), whereas *NR1I2* C4356T was strongly linked to A-601G SNP (D'=0.785). No significant linkage was found between other individual combinations of SNPs.

According to the haplotype analysis, the frequencies of the three major haplotypes (1236-2677-3435) of *ABCB1* were 32.3% for TTT, 25.7% for TGC and 20.4% for CGC. The remaining haplotypes constituted 21.6% of the patients' haplotypes.

Association between *ABCB1* genotype and CsA dose-adjusted trough blood concentrations

A significantly higher CsA C_0/D was observed in *ABCB1* 2677 TT carriers than in the other 2677 genotype carriers [90.4±24.5 *vs* 67.8±26.8 (ng/mL)/(mg/kg), P=0.001] (Figure 1A).

Similarly, carriers of the *ABCB1* 3435 TT genotype also had a significantly higher CsA C_0/D than carriers of the 3435 CT together with CC genotypes [92.0±24.0 *vs* 68.4±26.5 (ng/mL)/(mg/kg), P=0.002] (Figure 1B).

Although patients with the *ABCB1* 1236TT genotype tended to have higher CsA C_0/D than those with 1236CC or 1236CT genotypes, no significant association was observed between the CsA C_0/D and *ABCB1* C1236T genotype (P=0.097).

When combining the effects of *ABCB1* C1236T, G2677T/A and C3435T, carriers of the 1236TT-2677TT-3435TT haplotype had a considerably higher CsA C_0/D than carriers of other genotypes [97.2±21.8 *vs* 68.7±26.9 (ng/mL)/(mg/kg), P=0.001] (Figure 1C).

Association between *NFKB1* genotypes and CsA dose-adjusted trough blood concentrations

Patients with the *NFKB1* -94 ATTG ins/ins genotype typically had a higher CsA C_0/D than those with the *NFKB1* -94 ATTG del/del genotype [78.5±32.8 *vs* 61.1±19.4 (ng/mL)/(mg/kg)], but the difference was not statistically significant (P=0.069) (Figure 2A).
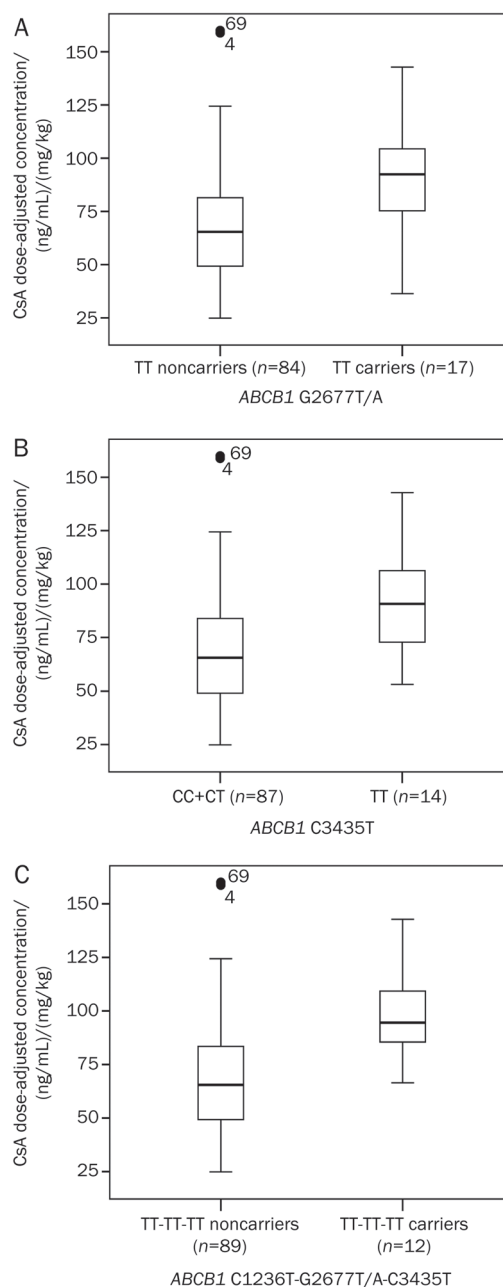

Figure 1. Correlation of the *ABCB1* (A) 2677TT genotype, (B) 3435TT genotype and (C) 1236TT-2677TT-3435TT haplotype with CsA C_0/D on d 7–18 after transplantation in renal transplant recipients.

Association between *CYP3A5*, *CYP3A4*, and *NR1I2* genotype and CsA dose-adjusted trough blood concentrations

Carriers of the *CYP3A5*1/*1* and **1/*3* genotypes were combined as CYP3A5 expressers, and carriers of the *3/*3* genotype were defined as non-expressers. No significant difference in CsA C_0/D was observed between CYP3A5 expressers and non-expressers. *CYP3A4*1G* SNP was also found not to influence CsA C_0/D. When the effects of *CYP3A5*3* and *CYP3A4*1G* were combined, we were still unable to observe any effect of the *CYP3A4-CYP3A5* haplotype on CsA C_0/D.
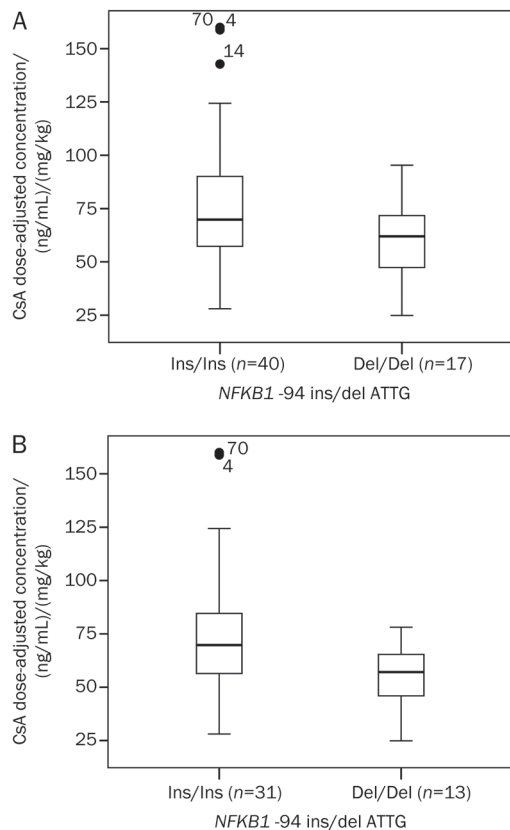
Figure 2. Correlation of *NFKB1* genotypes with the dose-adjusted trough concentration of cyclosporine. (A) Influence of the *NFKB1* -94 ins/del ATTG genotype on CsA C_0/D on d 7–18 after transplantation in renal transplant recipients. (B) Influence of the *NFKB1* -94 ins/del ATTG genotype on CsA C_0/D on d 7–18 after transplantation in non-carriers of the *ABCB1* 2677 TT and 3435 TT genotypes.

Although numerous SNPs within the *NR1I2* gene were included in this study, no association was observed between CsA C_0/D and any of the *NR1I2* SNPs (data not shown).

Stratification analysis

Stratification analysis was performed to eliminate any confounding effects of the genes that were included in this study. This study was designed to explore an independent effect of *CYP3A4*, *CYP3A5*, *NFKB1*, and *NR1I2* genotype on CsA C_0/D after 'standardization' for P-gp activity.

Among non-carriers of the *ABCB1* 2677 TT genotype and 3435 TT genotype, carriers of the *NFKB1* -94 ATTG ins/ins genotype had a significantly higher CsA C_0/D than carriers of the -94 ATTG del/del genotype [75.9±32.9 *vs* 55.1±15.1 (ng/mL)/(mg/kg), P=0.026] (Figure 2B).

Discussion

The current study is a comprehensive study on polymorphisms of drug metabolic enzymes (CYP3A4 and CYP3A5), a transporter (P-gp) and upstream regulators of them (PXR and NF-κB), to explore the cause of the large inter-individual varia-

tion in cyclosporine concentration observed in renal transplant recipients. Moreover, the current study is also directly evaluate the potential effect of NF-κB-related polymorphisms on drug metabolism.

ABCB1 C1236T, G2677T/A, and C3435T were the most commonly and extensively studied SNPs of *ABCB1*, which can form different haplotypes. Carriers of the TT genotype of *ABCB1* C3435T or G2677T/A have been reported to have significantly minimized P-glycoprotein activity compared to wild type activity[18, 19], suggesting a higher CsA C_0/D in carriers of the *ABCB1* 3435 TT and 2677 TT genotypes.

Separate studies on 44 Caucasian liver transplant recipients[20] and 88 Middle Eastern renal transplant recipients[21] up to 1 month after transplantation both found carriers of the *ABCB1* 3435 TT genotype had a higher CsA C_0/D than carriers of 3435 CC or CT genotype. By contrast, in 103 Chinese renal transplant recipients, Qiu *et al* reported a lower C_0/D in carriers of the *ABCB1* 2677GG wild-type genotype than in carriers of the *ABCB1* 2677TT variant genotype in the first 8 to 30 d after transplantation[22, 23]. Consistent with these studies, we found that the *ABCB1* 2677TT and 3435TT genotypes and 1236TT-2677TT-3435TT haplotype were closely correlated with a higher cyclosporine dose-adjusted C_0. Moreover, a more obvious influence was found in the 1236TT-2677TT-3435TT haplotype than in each SNP independently; the 1236TT-2677-TT-3435TT haplotype contributes more to the variation in CsA concentrations, as determined by multiple linear regression analysis. These findings, which are in agreement with previous observations, suggest that the use of the *ABCB1* haplotype is superior to SNP analysis for predicting concentrations of cyclosporine[24].

As a nuclear factor that takes part in the control of as many as 150 target genes, including many inflammatory genes, NF-κB is a focal point and its related pathways and relationships with many autoimmune diseases and cancers have been widely studied. The majority of studies on NF-κB-related polymorphisms, such as the *NFKB1* -94 ins/del ATTG mutation, have been focused on their association with the incidence of tumors and inflammatory diseases[25]. Although it has long been observed that inflammatory responses and infections decrease drug metabolism capacity in human, a correlation between NF-κB-related polymorphisms and drug metabolism has never been reported. In a previous *in vitro* study, Gu *et al* reported that the structural component of NF-κB, p65, can competitively bind to RXR, which is necessary to form PXR-RXR heterodimers. The competitive binding of p65 to RXR, therefore, disrupts the interaction of PXR-RXR heterodimers with the consensus DNA sequences in the regulatory regions of target genes, thus significantly suppressing gene expression[11]. It is possible that NF-κB may have an effect on CsA concentrations by suppressing PXR-mediated regulation of *CYP3A4/ABCB1*. Hence, we speculated that the *NFKB1* polymorphism might result in altered NF-κB expression, leading to reduced *CYP3A4/ABCB1* expression and increased CsA concentrations.

As reported previously, the del ATTG allele may result in

decreased *NFKB1* transcript levels and therefore decreased p50 protein production[12]. Thus, the ins/ins ATTG genotype is speculated to be associated with a higher NF-κB protein content, and consequently lower CYP3A4 expression and ultimately higher CsA concentrations than the levels in carriers of the del/del ATTG genotype. This finding is consistent with our results that carriers of the -94 ATTG ins/ins genotype had a significantly higher CsA C_0/D than carriers of the -94 ATTG del/del genotype in non-carriers of the *ABCB1* 2677 TT and 3435 TT genotypes. These observations suggest that immunoregulation has a notable effect on CsA concentrations. Further study is needed to assess our speculations and reveal the underlying mechanisms.

Although the importance of *CYP3A5**3 in tacrolimus concentrations has been fairly obvious, its influence on CsA concentrations is still in dispute. Several researchers[26-28] have observed a significant association between *CYP3A5**3 and CsA trough concentrations. However, no association between *CYP3A5**3 and CsA concentrations was found in this study, which is in consistent with some previous reports[29-31]. *CYP3A4**1G is a *CYP3A4* SNP with the highest occurrence in Chinese populations, but a definitive function has not been reported. A series of studies examined the relationship between the *CYP3A4**1G polymorphism and drug metabolism but resulted in inconsistent findings[32-34]. In agreement with our observation, *CYP3A4**1G was not associated with cyclosporine concentrations in 126 renal recipients[27]; however, Qiu *et al*[22] and Hu *et al*[35] found that it was associated with a lower CsA concentration. Differences in study populations, sample size, ethnicities and particularly in the duration after transplantation might be possible confounding factors. During the early stage after transplantation, steroids were used at a high dose, which might have induced CYP3A.

Although they were reported to be functional in previous studies, none of the *NR1I2* SNPs were found to be correlated with CsA concentrations in our study[5, 6, 8]. However, most of these studies were *in vitro*, without the consideration of many possible influences caused by other factors *in vivo*. Another caveat to be taken into account is the induction of PXR by steroids[36] because the recipients in this study, who were at an early stage after transplantation, routinely received a high dose of steroids. Therefore, the impact of *NR1I2* SNPs on cyclosporine concentrations at a stable stage after transplantation in patients who received a lower dose of steroids needs to be studied.

In summary, this study reports the potential effect of NF-κB-related polymorphisms on clinical drug metabolism. We also found that the *ABCB1* 1236TT-2677TT-3435TT haplotype was most significantly correlated with CsA concentrations in Chinese renal transplant recipients. Patients who carry the *ABCB1* 1236TT-2677TT-3435TT haplotype may be at a greater risk of high CsA concentrations that could lead to hepatotoxicity and nephrotoxicity. *NFKB1* polymorphisms were identified as a minor significant factor associated with CsA concentrations in this study, and patients with the *NFKB1* ins/ins ATTG genotype tended to have higher CsA concentrations. We also propose that pre-transplant genotyping of the *ABCB1* C1236T, G2677T/A, C3435T, and *NFKB1* ins/ins ATTG genotypes may be used as an index for CsA dosing in clinical practice and that the initial CsA dose for carriers of the *ABCB1* 1236TT-2677TT-3435TT haplotype and *NFKB1* -94 ins/ins ATTG genotype be lower to prevent possible toxicities during the early stage of transplantation.

Acknowledgements

Author contribution

Yu ZHANG, Jia-li LI, Min HUANG, and Chang-xi WANG designed the research. Yu ZHANG, Jia-li LI, Xue-ding WANG, Qian FU, and Long-shan LIU performed the research. Yu ZHANG, Jia-li LI, and Wen-ying SHU contributed new reagents and analytic tools. Yu ZHANG and Zhuo-jia CHEN analyzed the data. Yu ZHANG, Jia-li LI, and Wen XIE wrote the paper.

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