The POR rs1057868–rs2868177 GC-GT diplototype is associated with high tacrolimus concentrations in early post-renal transplant recipients

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Introduction

The calcineurin inhibitor tacrolimus (FK506; Prograf) is the first-line immunosuppressant that is widely used in solid-organ transplant recipients for prophylaxis against allograft rejection[1]. However, the narrow therapeutic window (trough concentration should be controlled in the range of 5 to 8 ng/mL in the first 3 months post-transplant in our routine[2]) and large inter-individual pharmacokinetic variability are the major therapeutic challenges. Over-immunosuppression causes toxicity and under-immunosuppression leads to rejection, characterizing the highest risks during the initial period, especially within the 7 d post-transplantation[3]. Therefore, identifying key predictors for earlier tacrolimus concentration and achieving individualized dosing have great potential for improving the procedure’s safety and efficacy.

Tacrolimus is predominantly metabolized by CYP3A5, and its intrinsic clearance is 1.6- to two-fold higher for CYP3A5 than for CYP3A4[4]. It is now generally accepted that tacrolimus pharmacokinetics is closely correlated with a

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Methods

A total of 154 renal transplant recipients were enrolled. Genotyping of CYP3A5*3 and 6 POR SNPs was performed. All patients received a triple immunosuppressive regimen comprising tacrolimus, mycophenolate mofetil and prednisone. Dose-adjusted tacrolimus trough concentrations were obtained on d 7 (C0D7) after transplantation when steady-state concentration of tacrolimus was achieved (dosage had been unchanged for more than 3 d). Results: Tacrolimus C0D7 in CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplototype carriers was 1.62- and 2.72-fold higher than those in CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplototype non-carriers and CYP3A5*1 carriers (220.17±48.09 vs 135.69±6.86 and 80.84±5.27 ng/mL/mg/kg, respectively, P<0.0001). Of CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplototype carriers, 85.71% exceeded the upper limit of the target range (8 ng/mL), which was also significantly higher compared with the latter two groups (14.29% and 0.00%, respectively, P<0.0001). The CYP3A5*3 and POR rs1057868–rs2868177 GC-GT diplototype explained 31.7% and 5.7%, respectively, of the inter-individual variability of tacrolimus C0D7/D, whereas the POR rs1057868–rs2868177 GC-GT diplototype could explain 10.9% of the inter-individual variability of tacrolimus C0D7/D in CYP3A5 non-expressers.

Conclusion: The CYP3A5*3 and POR rs1057868–rs2868177 GC-GT diplototype accounted for the inter-individual variation of tacrolimus C0D7/D. Genotyping of POR rs1057868–rs2868177 diplotypes would help to differentiate initial tacrolimus dose requirements and to achieve early target C0 ranges in Chinese renal transplant recipients.
nonfunctional splicing-defect in CYP3A5*3 allele (rs776746; 6985A>G) [8–10]. CYP3A5*3/*3 carriers (CYP3A5 function deficient, denominated as CYP3A5 nonexpressers) require a lower dose of tacrolimus to reach target concentrations compared with CYP3A5*1 allele carriers (CYP3A5 expressers). However, CYP3A5*3 genotype can explain only one-third of the inter-individual variability in tacrolimus pharmacokinetics [6].

Moreover, there are still obvious differences among both CYP3A5 nonexpressers and expressers. Hence, exploring the unaccounted genetic variation in tacrolimus pharmacokinetics on top of what is already known on CYP3A5 variants is needed.

Cytochrome P450 oxidoreductase (POR) is known as the unique electron donor to all microsomal cytochrome P450 (CYP) enzymes in human [8]. Because POR’s activity is necessary for CYP functions, it is reasonable to infer that genetic variations in POR gene could affect the functions of broad ranges of CYPs and thus alter therapeutic efficiency and toxicity of many drugs. The contribution of POR*28 (rs1057868C>T; A503V) to inter-individual variability of tacrolimus metabolism has been studied, but the results are conflicting. In addition to POR*28, several other POR SNPs have also been reported to be associated with altered cytochrome P450 activity [9, 12]. Because the distribution profile of POR SNPs in the Chinese population has rarely been reported, we performed a pilot study to assess the variant frequencies of 11 functional candidate SNPs in 96 healthy Chinese volunteers. The results are shown in Supplementary Table S1. Five SNPs—rs2302429, rs17685, rs2868177, rs2286823, and rs41301394—were prevalent in our healthy volunteers. Intronic SNPs-rs2302429, rs2286823 (IVS11±20G>A) and rs41301394 (831-35C>T) have been significantly associated with the altered CYP1A2, CYP2C19, or CYP3A4 activities [13, 14]. Rs2868177 has been found to be associated with the cardiotoxicity induced by daunorubicin, which is also a substrate of CYP3A [15]. Moreover, rs2868177 and other two SNPs (rs17148944 and rs17685) have been brought into warfarin pharmacogenetic research and reported to be correlated with warfarin maintenance dose [16]. Interestingly, a number of POR genetic variants have been shown to have substrate-dependent effects on CYP enzyme activities, probably owing to conformational changes induced by the substrate, depending on its size, electrical charge and chemical structure [17]. It suggests that although not all the SNPs mentioned above were related to CYP3A activity in the previous studies, they might influence CYP3A-mediated tacrolimus metabolism; a comprehensive investigation is needed.

The objective of this study was to comprehensively evaluate the influence of the popular studied POR*28 and other 5 common POR SNPs on tacrolimus concentrations during the initial period after transplantation in a group of Chinese renal transplant recipients.

Materials and methods

Ethics statement

The study was performed in accordance with the Declaration of Helsinki and guidelines on good clinical practice, and ethical approval of this study was obtained from the ethics committee of the First Affiliated Hospital of Sun Yat-Sen University (No 200823). Written informed consent was obtained from all subjects before participation.

Patients and therapy

We reviewed the medical records and laboratory results of patients who underwent renal transplantation in the Kidney Transplant Department of the First Affiliated Hospital of Sun Yat-sen University between July 2008 and December 2013 and received maintenance treatment with tacrolimus afterward. Adult male and female recipients undergoing single primary renal transplantation were eligible. Patients who received medication known to affect tacrolimus blood levels other than prednisone, such as verapamil, ketoconazole, itraconazole, erythromycin, clarithromycin, or diltiazem, were excluded. Patients with abnormal hepatic function and combined organ transplantations were also excluded. A total of 154 renal transplant recipients were enrolled.

All patients received a triple immunosuppressive regimen comprising tacrolimus (Prograft™, Astellas, Killorglin, Ireland); mycophenolate mofetil (Cellcept™, Roche, Basel, Switzerland), 1.0–1.5 g per day; and prednisone (Guangdong Huanan Pharmacy Ltd, Dongguan, China), 30 mg per day. According to the routine in the Kidney Transplant Department at the First Affiliated Hospital of Sun Yat-sen University, the initial dose of tacrolimus (0.05–0.075 mg/kg twice daily) was given from the morning of the first day post-transplant, and doses were subsequently adjusted to achieve a target trough concentration (C₀) of 5–8 ng/mL in the first 3 months post-transplant.

Data collection

Body weight, tacrolimus dosage and whole-blood trough concentrations (C₀) were obtained on d 7 after transplantation when steady-state concentration of tacrolimus was achieved (dosage had been unchanged for more than 3 d). Venous blood samples (2 mL) were collected before 8:00 AM, prior to administering the morning dose. The quantification of tacrolimus in human whole blood was achieved by chemiluminescent microparticle immuno assay of Abbott ARCHITECT tacrolimus assay (AbbottDiag, Chicago, IL, USA). The dose-adjusted tacrolimus trough concentration on d 7 after transplantation is the ratio of the measured tacrolimus trough concentration divided by dose expressed as mg/kg body weight (C₀/D). The corresponding laboratory parameters including alanine aminotransferase, aspartate aminotransferase and serum creatinine were also obtained.

DNA extraction and genotyping

After informed consent had been obtained, 2 mL venous blood samples for genotyping were collected in the outpatient clinic. Total genomic DNA was extracted from the peripheral leukocytes according to a previously described method [18]. CYP3A5*3 was determined by using published polymerase chain reaction restriction-fragment length polymorphism
(PCR-RFLP) methods\textsuperscript{[5, 19]} 6 POR SNPs were detected by Agena Bioscience MassARRAY\textsuperscript{®} system (Agena Bioscience, San Diego, CA, USA).

**Statistical analysis**

The Hardy-Weinberg equilibrium test was performed using $\chi^2$ test or Fisher’s exact test (two-sided). Linkage disequilibrium (LD) maps were constructed using online software SHEsis\textsuperscript{[20]}. Haplotypes were statistically inferred using an algorithm based on Bayesian inference by the program PHASE 2.1\textsuperscript{[21]}. For analysis of continuous pharmacologic variables, we used patient genotypes as categorical independent variables. Mann-Whitney $U$-test was used for comparisons between two groups and the Kruskal-Wallis $H$-test for comparisons among several groups. Multiple linear regression models considering the contribution of the genetic factors on tacrolimus $C_{0\text{D7}}$/D were evaluated using a stepwise variable selection method; only the variables with a $P$ value of less than 0.1 in the univariate analysis were included in the multivariate analysis. Tacro

\[C_{0\text{D7}}/D\] ratios were log-transformed to reduce skewness of the distribution. The statistical significance of the differences of the target tacrolimus $C_{0}$ achievement ratio between groups was calculated by $\chi^2$ test or Fisher’s exact test (two-sided). Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) software (version 21; SPSS, IBM, Armonk, NY, USA). Data are expressed as the median and range or mean±SD, depending on data type. Statistical power of the sample size was calculated by using software PASS (Power Analysis and Sample Size) software (version 11.0.7; PASS, NCSS, LLC). All the results met the requirement to have more than 80% power to detect the difference within/between groups with two-sided type one error at 5%.

**Results**

**Patient characteristics and POR polymorphisms**

The average age of the patients was 40.00±10.93 years (range 18–68 years), and the average body weight was 59.80±10.67 kg (range 35–105 kg). Age, gender, hepatic function and renal function were not found to be correlated with tacrolimus $C_{0\text{D7}}$/D (data not shown).

The MA(F) (minor allele frequency) of CYP3A5*3, POR rs1057868, rs2302429, rs17685, rs2868177, rs2286823, and rs41301394 were 25.2%, 34.3%, 30.8%, 39.3%, 38.7%, 40.1%, and 45.0%, respectively. All SNPs were in Hardy-Weinberg equilibrium. Genotype frequencies of CYP3A5*3 and POR rs1067868 were in agreement with those reported in previous publications in Chinese healthy volunteers or patients\textsuperscript{[6, 21]}. There was a linkage disequilibrium between POR rs2868177 and rs1057868. The $r^2$ and $|D'|$ values between the two SNPs were 0.189 and 0.651, respectively. Haplotypes were constructed, and the frequencies were 25.4% for A–C, 29.3% for A–T, 39.5% for G–C and 5.8% for G–T, respectively.

**Influence of CYP3A5*3, POR genotypes on tacrolimus $C_{0\text{D7}}$/D**

A total of 7 SNPs were examined. In accord with our earlier study\textsuperscript{[6]}, CYP3A5 nonexpressers had a significantly higher tacrolimus $C_{0\text{D7}}$/D compared with CYP3A5 expressers [137.96±8.40 vs 89.16±7.02 (ng·mL$^{-1}$)/(mg·kg$^{-1}$), $P<0.0001$] (Figure 1), and stratification analysis was performed to eliminate the confounding effect of CYP3A5*3.

A significantly higher tacrolimus $C_{0\text{D7}}$/D was observed in POR rs2868177 and (C) POR rs1057868–rs2868177 GC-GT diplotypes on tacrolimus trough concentration on d 7 after transplantation. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. $C_{0\text{D7}}$/D: Dose-adjusted tacrolimus trough concentration on d 7 after transplantation.

**Influence of POR haplotypes on tacrolimus $C_{0\text{D7}}$/D**

When the effects of POR rs1057868 and rs2868177 were...
combined, the $C_{077}/D$ in each diplotype of POR rs2868177–rs1057868, with or without stratifying for the CYP3A5*3, were as shown in Table 2. Carriers of the POR rs1057868–rs2868177 GC-GT diplotype had a considerably higher tacrolimus $C_{077}/D$ than noncarriers (220.17±48.09 vs 109.88±5.15 (ng·mL$^{-1}$)/(mg·kg$^{-1}$)), $P=0.002$ in the total cohort (Figure 1). The POR rs1057868–rs2868177 GC-GT diplotype was not detected in CYP3A5 expressers. In CYP3A5 nonexpressers, tacrolimus $C_{077}/D$ was also significantly higher in POR rs1057868–rs2868177 GC-GT diplotype carriers than in noncarriers (220.17±48.09 vs 135.69±6.86 (ng·mL$^{-1}$)/(mg·kg$^{-1}$)), $P=0.01$.

Multiple variable analysis for association with tacrolimus $C_{077}/D$

When the separated POR SNPs were considered, a multiple linear regression model for log-transformed tacrolimus $C_{077}/D$ was developed that included CYP3A5*3 and POR rs2868177.

Combined influence of CYP3A5*3 and POR diplotypes on tacrolimus $C_{077}/D$

Considering the combined effects of CYP3A5*3 and POR rs1057868–rs2868177 diplotypes, we divided patients into three groups.
groups: fast metabolizers (CYP3A5*1 carriers), slow metabolizers (CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplotype noncarriers) and ultra-slow metabolizers (CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplotype carriers).

Tacrolimus $C_{\text{O0D7}}$ was $220.17\pm48.09$ ng/mL/mg/kg for ultra-slow metabolizers, which was 1.62-fold higher than for slow metabolizers ($135.69\pm6.86$ ng/mL/mg/kg, $P<0.001$) and 2.72-fold higher than for fast metabolizers ($80.84\pm5.27$ ng/mL/mg/kg, $P<0.0001$) (Figure 2). Achievement of target ranges was analyzed on d 7 after transplantation; tacrolimus trough concentration in ultra-slow metabolizers ($10.92\pm2.90$ ng/mL) was also significantly higher than that in slow metabolizers ($7.29\pm2.02$ ng/mL, $P<0.001$) and in fast metabolizers ($5.46\pm2.04$ ng/mL, $P<0.0001$). A total of 39.6% of fast metabolizers did not achieve the lower limit of target tacrolimus $C_{\text{O0}}$ of 5 ng/mL compared with 7.4% of slow metabolizers and 0% of ultra-slow metabolizers. Conversely, 85.71% of ultra-slow metabolizers exceeded the upper limit of the target range (8 ng/mL) in comparison with 29.63% of slow metabolizers and 10.42% of fast metabolizers ($P<0.0001$) (Table 4).

**Discussion**

In the current study, the associations of 6 POR SNPs with tacrolimus $C_{\text{O0D7}}$ were examined. The positive role of POR rs1057868–rs2868177 GC-GT diplotype combinations on tacrolimus trough concentration on d 7 after transplantation was first observed, which
Table 4. Tacrolimus trough concentrations (C_{0D7}) and achievement of target C_{0} ranges according to CYP3A5*3 and POR rs1057868–rs2868177 haplotypes combinations.

| Fast metabolizers (n=70) | Slow metabolizers (n=77) | Ultra-slow metabolizers (n=7) | P value |
|--------------------------|--------------------------|-------------------------------|---------|
| C_{0D7} (ng/mL) | 5.46±2.04 | 7.29±2.02 | 10.92±2.90 | <0.0001 |
| Proportion of patients with tacrolimus C_{0} on d 7 (%) | | | |
| C_{0D7}<5 (ng/mL) | 39.58 | 7.41 | 0.00 | <0.0001 |
| C_{0D7}: 5–8 (ng/mL) | 50.00 | 62.96 | 14.29 |
| C_{0D7}>8 (ng/mL) | 10.42 | 29.63 | 85.71 |

Fast metabolizers: CYP3A5*1 carriers. Slow metabolizers: CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplotype non-carriers. Ultra-slow metabolizers: CYP3A5*3/*3/ POR rs1057868–rs2868177 GC-GT diplotype carriers. n: the numbers of observation.

could explain 5.7% and 10.9% of inter-individual variability of tacrolimus C_{0D7}/D in the entire cohort and in CYP3A5 nonexpressers, respectively. POR rs1057868–rs2868177 GC-GT diplotype carriers also represent a higher-risk group because they have a significantly higher likelihood of exceeding the upper limit of the target range. Therefore, genotyping of POR rs1057868–rs2868177 diplootypes, would help to further differentiate initial tacrolimus dose requirements and control the toxicity of over-immunosuppression.

POR is the unique electron donor to all microsomal CYPs, and the possibility of POR as a potential rate-limiting step in CYPs-mediated drug metabolism has been considered since 1969[22]. Thus, the contribution of common POR variants to variability of tacrolimus metabolism is of great interest. POR*28, the most common sequence variant in POR gene, induces an amino acid substitution (C>T, p.Ala503Val) and influence the electron binding moiety of POR[23]. A previous in vitro study showed that this mutation could decrease CYP3A4 activity. By contrast, POR*28 has been reported to be associated with lower tacrolimus concentrations in CYP3A5 expressers[24, 25] or nonexpressers[26, 27], suggesting that POR*28 variant may enhance CYP3A4 or CYP3A5 enzyme activity. Moreover, no significant association between POR*28 and tacrolimus concentration was found in the present study, which is in line with a study also conducted in Chinese early post-renal transplant recipients[28] and two studies conducted in Caucasian early or stable renal transplant recipients[29, 30]. The reason for the discrepancy among these studies is not clear, but several possibilities may be involved. First, different doses or types of corticosteroids were co-administered with tacrolimus in different studies. For instance, prednisone is a CYP3A and P-gp inducer[30, 31], whereas prednisolone is a weak competitive inhibitor of CYP3A4[32]. Thus the different influences on tacrolimus metabolism between prednison and prednisolone may interfere with the analysis of the effect of POR*28. Furthermore, the dose of co-administered corticosteroids is reduced gradually during the post-transplant period; thus, the extent of drug-drug interaction between corticosteroids and tacrolimus may also be a confounding factor. Second, Saito et al[33] found that there were significant interethnic differences in the haplotype profile of the POR gene containing POR*28. POR*28 may be associated with other functional polymorphisms, and the interethnic haplotype difference may lead to different results. We speculated that these confounding effects may complicate the influence of POR*28 on tacrolimus, and further investigation is required.

The tacrolimus C_{0D7}/D was higher in POR rs2868177 GG genotype carriers than in AA plus AG carriers (P=0.041). We speculated that POR rs2868177 GG genotype may be associated with decreased in vivo CYP3A activity and lead to increased tacrolimus concentration. In a genome-wide association study (GWAS) in acute myeloid leukemia patients, the minor allele (G) was found to be associated with a higher incidence of cardiotoxicity induced by daunorubicin, which is also a substrate of CYP3A, suggesting that the G allele could lead to slower daunorubicin metabolism[34]. However, the underlying mechanism needs further clarification.

Interestingly, when the effects of POR rs1057868 and rs2868177 were combined, a more obvious correlation was observed, namely, that tacrolimus C_{0D7}/D in carriers of the POR 1057868–rs2868177 GC-GT diplotype was approximately two-fold higher than in noncarriers (220.17±48.09 vs 109.88±5.15 (ng·mL⁻¹)/(mg·kg⁻¹), P=0.002). Moreover, in the multiple linear regression analysis, the POR 1057868–rs2868177 GC-GT diplotype was the only contributor accounting for the inter-individual variation of tacrolimus C_{0D7}/D besides CYP3A5*3. Such findings suggest that haplotype analysis may be superior to SNP analysis in describing genotype-phenotype associations; this was confirmed in MDR1 pharmacogenetic studies[35, 36]. However, further study with a larger sample size is warranted.

Among CYP3A5 nonexpressers, the POR 1057868–rs2868177 GC-GT diplotype could explain 10.9% of total variation in tacrolimus C_{0D7}/D. Because the metabolism of tacrolimus depends mainly on CYP3A4 activity in CYP3A5 nonexpressers[37], we presumed that the POR 1057868–rs2868177 GC-GT diplotype might be associated with decreased CYP3A4 enzymatic activity with tacrolimus as a substrate. Further studies identifying the functional characterization of the POR rs1057868–rs2868177 diplootypes and their impacts on the
metabolism of tacrolimus are also needed.

It is now generally accepted that patients who carry CYP3A5*3/*3 genotype are slow metabolizers for tacrolimus. However, in this study, we separated a new sub-group from conventional slow metabolizers: CYP3A5*3/*3 and POR GC-GT diplotype carriers, who have been found to have the highest C_{\text{crit}}/D and need 38.2% and 63.2% dosage reduction to obtain the same level of C_{\text{\text{peak}}}, respectively. Therefore, the term “ultra-slow metabolizers” was used to dissociate this group from the conventional “slow metabolizers.” Moreover, 85.71% of ultra-slow metabolizers exceeded the upper limit of the target range (8 ng/mL) despite concentration-controlled dose adjustments. It has been found that tacrolimus C_{\text{\text{peak}}} recorded within 7 d post-transplantation was significantly associated with rates of rejection and toxicity and a significant trend for increasing toxicity with increasing maximum tacrolimus C_{\text{\text{peak}}}.[3]

Therefore, timely dosage reduction is in urgent need for the ultra-slow metabolizers who have the highest risk of over-immunosuppression and toxicity, and identifying the POR rs1057868–rs2868177 GC-GT diplotype before tacrolimus administration may have great potential for improving the procedure’s safety.

In conclusion, this observational pharmacogenetic analysis demonstrated for the first time that POR rs1057868–rs2868177 GC-GT diplotype significantly increased tacrolimus C_{\text{\text{peak}}}/D in recipients in the early stage after renal transplantation. Patients who are CYP3A5 nonexpressers and carry POR rs1057868–rs2868177 GC-GT diplotype might be at an elevated risk of early tacrolimus overexposure and need timely dosage adjustment. The results could be useful for personalized medicine for organ-transplant recipients.

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Author contribution
Jia-li LI and Chang-xi WANG were involved in study design and manuscript writing, and they are the corresponding authors; Shu LIU was involved in study design, research performance, data analysis and manuscript writing; Qian FU and Jun LI were involved in data collection and analysis; Yu ZHANG, Xue-ding WANG, Ling-yan CHEN, and Xiao-man LIU were involved in laboratory analysis; Rong-xin CHEN, Min HUANG and Hong-bing HUANG were involved in data analysis.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

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