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RESEARCH ARTICLE

The Genetic Polymorphism of *CYP3A4* rs2242480 is Associated with Sirolimus Trough Concentrations Among Adult Renal Transplant Recipients

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Abstract: ***Background*:** The large interindividual variability in the genetic polymorphisms of sirolimus (SIR)-metabolizing enzymes, transporters, and receptors can lead to qualitatively and quantitatively distinct therapeutic responses.

***Objective*:** We examined the impact of numerous candidate single-nucleotide polymorphisms (SNPs) involved in the trough concentration of SIR-based immunosuppressant regimen.

***Method*:** This is a retrospective, long-term cohort study involving 69 renal allograft recipients. Total DNA was isolated from recipient blood samples and trough SIR concentrations were measured by microparticle enzyme immunoassay. Genome sequence reading was targeted based on next-generation sequencing. The association of tagger SNPs to SIR trough concentrations with non-genetic covariate adjusting was analyzed using logistic regression.

***Results*:** A total of 300 SNPs were genotyped in the recipient DNA samples using target sequencing analysis. Only the SNP of CYP3A4 (Ch7: 99361466 C> T, rs2242480) had a significantly higher association with SIR trough concentration as compared to the other 36 tagger SNPs. The mean trough SIR concentration of patients in the CYP3A4 rs2242480-CC group was more significant compared to that of the CYP3A4 rs2242480-TC and TT group, respectively 533.3; 157.4 and 142.5 (ng/ml)/mg/kg, P<0.0001. After adjusting the SNPs, there was no significant association between clinical factors such as age, follow-up period, the incidence of delayed graft function, immunosuppression protocol, and sex with SIR trough concentration.

***Conclusion*:** These findings indicated a significant association of polymorphism in the CYP3A4 (Ch7: 99361466 C> T, rs2242480) with SIR trough concentration after 1-year administration in patients who have undergone kidney transplantation.

Keywords: Renal transplantation, sirolimus, trough concentration, pharmacogenetics, targetted sequencing.

1. INTRODUCTION

Kidney transplantation is an important recommendation, as there is no other treatment strategy for irreversible chronic kidney disease. Patients who undergo kidney transplantation achieve better results, longer life expectancy, and much better quality of life than patients who receive only therapeutic intervention. In the last decade, the survival rate of kidney transplantation patients gradually increased compared to that of maintenance dialysis patients [1-3]. In fact, transplantation enhances access to and reduces the overall cost of successful therapy management of end-stage renal disease [4].

Although the overall success rate of kidney transplantation has increased significantly, problems may occur after the transplantation. Patients should be closely monitored for an extended period to avoid complications and adverse effects. A direct reaction after transplantation can lead to acute organ rejection [5]. Therefore, immunosuppressive therapy is necessary to reduce its incidence [6].

Several *CYP3A* enzymes have a pivotal role in primary phase 1 of metabolic sirolimus (SIR) reactions and influence the dose requirement [7]. *CYP3A4*, *CYP3A5* and *CYP2C8* are the major phase 1 enzymes, contributing to the intrinsic clearance of SIR [8]. Other factors, including drug transport activity such as drug efflux pump P-glycoprotein, encoded by the *ABCB1* gene, and receptor sensitivity in the nucleus, can affect the SIR biotransformation [9-12]. An increase or decrease in blood SIR levels due to various gene polymorphisms can lead to poor efficacy and safety. Therefore, the multiple gene polymorphisms in these mechanism pathways can lead to differences in patients' response to pharmaceutical therapy [11].

Among these types of polymorphisms, the *CYP3A4* gene family plays a significant role in SIR metabolism [13, 14]. Several studies have confirmed the role of novel single-nucleotide polymorphisms (SNPs) in the enzyme activity level, including the *CYP3A4**1G (rs2242480, G>A, intron 10 at position 82266) allele, rs4646437 C>T intron 7 and *CYP3A4**22 (rs35599367, intron 6 C>T) as a biomarker of *CYP3A4*- drug metabolization [15-17]. These studies have established that recipients with *CYP3A4* polymorphism show different patient-to-patient responses to the appropriate drug dosage that impact therapeutic outcomes.

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Others have also investigated the relationship between various genetic polymorphisms with SIR responsiveness, but the results have been inconsistent [18-23]. The *ABCB1* 3435CT/TT genotypes, *IL-10* -1082GG homozygotes, and *CYP3A5* non-expressers (*CYP3A5*3/*3* carrier) have deficient enzymatic activity, suggesting lower SIR daily dose [19, 24-26]. Moreover, *CYP3A4*1B* is associated with enhanced liver metabolism and lower SIR concentration/dose ratio [18]. Several new polymorphisms of *CYP3A4*22*, *POR*28* rs1057868 C>T and *PPARA* rs4253728 G>A show no significant influence on SIR pharmacokinetics in renal transplant recipients [27]. Consequently, genetic polymorphism studies based on ideal immunosuppressive drug use in kidney transplantation are still ongoing to show the best current evidence. It is clear that pharmacokinetics approaches based on individualized therapy should be applied for kidney transplant patients based on individualized SIR therapy. The aims of our study lie in the targetting of numerous genes by next-generation sequencing to analyse their effects together with other variants on SIR trough concentration in renal transplant recipients. To our knowledge, this is the first study to demonstrate the association of gene polymorphisms coding for metabolism enzymes in the Chinese renal transplant patients on the long-term use of SIR. This study will undoubtedly greatly benefit long-term SIR-based immunosuppressive therapy to kidney transplantation recipients.

2. MATERIALS AND METHODS

2.1. Study Design and Population

We performed a retrospective, single-center cohort study to examine the effect of SNPs of multiple genes on the SIR trough concentrations (C0) in renal transplant recipients under long-term observation. For SIR level collection, sample size calculation was performed according to the desired power of 0.80 ($\beta = 0.20$) and α of 0.05.This study was strictly limited to the living-related transplantation of kidney donors to lineal or collateral relatives not beyond the third degree of kinship, or kidney transplantation from cadaveric allograft donors after cardiac death. During the period of 1 February 2011 and 1December 2015, a total of 300 kidney transplant patients admitted to The First Affiliated Hospital of Nanjing Medical University were screened in this study. According to the study criteria, we included patients who: (1) were aged > 18 years or up to 60 years; (2) had received first kidney transplantation; (3) had received SIR for at least 12 months as the primary immunosuppressant; (4) who have been observed for up to 12 months; and (5) volunteered to participate in this study. In addition, the exclusion criteria were patients who (1) did not meet the inclusion criteria; (2) participated in other clinical trials; (3) had chronic viral infections, such as HIV and chronic hepatitis B and C virus; (4) were pregnant women; and (5) whose extracted DNA samples did not meet SIR requirements.

The adjusted minimum dose level of SIR and adjusted body mass index (BMI) SIR (= C0/dose/BMI) were defined as the outcome variable (the SIR pharmacokinetic index) in this study. SIR doses and C0 were obtained at 12 months from patients in stable conditions [defined as stable serum creatinine (Scr) value <120 μmol/L or with fluctuations <20% to be collected and also no episodes of acute rejection (AR), delayed graft function (DGF) or opportunistic infections after kidney transplantation]. Other clinical data from the medical records of the included patients, such as age, sex,, weight, height, frequency of AR or DGF and immunosuppressive protocols, were critically reviewed and extracted by two doctors (M Zheng and X Zhang). The medical records of the included patients were independently reviewed by two doctors (ZJ Wang and RY Tan). Importantly, if the recipients experienced DGF, AR, or opportunistic infections, C0 detection and SIR dosage were delayed until they reached a stable allograft status.

2.2. Immunosuppressant Protocol

All recipients received maintenance immunosuppressive protocols, which include four drugs: mycophenolate mofetil (MMF), sirolimus (SIR), prednisone (Pred), and tacrolimus (TAC) or cyclosporine (CsA). Next, the SIR adjustment oral dosage was determined by the Scr level to maintain target blood levels between 3 ng/mL and 12 ng/mL. The initial dose of SIR is 0.2 mg kg-1 day-1. MMF was given intravenously 24 to 48 hours after transplantation with an initial dose of 0.75 to 1.0 g / day (BID). In patients with episodes of AR, methylprednisolone is given intravenously at a dose of 200 mg/ day for 3-5 days.

2.3. SIR Trough Concentration Measurement

Blood samples from each registered patient were recorded at 12 months after a kidney transplant. SIR total blood levels were obtained using a microparticle enzyme immunoassay. The samples were transferred to ambient temperature in an EDTA tube, extracted with a protein precipitating reagent and centrifuged. The supernatant was decanted for testing with ARCHITECT Sirolimus Assay (Abbot Diagnostics, Lake Forest, IL, USA), a chemiluminescent microparticle immunoassay (CMIA) for quantitative measurement on the ARCHITECT system quantitatively. In combination with the anti-SIR-coated paramagnetic microparticles, the conjugate-labeled SIR-acridinium was added to the reaction mixture. After incubation, the microparticles were washed and a pre-trigger/ trigger solution was added to the reaction mixture. The produced chemiluminescent reaction was measured as a relative light unit (RLU). Using an indirect connection between the number of SIRs and RLUs obtained by the Architect I2000 optical acquisition system, a calibration curve was produced using the 4-parameter logistic curve fit (4PLC, Y-weighted) method, and SIR trough concentrations were measured and reported C0. The dosage and SIR C0 adjusted for body weight (C0/dose/ weight) as SIR-PK index were considered as the main variables for the results [formula: C0/-dose/weight = C0/(dose/weight)].

2.4. Sample Preparation, Data Quality Control and Targeted Sequencing Analysis (TS)

We extracted DNA from the peripheral blood samples of each recipient using a QIAamp-DNA mini kit (Qiagen, Hilden, Germany), and calculated the concentration and purity of genomic DNA (gDNA) using a NanoDrop ND2000 (ThermoFisher Scientific, Waltham, MA, USA). Gene integrity was evaluated by agarose gel electrophoresis. We selected the gDNA hybrids then fragmented and measured them using a Diagenode Bioruptor (Liège, Belgium) to ensure that the average fragment size was 150-250 bp. The Illumina PhiX control was added to lane 8 of each flow cell. Two-sided end reads (PE150) were produced by sequencing using the Illumina HiSeq2000 platform according to the manufacturer's instructions.

We analysed the sequencing data *i.e.* the number of mutated chromosomes, changes in the genome, and depth of the sequence range. All analyses were based on the UCSC build hg19 human reference sequence (NCBI build 37.2) using Burrows-Wheeler [28]. Besides, suspected somatic variants suspected detected by MuTect 1.1.5 and VarScan 2.3.6 were identified by pairing each sample with the corresponding blood sample [29, 30].

2.5. Statistical Analysis

Minor allele frequencies (MAF), Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium (LD) were determined using

Haploview 4.2 (Broad Institute, MA, Cambridge, USA) [31]. Variant genes with MAF <0.05 and/or HWE below the adjusted *P*-value (P <0.05) as rare/low-frequency variants were excluded from further analysis. Tagger SNPs used for logistic regression analysis and association analysis were selected by Haploview 4.2 (Broad Institute, MA, Cambridge, USA) [32]. For SNP analysis in a single-site association, SIR drug levels between two and three genotypes were compared using Student's *t*-test and analysis of variance (ANOVA). The association of tag SNPs between the natural log (ln)-transformed dose and BMI-normalized SIR C0 was examined using the general linear model (GLM). The genotypic distributions of the SNPs in recipients were explored using forward/stepwise methods of logistic regression models by adjusting clinical factors such age, sex, follow-up period, frequency of DGF, AR, and immunosuppressive protocol with a *P*-value of 0.10. Data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1. Participants and Baseline Characteristics

In total, 300 cases underwent primarily screening for sample collection. Among these, a total of 90 cases were excluded because they declined to participate or had long-term follow-up (76 cases) and sample quality issues (14 cases). Therefore, a total of 210 cases were obtained for secondary screening. Based on the exclusion criteria and adequate clinical assessment, a total of 69 cases were presented for next-generation targeted sequencing in our association study analysis. (Fig. **1**) shows further details on the reasons for participants' selection.

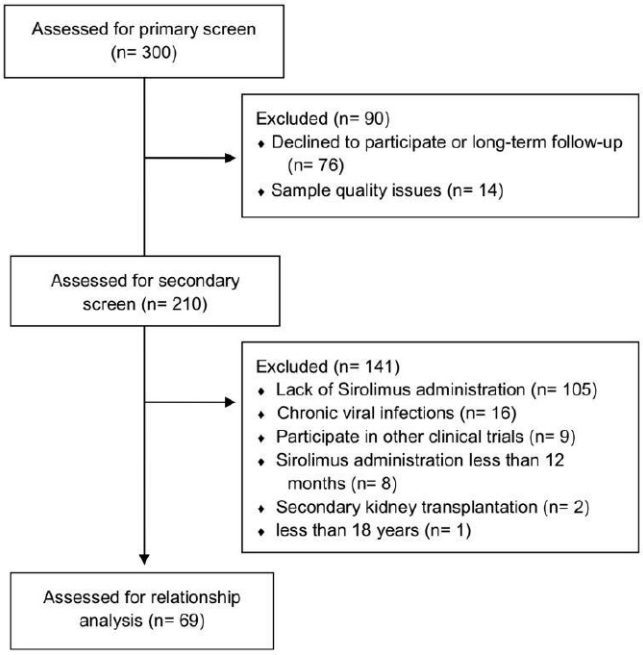

Fig. (1). Flow diagram for the selection of participants in our study.

Table (**1**) shows that most recipients were males (73.91%), while females were 26.09%. The mean (± SD) weight and age of participants were 63.22 ± 10 kg; 35.22 ± 10 years, respectively. Of the patients in this cohort, 92.5% were referred to as cardiac arrest and 100% of them were primary renal transplants. The most commonly used immunosuppressive therapies were Pred+MMF+TAC (53.62%), and Pred+MMF+CsA (46.38%). The incidence of DGF was 36.23%AR episodes were 53.62%. The 69 renal transplant patients showed a mean (± SD) duration after renal transplant of 1964 ± 674 days.

Table 1. Baseline clinical characteristics in the cohort.

Clinical Variables	Value
Case number (n)	69
Age (years; mean ± SD)	35.22 ± 10
Gender, n (%)	
Male	51 (73.91)
Female	18 (26.09)
Weight (kg, mean ± SD)	63.22 ± 10
Duration after renal transplant (days, mean ± SD)	1964 ± 674
PRA before renal transplant (%)	0
Primary/secondary renal transplant	69/0
Type of donor, n (%)	
DCD	64 (92.76)
Living-related	5 (7.25)
ISD protocol	
Prednisone + MMF + Tacrolimus	37
Prednisone + MMF + CsA	32
Incidence of DGF episodes, n (%)	25 (36.23)
Incidence of AR episodes, n (%)	37 (53.62)

Abbreviations: AR, Acute Rejection; CsA, Cyclosporine; DCD, Donation after Cardiac Death; DGF, Delayed Graft Function; ISD, immunosuppressive drugs; MMF, Mycophenolate Mofetil; PRA, Panel Reactive Antibodies; SD, Standard Deviations.

3.2. Linkage Disequilibrium Analysis

Target sequencing (TS) analysis based on next-generation sequence obtained a total of 300 SNPs for all genes (*CYP3A4*, *CYP3A5*, *CYP2C8*, *CYP2C19*, *POR*, *PPARA*, *ABCB1*, *HSD11B1*, *NR3C1*, *UG/T1A8*, *UG/T2B7* and *UG/T1A9*) including 20 SNPs in *CYP3A4*, 20 SNPs in *CYP3A5*, 27 SNPs in *CYP2C8*, 27 SNPs in *CYP2C19*, 63 SNPs in *POR*, 18 SNPs in *PPARA*, 58 SNPs in *ABCB1*, 8 SNPS in *HSD11B1*, 22 SNPs in *NR3C1*, 8 SNPs in *UG/T1A8*, 4 SNPs in *UG/T1A9*, 22 SNPs in *UG/T2*B7 and 4 novel SNPs with undetermined genotype names (Supplemental Table **1**).

We observed several SNPs in 15 haplotype blocks with r2>0.8 using the confidential interval methods [33] in LD analysis Haploview 4.2 software. Supplemental Fig 1 shows that several genes had extremely strong LD, namely: 11 SNPs in *POR* in three LD blocks ((Block 4: Ch7:75612770, Chr:75612783, Block 5: Chr7:75613998, Chr7:75614029, Chr7:75614082, Chr7:75614288, Chr7:75614296, Chr7:75614777 and Block 6: Chr7:75614863, Chr7:75614864, Chr7:75614953)), 8 SNPs of *UG/T2B7* in two LD blocks (Block 1: Chr4:69962449, Chr4:69962610 and Block 2: Chr4:69964180, Chr4:69964209, Chr4:69964337, Chr4:69964338, Chr4:69972949, Chr4:69973044), 5 SNPs of *CYP2C8* in two LD blocks (Block 11: Chr10:96802598, Chr10:96805371, Chr10:96818362 and Block 12: Chr10:96824406, Chr10:96824738). Multiallelic pairs of those genes (*UG/T2B7*, *POR*, *CYP2C8*) had a D´value equal to 1.0, which implies a tight correlation in LD. This means that the chromosomes that contain those genes display no evidence of historical recombination. After removing the SNPs with HWE <0.05 and MAF <0.05, we found 13 genotypes with 80 SNPs: *ABCB1* (15 SNPs), *PPARA* (2 SNPs), *NR3C1* (8 SNPs), *UG/T1A8* (3 SNPs), *POR* (20 SNPs), *CYP2C19* (7 SNPs), *CYP2C8* (9 SNPs), *UG/T2B7* (8 SNPs), *CYP3A4* (2 SNPs), *CYP3A5* (4 SNPs), and *HSD11B1* (2 SNP). When considering all 69 recipients, 80 SNPs with MAF≥0.05 were included in further single site association analysis (Supplemental Table **2**).

Table 2. Performance of genetic factors influencing the sirolimus trough concentrations using a general linear model.

No	Chromosome	Position	Reference Allele	Alternative Allele	Gene Name	Function	avsnp144	F value	*P*-value
1	Chr7	99260362	C	A	CYP3A5	intronic	rs4646453	3.54	0.036
2	Chr7	99361466	C	T	CYP3A4	intronic	rs2242480	5.24	0.008
3	Chr7	99245914	A	G	CYP3A5	UTR3	rs15524	3.94	0.026
4	Chr22	46615625	G	A	PPARA	intronic	rs1800246	3.24	0.077

3.3. Association Analysis of SNPs, Genotype and SIR PKs

We identified 80 SNPs with HWE and MAF ≥ 0.05, and selected 36 tag SNPs for single-site analysis. Tag SNPs that capture information on other variants with MAF ≥0.05 were selected using the Tagger program (BROAD Institute, implemented in Haploview) [34]. We found 36 tag SNPs, including 8 SNPs in *ABCB1*, 4 SNPs in *CYP2C19*, 4 SNPs in *CYP2C8*, 1 SNP in *CYP3A4*, 3 SNPs in *CYP3A5*, 1 SNP in *HSD11B1*, 3 SNPs in *NR3C1*, 5 SNPs in *POR*, 2 SNPs in *PPARA*, 3 SNPs in *UG/T1A8*, and 2 SNPs in *UG/T2B7* that were examined (Supplemental Table **3**). We analysed the relationship between each SNP and 12 months of SIR PKs to observe a significant difference. GLM analysis showed that four SNPs positioned in Chr7:99361466 intronic C>T rs2242480 (*P*=0.008, *CYP3A4*), Chr22:46615625 intronic G>A rs1800246 (*P*=0.077, *PPARA*), Chr7:99245914 UTR3 A>G rs15524 (*P*=0.026, *CYP3A5*) and Chr7:99260362 intronic C>A rs4646453 (*P*=0.036, *CYP3A5*) were significantly associated with the dose- and BMI- normalized SIR concentrations Table (**2**).

In a total of 69 recipients, *CYP3A4* rs2242480 had a greater proportion than of the CC genotype than TC and TT genotypes (56.9% *vs* 37.3% *vs* 3.4%). (Fig. **2**) shows the genotype polymorphisms that influenced the weight-adjusted SIR C0/dose. In the homozygote dominant variant CC, the mean C0 of the *CYP3A4* rs2242480 was significantly higher than that for the heterozygote TC and homozygote recessive TT (*P*<0.0001) (533.3, 157.4, 142.5 (ng/ml)/mg/kg, respectively). The difference in the mean ± SD value between theweight-adjusted SIR C0/dose of the CC and TC group *CYP3A4* rs2242480 was 375.9±35.27 (ng/ml)/mg/kg) (*P*<0.0001). The most significant change in SIR C0 was for homozygote dominant CC, which increased to more than 3.4-fold compared with heterozygote TC and homozygote recessive TT.

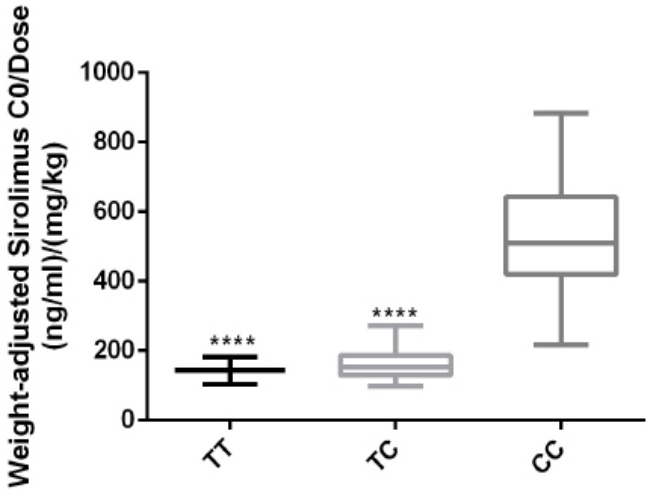

Fig. (2). Influence of *CYP3A4* rs2242480 of TT, TC and CC genotype in weight-adjusted SIR C0/Dose (****P<0.0001 when compared with CC group).

3.4. Multivariate Association of SNPs and Clinical Factors with SIR PKs.

We analysed the clinical variables influencing the SIR trough concentrations in the combined effect analysis using a multivariable GLM. Clinical factors such as age (*P*=0.999), follow up duration (*P*=1), the incidence of DGF (*P*=0.999), immunosuppressant protocol (*P*=0.991), sex (*P*=0.987) and AR (*P*=0.954) showed no significant association with SIR C0/Dose/BMI levels using those models (*P*>0.1) (Supplemental Table **4**). The forward/stepwise logistic regression analysis between genetic and clinical factors identified a significant difference of *CYP3A4* (Ch7: 99361466 C>T, rs2242480, *P*=0.02) toward SIR C0. We also found that all clinical factors did not influence the SIR C0.

4. DISCUSSION

We successfully collected blood samples from 69 renal transplant patients treated with SIR-based therapy to measure their plasma trough concentrations. Unfortunately, there remains a lack of association studies on genetic variants with long-term SIR use in renal transplant patients. For this purpose, our analysis focused on investigating the significance of the impact SNPs and SIR trough concentration on ensuring long-term renal graft survival in the patient. Here, we measured SIR trough concentration at 1 year after transplantation. The aim of taking long-term immunosuppressive agents in renal transplantation plays a role in maintaining patient survival of late acute rejection or chronic rejection [35-37].

Here, we used targeted sequencing technology and obtained 300 SNPs mapped from a total of 69 kidney transplant patients. We successfully identified several genes: *CYP3A4*, *CYP3A5*, *CYP2C8*, *CYP2C19*, *POR*, *PPARA*, *ABCB1*, *HSD11B1*, *NR3C1*, *UG/T1A8*, *UG/T1A9*, and *UG/T2B7*. After adjusting HWE> 0.05 and MAF> 0.05, 80 SNPs were obtained. Subsequently, 36 tag SNPs identified genotype details for *CYP3A5*, *CYP2C8*, *PPARA*, *NR3C1*, *UG/T1A8*, *POR*, *CYP2C19*, *ABCB1*, *UG/T2B7*, and *HSD11B1*. All of these genes have an important role in determining the therapeutic outcome of drug levels in the blood. The GLM showed that 4 SNPs had a significant effect on the SIR steady-state concentration measured after 1 year of transplantation: *CYP3A5* (Chr7: 99260362 C>A, rs4646453 and Ch7: 99245914 A> G, rs15524), *CYP3A4* (Ch7: 99361466 C>T, rs2242480) and *PPARA* (Chr22: 46615625 G> A, rs1800246) .

Multivariable analysis after adjustment for patient's clinical factors showed that *CYP3A4* (Ch7: 99361466 C>T, rs2242480) was independently associated with the trough blood level of SIR compared to the other four significant tag SNPs. We also plotted the association of the *CYP3A4* rs2242480 genotype and found that subjects in the CC group had the highest average increase in SIR levels of 533.3 ng/mL (*P*<0.0001). In comparison, subjects in the TT group had an average 142.5 ng/mL decrease in SIR levels (*P* <0.0001). Our study confirms the influence of *CYP3A4* rs 2242480 on SIR blood levels in the renal transplant setting. The rs 2242480 CC genotype is a novel polymorphism of *CYP3A4* that leads to decreased metabolism rate and results in increased SIR blood levels. In other words, patients with the TT genotype of *CYP3A4**1G tend to have lower blood levels of SIR at the same drug dose compared

with those who express the CC genotype of *CYP3A4**1G. New evidence for another immunosuppressant of tacrolimus shows that *CYP3A4**1/*1G or *CYP3A4**1G/*1G—an allele variant of the *CYP3A4* gene that results in higher enzymatic activity—is associated with higher tacrolimus clearance and lower maintenance dose requirements than *CYP3A4**1/*1, the wild-type genotype associated with lower enzyme expression [38]. Based on these findings, genotyping *CYP3A4* to optimize SIR dosing would be the most promising application of pharmacogenetics to renal transplant medicine. Therefore, it is unlikely that *CYP3A4* genotyping will prove to be of value in predicting SIR dosing in the clinical practice.

CYP3A4 is the most important enzyme in the metabolism of immunosuppressive drugs such as cyclosporin, tacrolimus and sirolimus. However, little is known about the functional role of this enzyme's polymorphism [39, 40]. Some are due to low-frequency polymorphisms (<1%: *CYP3A4* *2,*4,*5,*6, *8,*9,*11-21*) and unclear enzyme activity (*CYP3A4* *1B, *3, *10) [41]. In addition, the high frequency of *CYP3A4*□1G variants has been in patients of Han Chinese ethnicity [42]. The frequency of the *CYP3A4**1G allele genotype is very high in the Chinese population, but the definitive function of this gene has never been described [38, 43, 44]. In the present study, we found that *CYP3A4* (Ch7: 99361466 C>T, rs2242480) primarily showed a significant effect on steady-state concentrations of SIR measured at month 12 after transplantation. He *et al.*, reported that patients with *CYP3A4* *1G alleles (rs2242480, 20230 C>T) showed high CYP3A4 enzyme activity [16]. Whereas in previous studies, Miura *et al.*, stated that *CYP3A4* *1G could influence the difference in tacrolimus pharmacokinetic (PK) response in *CYP3A5* expressors. *CYP3A4* *1G alleles can increase the metabolic activity of *CYP3A4* substrates so that patients with the *CYP3A4* *1G allele genotype have a significantly lower dose-adjusted tacrolimus C0 than the wild type (*CYP3A4* *1/ *1) [45]. Hu *et al.*, reported that *CYP3A4* *1G could affect the oral clearance (CL/F) of tacrolimus in *CYP3A5* expressors or non-expressers [46]. Zhang *et al.*, demonstrated that healthy Chinese patients who carried *CYP3A4**1G had significantly lower Cmax (maximum concentration) and AUC (area under curve) values than CYP3A4*1/*1 homozygous subjects [47].

The effect of *CYP3A4* *1G on protein/mRNA levels remains unclear. Although there are mixed data for *CYP3A4**1G, which is located in intron 10, most studies have reported decreased clearance. A study using gene assays on heterogeneous networks showed that the minor G alleles in *CYP3A4**1G are related to decreased transcription, resulting in the loss of function. In addition, the minor G alleles will cause a reduction in the tacrolimus dose-adjusted blood level (AUC) [45]. Zhou *et al.*, also concluded that healthy patients with the wild-type gene had significantly higher dose-corrected 0-24 hours AUC that was 1.35-fold higher than that in *CYP3A4**1G carriers [48]. Moreover, *CYP3A4* *1G had a high LD with *CYP3A5* *1 in Japanese patients [49]. Therefore, the level of *CYP3A5* mRNA expression is also related to the *CYP3A4* * 1G genotype.

In addition to investigating genetic variation in *CYP3A4*, we also explored the role of *PPARA* regulators, which contribute to differences in the SIR trough concentrations of kidney transplant patients. Genotype changes that occur in *PPARA* can also affect P450 enzyme activity. *PPARA* can influence SIR PK through *CYP3A4* activity by either directly activating the *CYP3A4* gene transcription or indirectly through another nuclear receptor, *PXR*. It also can inhibit *CYP3A4* downregulation through its anti-inflammatory properties [50]. In the present study, the *PPARA* genotype (Chr22: 46615625 G>A, rs1800246) had a significant effect on the steady concentration of SIR given over a long period. Si *et al.*, also reported that *PPARA* exons did not show any impact on tacrolimus metabolism [32].

Drug concentration can also be affected by other non-genetic factors. In the present study, we assessed the effects of age, sex, length of follow-up, AR, DGF, immunosuppressive protocols on blood levels of SIR. The multivariable regression analysis showed that all evaluable clinical factors had no significant differences in SIR blood level by considering the SNPs effect. Another study has shown that patients are highly vulnerable to acute organ rejection after undergoing transplantation therapy. Uesugi *et al.*, have also shown that the incidence of acute cellular rejection in liver transplants with *CYP3A4* *1G alleles tends to be higher than in *CYP3A4* *1/*1 alleles. The author reported that adult liver and intestine transplant patients with *CYP3A4* * 1G alleles have a higher risk of acute cellular rejection than those with *CYP3A4* *1/*1 [51]. Although the evidence shows that SIR-based immunosuppressant combination therapy can reduce the incidence of acute renal allograft rejection episodes compared to other immunosuppressant groups [51, 52], the effect of SNPs on drug concentrations can also affect the incidence of AR. Patients with drug concentrations below the therapeutic limit due to polymorphism would not experience a preventative effect on the incidence of acute organ rejection.

However, further functional research is needed to confirm these genes. Moreover, the molecular mechanism of the effect of the *CYP3A4* *1G genotype on drug metabolism activity in the liver is unknown. As the *CYP3A4* * 1G genotype is an intronic SNP, the SNP molecular effects are at the level of mature *CYP3A4* or *CYP3A5* expression. The *CYP3A4* *1G genotype is associated with *CYP3A5* mRNA expression rather than *CYP3A4* [48, 51]. Nevertheless, a molecular mechanistic explanation for clarifying the role of *CYP3A4* *1G phenotype in the function of *CYP3A5* is still required. Therefore, the molecular relationship of the *CYP3A4* *1G and *CYP3A5* *3 genotypes requires further research on several drug substrates other than SIR.

Our single center retrospective cohort study is limited by the relatively small sample size for measuring genetic polymorphism only the trough concentration of SIR in the blood. Although our study involved a relatively small number of samples, we were able to identify multiple SNPs that could potentially influence the blood levels of SIR.

CONCLUSION

The large inter-individual differences in SIR trough concentration might be partly explained by genetic factors. We demonstrate that a strong correlation exists between *CYP3A4 (*Ch7: 99361466 C>T, rs2242480) and SIR dose requirement in long-term renal transplant patients treated with SIR-based therapy. Patients carrying the *CYP3A4 (*Ch7: 99361466 C>T, rs2242480) homozygote CC genotype require significantly less SIR to achieve adequate blood trough concentrations.

CURRENT & FUTURE DEVELOPMENT

This study will be expanded by a broader and independent prospective patient population to validate the multiple genes to the specific SIR PK profiles *in vitro* or *in vivo*. This can yield detailed results related to the identification of novel SNP candidates in gene variants involved in SIR PK. The identification of these SNP candidates will be useful in determining the PK profile and dosage regimen for applying genomic-based therapy.

LIST OF ABBREVIATION

ABCB = ATP-binding Cassette Sub Family B

ANOVA = Analysis of Variance

AR = Acute Rejection
BID = Bis in Die
BMI = Body Mass Index
Chr = Chromosome
CsA = Cyclosporine
CYP450 = Cytochrome P-450
DGF = Delayed Graft Function
DNA = Deoxyribonucleic Acid
EDTA = Ethylene Diamine Tetraacetic Acid
gDNA = Genomic DNA
GLMs = General Linear Models
HIV = Human Immunodeficiency Virus
HSD = Hydroxysteroid Dehydrogenases
HWE = Hardy-weinberg Equilibrium
IL-10 = Interleukin 10
LD = linkage Disequilibrium
MAF = Minor Allele Frequency
MMF = Mycophenolate Mofetil
NR = Nuclear Receptor
PK = Pharmacokinetic
POR = P-450 Oxidoreductase
PPARA = Peroxisome Proliferator Activated Receptor Alpha
Pred = Prednisone
PXR = Pregnane X Receptor
RLU = Relative Light Unit
Scr = Serum Creatinine
SD = Standard Deviation
SIR = Sirolimus
SNPs = Single Nucleotide Polymorphisms
TAC = Tacrolimus
TS = Target Sequencing
UG/T = Uridine 5′-diphospo-glucuronosyl Transferase
UTR = Untranslated Region

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocols followed were approved by the local ethics committee of the First Affiliated Hospital with Nanjing Medical University (reference no: 2016-SR-029). The procedures followed in our study were in accordance with the ethical standards of the Declarations of Helsinki and Istanbul. We obtained written informed consent from all transplant recipients.

HUMAN AND ANIMAL RIGHTS

All manuscripts reporting data involving participants, formal review, and procedures followed in our study were approved by the First Affiliated Hospital with Nanjing Medical University institutional review board in accordance with the ethical standards of the Declarations of Helsinki principles.

CONSENT FOR PUBLICATION

Consent from all participants has been obtained prior to publishing.

AVAILABILITY OF DATA AND MATERIALS

Genetic expression files are posted on the Sequence Read Archive (SRA; url: https://www.ncbi.nlm.nih.gov/sra database (Accession: PRJNA432695, ID:432695).

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material isavailable on the publisher’s web site along with the published article.

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