Received: 15 April 2022 | Revised: 3 July 2022 | Accepted: 3 August 2022

DOI: 10.1111/cts.13388

ARTICLE

Genomewide association study identifies a novel variant associated with tacrolimus trough concentration in Chinese renal transplant recipients

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Funding information
Guangdong Basic and Applied Basic Research Foundation, Grant/Award Number: 2019A1515010100; Science and Technology Projects in Guangzhou, Grant/Award Number: 202201011752; Southern Medical University Innovation Training Program for Undergraduate Students, Grant/Award Number: S202112121123 and S202212121153

Abstract

Tacrolimus (TAC) is an immunosuppressant widely used in kidney transplantation. TAC displays considerable interindividual variability in pharmacokinetics (PKs). Genetic and clinical factors play important roles in TAC PKs. We enrolled a total of 251 Chinese renal transplant recipients and conducted a genomewide association study (GWAS), linkage disequilibrium (LD), and one-way analysis of variance (ANOVA) to find genetic variants affecting log-transformed TAC trough blood concentration/dose ratio (log[C_0/D]). In addition, we performed dual luciferase reporter gene assays and multivariate regression models to evaluate the effect of the genetic variants. The GWAS results showed that all 23 genomewide significant single-nucleotide polymorphisms ($p < 5\times10^{-8}$) were located on chromosome 7, including *CYP3A5*3*. LD, conditional association analysis, and one-way ANOVA showed that rs75125371 T > C independently influenced TAC log(C_0/D). Dual luciferase reporter gene assays indicated that rs75125371 minor allele (C) was significantly associated with increased normalized luciferase activity than the major allele (T) in the Huh7 cells ($p = 1.2\times10^{-5}$) and HepaRG cells ($p = 0.0097$). A model inclusive of age, sex, hematocrit, *CYP3A5*3*, and rs75125371 explained 37.34% variance in TAC C_0. These results suggest that rs75125371 T > C is a functional and population-specific variant affecting TAC C_0 in Chinese renal transplant recipients.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Tacrolimus (TAC) is an immunosuppressant widely used in kidney transplantation. Genetic and clinical factors lead to considerable interindividual variability in TAC pharmacokinetics (PKs). TAC PKs vary among recipients of different

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populations because of different variants by ancestry. There is still a large portion of TAC PK variability yet to be explained.

WHAT QUESTION DID THIS STUDY ADDRESS?

Can any population-specific variants underlie the unexplained TAC PK variability in Chinese renal transplant recipients? How much effect do they exert on TAC PK variability?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

All variants reaching genomewide significant association with TAC trough blood concentration (C_0) were located within or around the *CYP3A* loci and rs75125371 was a novel functional and population-specific variant affecting TAC C_0 in Chinese renal transplant recipients.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

These results show the rs75125371 should be taken into consideration for better personalized TAC dose in Chinese renal transplant recipients.

INTRODUCTION

Tacrolimus (TAC), also known as FK506, is an immunosuppressant widely used after solid organ transplantation, such as kidneys, liver, heart, and bone marrow transplantation.[1] TAC, classified as a calcineurin inhibitor (CNI), can inhibit calcineurin (CaN)-mediated phosphorylation, and block the activation and proliferation of T lymphocytes, thus exerting its immunosuppressive effect.[2] TAC has a narrow therapeutic index and wide-ranging interindividual variability in pharmacokinetics (PKs).[3] Over the last two decades, TAC has become the primary immunosuppressant used for allogenic renal transplantation. This is mainly due to its ability to improve renal transplant outcomes by increasing graft survival rates, reducing the frequency of acute rejection episodes, and improving renal function.[4] However, along with the benefits, the use of TAC also brings with its side effects, such as nephrotoxicity, neurotoxicity, hypertension, and post-transplant diabetes mellitus.[5] To alleviate these side effects, it is recommended for renal transplant recipients to monitor the blood trough concentration (C_0) of TAC-therapeutic drug monitoring (TDM): given an initial dose, then monitor the TAC C_0 every 2 or 3 days and adjust the dose according to the TAC C_0 until the target concentration range is reached.[6] TDM is crucial during this period, as unstable drug concentrations may cause severe side effects. A better understanding of personalized optimal initial dose of TAC based on genetic and clinical factors affecting the TAC PK variability could help to improve the outcomes of renal transplantation.[7,8]

TAC is mainly metabolized by the cytochrome P450 (CYP 450) 3A subfamily members, which mainly include CYP3A4, CYP3A5, and CYP3A7.[9,10] Several studies have demonstrated that the splicing variant *CYP3A5*3* (rs776746) that causes alternative splicing and protein truncation is associated with the higher TAC blood concentration in renal transplantation recipients.[11,12] Compared with the nonexpressers (*CYP3A5*3/*3*), the TAC initial dose of the expressers (*CYP3A5*1/*1* and *CYP3A5*1/*3*) needs to be increased by 1.5 to 2 times.[13] We and others found that *CYP3A5*3* and clinical variables can only explain part of TAC C_0 variability.[14,15] In addition, genetic variants, such as *CYP3A4*1B* (rs2740574),[16] *CYP3A4*1G* (rs2242480),[17,18] and *CYP3A4*22* (rs35599376)[12,19–22] were reported to be associated with TAC concentration. However, these genetic variants cannot fully explain the large interindividual TAC PK variability, thus there is still a large portion of TAC PK variability yet to be explained.

The genomewide association study (GWAS) has contributed to better our understanding of the genetic mechanisms of complex disease phenotypes.[23] In order to identify novel variants that account for the unexplained TAC C_0 variability, several studies have used GWAS to analyze the association between genetic variants and TAC PK in African American,[24] White,[22] Korean,[14] and diverse populations,[25] including adult and pediatric renal transplant recipients. In addition, Liu et al.[26] conducted a GWAS to analyze the association between exonic variants and TAC PKs in Chinese patients who underwent liver transplantation using exome chips. In these GWAS, *CYP3A5*3* was the top GWAS single-nucleotide polymorphism (SNP) or had high linkage disequilibrium (LD) with the top GWAS SNPs. These studies also identified several other *CYP3A* variants such as *CYP3A5*6* (rs10264272), *CYP3A5*7* (rs41303343), and *CYP3A4*22* (rs35599367), which can affect TAC PKs. However, the minor allele frequency (MAF) of these three variants is quite low (MAF < 1% in

the Han Chinese in Beijing (CHB) population, data from 1000 Genomes Project Phase III) in the Chinese population. Thus, the three *CYP3A* variants cannot further explain the TAC PK variability in the Chinese population. Due to the differential genetic architecture existing between populations, there might be some common and specific genetic variants besides *CYP3A5*3* that are associated with TAC C_0 in the Chinese population. However, no GWAS study has been conducted to analyze the effect between common variants and TAC C_0 in the Chinese adult renal transplant recipients. Here, we used a GWAS approach to evaluate the contribution of common genetic variants to TAC C_0 in 251 Chinese adult renal transplant recipients.

MATERIALS AND METHODS

Study cohort

This study cohort included Chinese renal transplant recipients at Nanfang Hospital from 2015 to 2020. The inclusion criteria were as follows: age ≥18 years; receiving TAC for maintenance immunosuppression; available TAC concentration, dose, and clinical information during hospitalization after transplantation. The exclusion criteria were as follows: age <18 years, second renal transplantation, liver and renal transplantation, systemic lupus erythematosus on long-term hormone therapy, and receiving drugs that had potential influence on TAC concentration. Finally, a total of 251 Chinese renal transplant recipients were included in this study. Genomic DNA was obtained from the peripheral blood of patients and extracted using the phenol-chloroform and TIANamp Blood DNA Kit (Tiangen Biotech). Written informed consent was obtained from all participants before study inclusion. This study was approved by the Ethical Committee of Nanfang Hospital and registered at www.ClinicalTrials.gov (NCT03083769).

Immunosuppressant regimens and clinical parameters

All renal transplant recipients in our study cohort received a triple immunosuppressive regimen composed of TAC, mycophenolate mofetil (MMF), and corticosteroid. The recipients were intravenously given 1000 mg methylprednisolone at the time of transplantation, then decreasing to 500, 250, and 250 mg on days 1, 2, and 3 after transplantation, respectively. On day 4, the recipients were treated with 30 mg of oral prednisolone, which was then decreased by 5 mg every week until a maintenance dose of 5 mg per day was reached. The recipients were orally administered TAC for the first time about 12 h after transplantation, and the initial dosage was calculated according to the weight of the patient (0.10–0.15 mg/kg body weight per day, twice a day), and further adjusted according to TAC C_0. The MMF was given orally 1000–1500 mg per day, twice a day. The TAC C_0 was routinely measured every 2–4 days after transplantation by enzyme multiplied immunoassay technique using the Viva-E instrument (Siemens AG). The stable maintenance TAC dose (D) was defined as the dose which patients received >2 consecutive days and TAC C_0 reached the target therapeutic range (10–12 ng/ml), and the TAC C_0 did not deviate from the range of 9–14 ng/ml during the following period. The dose would not be changed and considered to be the stable maintenance tacrolimus dose. During this period, the C_0 was standardized for dose and body weight ($C_0/D = C_0$/[dose/body weight]) using the corresponding 24-h dose on a mg/kg basis and the log-transformed TAC C_0/D ($\log(C_0/D)$) of renal transplant recipients were used as representative ratio parameters for statistical analysis. Detailed medical records were retrospectively collected, including age, sex, body weight, clinical characteristics, TAC daily dose, and TAC C_0.

GWAS genotyping and data quality control

Genotyping was performed using an East Asian-specific Affymetrix SNP chip: CBT_PMRA chip (CapitalBio Technology). This chip contains more than 773,000 SNPs, including ~630,000 common SNPs covering East Asian populations, and nearly 20,000 pharmacogenomics variants related to drug absorption, distribution, metabolism, and excretion. Data quality control was carried out with PLINK software (version 1.90). After excluding SNPs with the following criteria: genotype call rate <95%; MAF <1%; Hardy–Weinberg Equilibrium p value $<1\times10^{-4}$, the total number of SNPs analyzed from the SNP chip was 512,440. The Affymetrix SNP array raw data has been deposited in Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196772).

Genomewide association analysis and linkage disequilibrium analysis

Genotyping and data quality control were conducted as previously described. Genomewide association between 512,440 SNPs and TAC C_0 was analyzed using PLINK software (version 1.90). The Manhattan plot and QQ plot were

used to visualize the results of association analysis using the R 4.0.3 with qqman package. Pairwise LD values were estimated using Haploview version 4.2. A scatter plot was drawn with $-\log_{10}(p)$ as the X axis and LD R^2 as the Y axis to find possible SNPs which had low LD with other SNPs and significant GWAS p value with TAC $\log(C_0/D)$.

SNP genotyping

For SNPs not included in the GWAS chip, we performed the genotyping of these SNPs using high-resolution melting assays (Tiangen Biotech Co. Ltd.) and DNA Sanger sequencing.

Dual luciferase reporter gene assays

We amplified the predicted promoter or enhancer regions where the candidate SNPs were located within and cloned them into firefly luciferase reporter vectors (pGL3-Basic) to investigate the effect of the predicted regions on downstream gene expression. We used the WashU Epigenome Browser (http://epigenomegateway.wustl.edu/) to load the H3K4me1, H3K27ac, H3K4me3, and H3K9ac histone marks within 2 kb upstream and downstream of the candidate SNPs in the adult liver tissue and selected the histone marks' regions as the insertion fragments. The fragments were amplified and cloned into pGL3-Basic vectors, and the plasmids without inserted fragments were used as negative controls. The cloned firefly luciferase reporter vectors were transfected into the HEK-293 T cells, Huh7 cells, and HepaRG cells together with the renilla luciferase reporter vector (pRL-TK) as the internal control via the Lipofectamine 2000 Reagent (Invitrogen). Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) 36–48 h after transfection. Each experiment was repeated three times and contained three replicate wells per treatment. Sanger sequencing was performed on all plasmids to confirm sequences. Primer sequences for generating reporter plasmids are shown in Table S1.

Statistical analysis

Statistical analysis was performed using the software R version 4.0.3, the IBM SPSS Statistic 22 (SPSS), and GraphPad Prism 5 (https://www.graphpad.com/). The log-transformed blood C_0 normalized by body weight (W) and TAC dose (D) during the stable period was set as representative parameter for further statistical analysis. Genomewide association between 512,440 SNPs and TAC $\log(C_0/D)$ was analyzed using PLINK software (version 1.90). Linear regression model was used to test for associations between TAC $\log(C_0/D)$ and genotypes, age, sex, body mass index (BMI), and hematocrit (HCT) were included as covariates in this analysis using PLINK software. Genomewide significance was declared with association p value $<5\times10^{-8}$. The association between candidate SNP genotype and the representative parameter $\log(C_0/D)$ was analyzed using one-way analysis of variance (ANOVA) and the R package ggplot2 was used to create box-and-whisker plots. Unpaired t-tests were performed for luciferase reporter gene assays. A two-side p value <0.05 was considered statistically significant. A multivariate regression analysis was conducted to estimate the effect of clinical factors, *CYP3A5*3*, and candidate SNPs on the TAC $\log(C_0/D)$. The dummy variables were used as the category variables in the multivariate regression models.

RESULTS

Demographics of cohort

The study cohort included 251 renal transplant recipients from Nanfang Hospital, of these 168 (66.93%) were men. Demographics, TAC PK parameters, and clinical characteristics of the study cohort are shown in Table 1.

TABLE 1 Characteristics of the Chinese renal transplant recipients ($n = 251$)

Clinical characteristics	Mean ± SD
Age, years	42.32 ± 11.02
Sex, male/female, *n*	168/83
Weight, kg	59.28 ± 11.19
BMI, kg/m^2	21.50 ± 3.35
Dose, mg/day	8.38 ± 2.96
C_0, ng/ml	11.28 ± 1.82
$\log(C_0/D)$, ng/ml/mg/kg	1.92 ± 0.19
Hematocrit ‰	313.94 ± 46.75
Hemoglobin g/L	103.00 ± 15.51
Albumin g/L	39.22 ± 18.04
Alanine aminotransferase U/L	26.41 ± 26.37
Aspartate aminotransferase U/L	18.70 ± 11.27
Total bilirubin μmol/L	9.27 ± 3.80
Direct bilirubin μmol/L	3.54 ± 2.26
Indirect bilirubin μmol/L	5.82 ± 2.92
Serum creatinine μmol/L	182.64 ± 195.50

Abbreviations: BMI, body mass index; C_0, trough blood concentration; $\log(C_0/D)$, log-transformed TAC blood trough concentration normalized by dose and weight.

The mean age was 42.32 ± 11.02 years. The mean BMI was 21.50 ± 3.35 kg/m^2. The stable blood C_0 was 11.28 ± 1.82 ng/ml, and the maintenance TAC dose was 8.38 ± 2.96 mg/day. The mean log(C_0/D) of renal transplant recipients was 1.92 ± 0.19. Histograms showed that the TAC C_0/D exhibited a right-skew distribution, after logarithmic transformation, TAC log(C_0/D) exhibited a normal distribution (Figure S1).

Genomewide association study

We conducted a GWAS of 251 Chinese renal transplant recipients to find SNPs associated with TAC log(C_0/D). A total of 512,440 SNPs passed quality control and were included in the GWAS using the linear regression model, including age, sex, BMI, and HCT as covariates. The Manhattan plot revealed that all SNPs with a genomewide significance ($p < 5 \times 10^{-8}$) were located on chromosome 7 (Figure 1). The QQ plot is shown in Figure S2. Genomewide significant SNPs are shown in Table 2. The most significant SNPs were rs776746 (*CYP3A5*3*), rs2740565 and rs2257401 ($p = 1.169 \times 10^{-15}$). From our previous study, the rs776746 (*CYP3A5*3*) and rs2257401 (*CYP3A7*2*) variants were found to be significantly associated with TAC log(C_0/D).[25] The other top SNPs included rs4646450, and the variant was also found to be significantly associated with TAC C_0 in previous studies.[27,28] SNPs with p value <0.05 are shown in Table S2.

Linkage disequilibrium analysis and one-way ANOVA

The LD values among 23 SNPs with $p < 5 \times 10^{-8}$ on chromosome 7 were estimated, and rs75125371 was one of the SNPs that had relatively low LD values with other SNPs, including *CYP3A5*3* (Figure 2a). In addition, rs75125371 had a low p value (9.664×10^{-9}) with TAC log(C_0/D) and deviated from the linear regression line in Figure 2b. According to the regional association plot of *CYP3A* locus (Figure S3A), the LD R^2 value between rs75125371 and *CYP3A5*3* was smaller compared with other SNPs with significant p values at the genome level. By using age, sex, BMI, HCT, and *CYP3A5*3* as covariates, rs75125371 was the only SNP that showed significance ($p = 0.0265$) with TAC log(C_0/D) (Table 2). Figure S3B showed that rs917153 and rs141579238 had high LD ($R^2 > 0.7$) with rs75125371 within 500 kb upstream and downstream of rs75125371 in the CHB population. We conducted a linear regression model, including age, sex, BMI, HCT, and *CYP3A5**3 as covariates to analyze the association among the three SNPs and TAC log(C_0/D). The result shows that all three SNPs had significant association with TAC log(C_0/D) ($p < 0.05$; Table S3). However, WashU

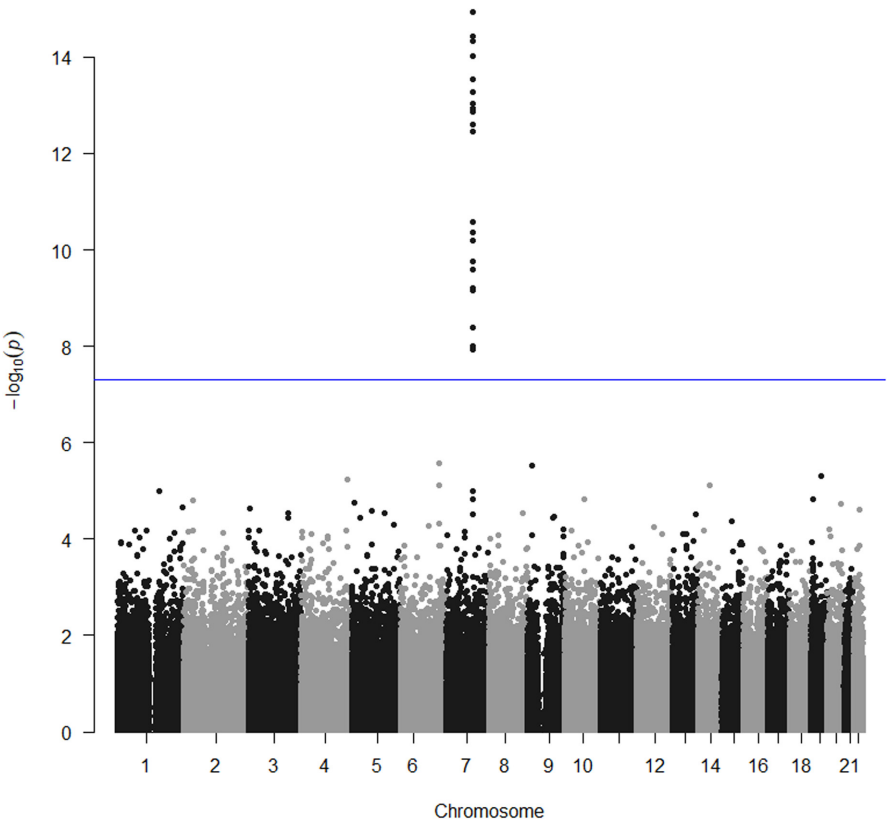

FIGURE 1 Manhattan plot of single-nucleotide polymorphisms associated with TAC log(C_0/D). All expression quantitative trait locus are shown in order from chromosome 1–22. The horizontal line indicates a p value of 5×10^{-8}.

TABLE 2 Significant SNPs ($p < 5 \times 10^{-8}$) and effect sizes associated with TAC log(C_0/D) in Chinese renal transplant recipients

Chr	Position (GRCh38.p13)	SNP	Gene	Effect allele	Non-effect allele	MAF	HWE, p	Call rate, %	Beta[a]	*p* value[a]	Beta[b]	*p* value[b]
7	99672916	rs776746	*CYP3A5*	T	C	0.2948	0.88	100	−0.1278	1.169E-15	NA	NA
7	99695852	rs2740565	*CYP3A51P*	A	T	0.2948	0.88	100	−0.1278	1.169E-15	NA	NA
7	99709062	rs2257401	*CYP3A7*	C	G	0.2948	0.88	100	−0.1278	1.169E-15	NA	NA
7	99668695	rs4646450	*CYP3A5*	A	G	0.2928	0.7605	100	−0.127	3.633E-15	NA	NA
7	99725451	rs2687139	*CYP3A7*	A	C	0.2928	1	100	−0.125	4.545E-15	NA	NA
7	99648291	rs15524	*CYP3A5*	G	A	0.3127	0.7689	100	−0.1229	9.383E-15	−0.02269	0.6525
7	99642556	rs10242455	–	G	A	0.3108	0.7699	100	−0.121	2.827E-14	−0.006813	0.8864
7	99647457	rs4646457	*CYP3A5*	C	A	0.314	1	99.6016	−0.1187	5.219E-14	0.005127	0.9144
7	99617051	rs115435341	*ZSCAN25*	T	G	0.25	1	98.805	−0.1252	9.489E-14	−0.01681	0.6443
7	99629549	rs1859690	*ZSCAN25*	G	A	0.3307	0.5687	100	−0.1166	1.155E-13	−0.01927	0.5799
7	99662739	rs4646453	*CYP3A5*	A	C	0.264	0.8708	99.6016	−0.1206	1.403E-13	−0.01134	0.7684
7	99647390	rs4646458	*CYP3A5*	G	T	0.282	0.8763	99.6016	−0.1195	2.494E-13	−0.01943	0.5305
7	99677460	rs4646446	*CYP3A5*	T	C	0.258	1	99.6016	−0.1217	3.594E-13	−0.005276	0.8936
7	99450809	rs2280600	*PTCD1*	C	T	0.3534	0.1263	99.2032	−0.1076	2.651E-11	−0.01593	0.5317
7	99484107	rs6962772	*ZNF394*	G	A	0.3506	0.1275	100	−0.1064	4.407E-11	−0.01161	0.6444
7	99379564	rs11762273	*ARPC1B*	G	A	0.3531	0.1612	97.61	−0.1065	6.553E-11	−0.03092	0.1472
7	99758352	rs3735451	*CYP3A4*	C	T	0.3008	0.8818	100	−0.1029	1.738E-10	0.03912	0.2142
7	99756491	rs12333983	*CYP3A4*	A	T	0.304	0.8811	99.6016	−0.1027	2.588E-10	0.03591	0.2465
7	99606030	rs10225314	*TMEM225B*	C	T	0.3016	0.7628	98.406	−0.1011	6.339E-10	−0.008303	0.7295
7	99434835	rs940336	*PTCD1*	T	C	0.3207	0.1114	100	−0.102	6.882E-10	−0.01777	0.4177
7	99356240	rs17161692	*ARPC1A*	T	C	0.356	0.1312	99.6016	−0.09512	3.986E-09	−0.02257	0.2466
7	99730059	rs75125371	*CYP3A7*	C	T	0.1116	0.7492	100	−0.1389	9.664E-09	−0.05675	0.02653
7	99321293	rs73709641	*ARPC1A*	T	C	0.3	0.2277	99.6016	−0.0954	1.143E-08	−0.02875	0.1096

Abbreviations: BMI, body mass index; HCT, hematocrit; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; NA, not applicable; SNP, single-nucleotide polymorphism; TAC log(C_0/D), log-transformed tacrolimus trough blood concentration/dose ratio.

[a]Beta and *p* value of SNPs associated with TAC log(C_0/D) in the linear regression model including age, sex, BMI, and HCT as covariates.

[b]Beta and *p* value of SNPs associated with TAC log(C_0/D) in the linear regression model including age, sex, BMI, HCT, and *CYP3A5*3* (rs776746) as covariates.

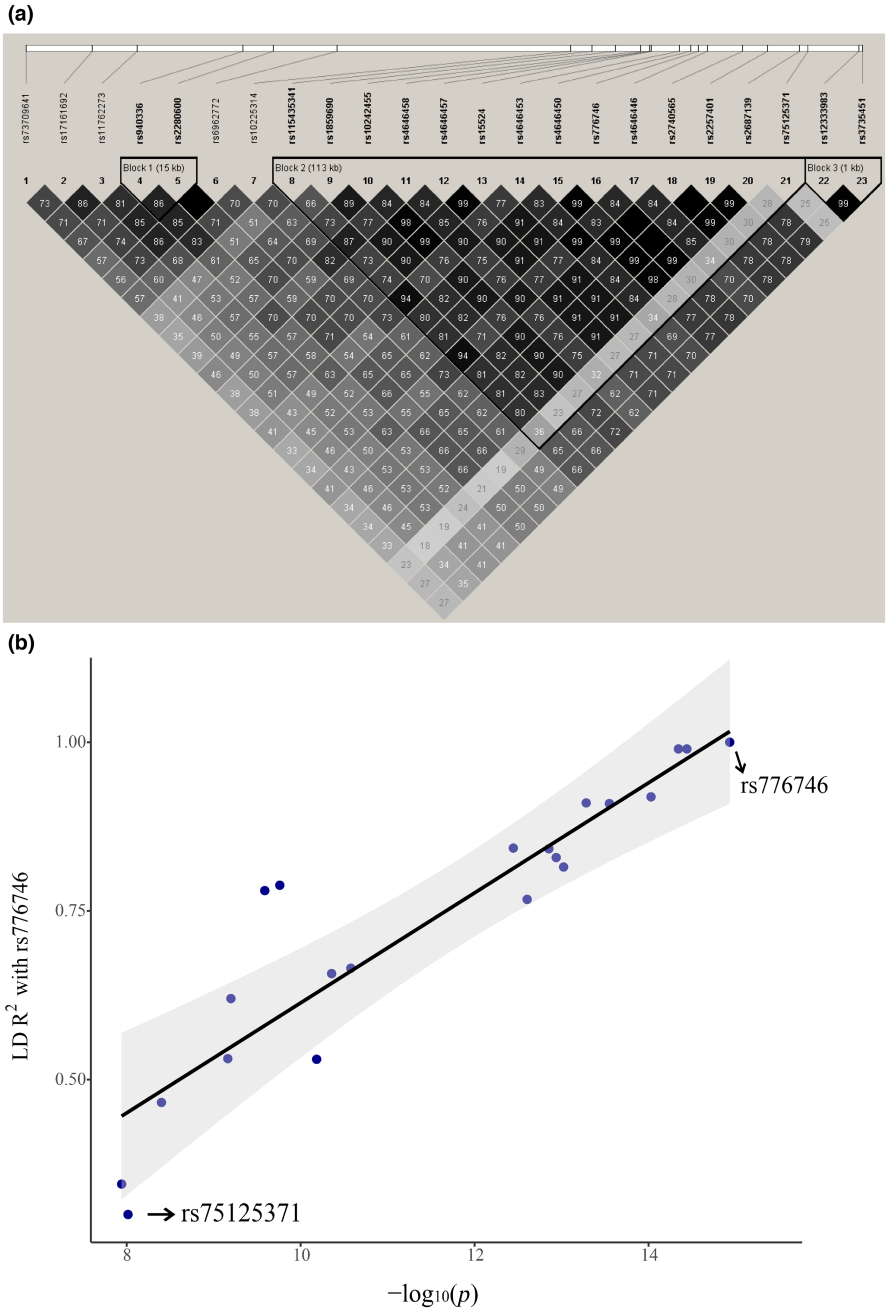

FIGURE 2 LD structure of 23 SNPs with $p < 5 \times 10^{-8}$ on chromosome 7. (a) Pairwise LD values estimated based on genotype data of renal transplant recipients. The R^2 value between every two SNPs was shown in the matrix. (b) The *X* axis represented negative log-transformed *p* value, and the *Y* axis represented the LD R^2 between each SNP and rs776746. LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

Epigenome Browser shows that only rs75125371 locates in the histone marks' regions in the adult liver tissue. Therefore, rs75125371 may be a functional variant that is independent of *CYP3A5*3*. Thus, rs75125371 was selected as the candidate SNP for further investigation. One-way ANOVA revealed that the functional variant *CYP3A5*3* (rs776746) was significantly associated with higher TAC blood concentration ($p = 3.54 \times 10^{-6}$; Figure 3a). To analyze whether rs75125371 influenced TAC log(C_0/D) independent of *CYP3A5*3*, TAC log(C_0/D) among the three genotypes of rs75125371 in *CYP3A5* different genotypes was compared using one-way ANOVA. The Box-and-whisker plot (Figure 3b) showed that the rs75125371 TT group had significantly higher TAC log(C_0/D) than the CT group or CC group among the three *CYP3A5* genotype groups. The *p* values were 0.011, 0.024, and 0.023, respectively. The results indicated that rs75125371 influenced TAC log(C_0/D) independent of *CYP3A5*3*.

Impact of rs75125371 in luciferase expression

Histone marks on rs75125371 were analyzed in liver tissues, and the genomic region carrying hepatocellular-specific histone marks was cloned into the pGL3-Basic vector (Figure S4). The constructed plasmids were verified as successfully cloned without other variants except rs75125371 by DNA sequencing. For constructs with

rs75125371, two types of constructs (carrying major allele [T] or minor allele [C], respectively) showed increased luciferase activity compared to the negative control in Huh7 cells, HepaRG cells, and HEK-293T cells (Figure 4). The construct containing the rs75125371 minor allele (C) had significantly greater luciferase activity than that of the major allele (T) in Huh7 cells ($p = 1.2\times10^{-5}$) and HepaRG cells ($p = 0.0097$; Figure 4a,b), and had greater luciferase activity than that of the major allele (T) ($p = 0.0587$) in HEK-293T cells (Figure 4c).

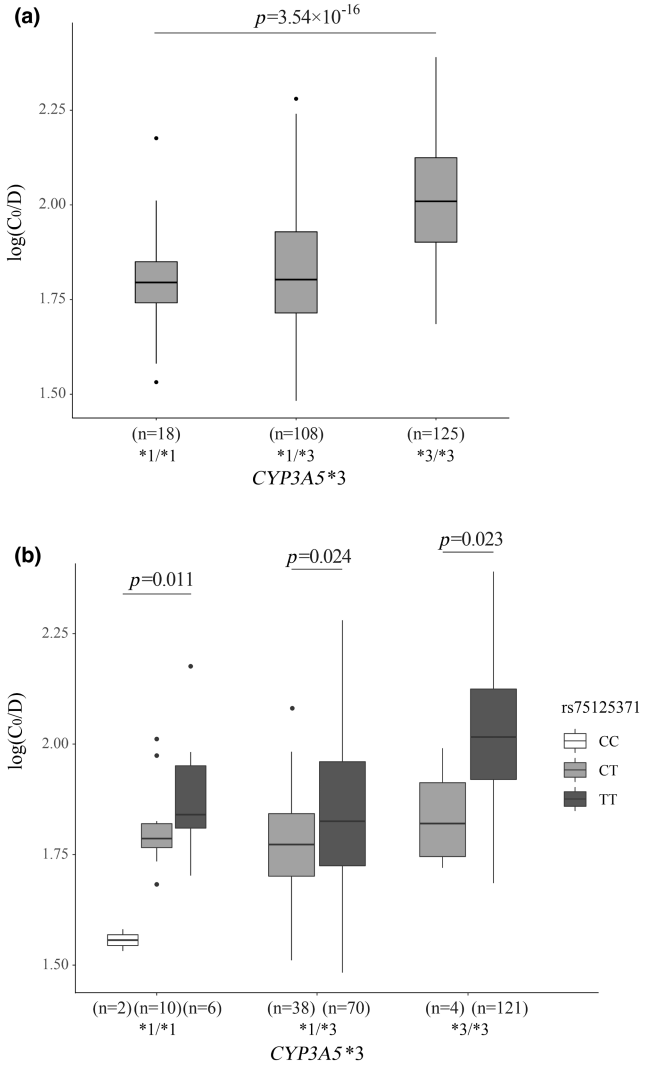

FIGURE 3 Box plots and one-way analysis of variance analysis of TAC log(C_0/D) among different genotypes of *CYP3A5*3* and rs75125371. (a) TAC log(C_0/D) among the *CYP3A5*1/*1*, **1/*3* and **3/*3* groups. (b) TAC log(C_0/D) among the three genotypes of rs75125371 in different *CYP3A5* genotypes.

Multivariate regression models

Using multivariate regression analysis, the effect of clinical covariates (age, sex, HCT, HGB, ALB, ALT, AST, DBIL, IBIL, and Cr), *CYP3A5*3* and rs75125371 on TAC log(C_0/D) were analyzed. The results indicated that age, sex, HCT, *CYP3A5*3*, and rs75125371 were significantly associated with TAC log(C_0/D) (p values were 1.09×10^{-4} for age, 9.78×10^{-5} for sex, 0.019 for HCT, 1.57×10^{-8} for *CYP3A5*3*, and 0.0035 for rs75125371 T > C), and other covariates have no significant association with TAC log(C_0/D). The estimates of variance in the TAC log(C_0/D) associated with the clinical covariates, *CYP3A5*3*, and rs75125371 T > C are shown in Table 3. The clinical covariates, including age, sex, and HCT, accounted for 17.89% of the variance in TAC log(C_0/D), *CYP3A5*3* accounted for 17.23% of the variance, rs75125371 T > C accounted for 2.22% of the variance, and a total of 37.34% variance was explained by age, sex, HCT, *CYP3A5*3*, and rs75125371 (Table 3).

DISCUSSION

TAC has a narrow therapeutic index and wide-ranging interindividual PK variability.[29] Reaching target TAC concentrations is critical for renal transplant recipients to reduce the risk of side effects. Genetic and clinical factors play important roles in PK variability of TAC.[30] A series of *CYP3A* variants, such as *CYP3A5*3*, **6*, **7*, *CYP3A4*22*, and *CYP3A7*2*, were discovered to be significantly associated with TAC C_0 in different studies.[11,20,27,31] However, clinical factors and *CYP3A* variants cannot fully explain

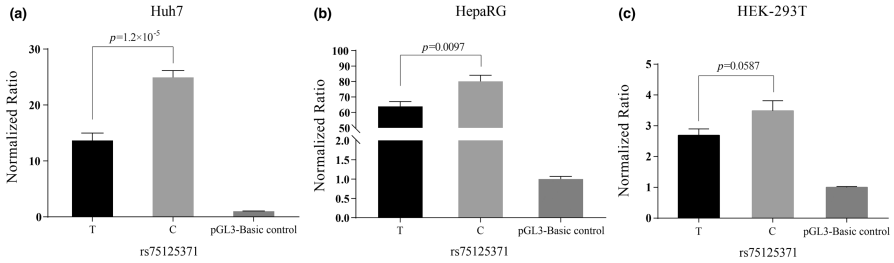

FIGURE 4 Luciferase Reporter gene activity assays. (a) Normalized ratio of luciferase activity in the rs75125371 T, C, and control groups in the Huh7 cells. (b) Normalized ratio of luciferase activity in the rs75125371 T, C, and control groups in the HepaRG cells. (c) Normalized ratio of luciferase activity in the rs75125371 T, C, and control groups in the HEK-293 T cells.

TABLE 3 Clinical covariates and genetic variants contributing to the variation of TAC log(C_0/D) in Chinese renal transplant recipients

Multivariate regression model (Enter)	R^2	R^2 change	Age	Sex	HCT	rs776746 (log-additive)	rs75125371 (log-additive)
log(C_0/D) ~ Age	11.03%	11.03%	0.332[a] (7.08×10^{-8}[b])				
log(C_0/D) ~ Age + Sex	15.76%	4.73%	0.330[a] (3.96×10^{-8}[b])	0.217[a] (2.37×10^{-4}[b])			
log(C_0/D) ~ Age + Sex + HCT	17.89%	2.14%	0.318[a] (9.41×10^{-8}[b])	0.201[a] (6.19×10^{-4}[b])	0.148[a] (1.19×10^{-2}[b])		
log(C_0/D) ~ Age + Sex + HCT + *CYP3A5*3*	35.12%	17.23%	0.224[a] (3.09×10^{-5}[b])	0.201[a] (1.26×10^{-4}[b])	0.132[a] (1.13×10^{-2}[b])	0.426[a] (2.87×10^{-14}[b])	
log(C_0/D) ~ Age + Sex + HCT + *CYP3A5*3* + rs75125371(T > C)	37.34%	2.22%	0.206[a] (1.09×10^{-4}[b])	0.202[a] (9.78×10^{-5}[b])	0.121[a] (1.91×10^{-2}[b])	0.344[a] (1.57×10^{-8}[b])	0.174[a] (3.53×10^{-3}[b])

Abbreviations: HCT, hematocrit; TAC log(C_0/D), log-transformed tacrolimus trough blood concentration/dose ratio.

[a]Beta.

[b]*p*-value.

TAC PK variability. In addition, TAC dose-normalized C_0s vary significantly among different ancestry groups.[32] The results indicate that there may be other ethnic-specific variants in the Chinese population that affect TAC blood concentrations. Therefore, we conducted a GWAS to evaluate the contribution of the common SNPs to TAC C_0 in Chinese renal transplant recipients.

Of the 512,440 SNPs, only 23 SNPs reached the genomewide significant level ($p < 5\times10^{-8}$; Table 2), and they were all located within or around the *CYP3A* loci. In addition to *CYP3A* loci, other studies also found some SNPs in genes encoding TAC transporter, and *CYP3A* transcriptional regulators to be associated with TAC blood concentration. These included P-glycoprotein efflux transporter (*ABCB1*) *ABCB1* 3435C>T (rs1045642),[33] cytochrome p450 oxidoreductase (*POR*) *POR**28 (rs1057868),[34] pregnane X receptor (*NR1I2*) *NR1I2* 8055C>T (rs2276707),[35] and peroxisome proliferator activated receptor alpha (*PPARA*) *PPARA* SNP rs4253728.[36] However, we found that these SNPs were not significantly associated with TAC log(C_0/D) in our study cohort. In the other three reviewed GWASs, the genetic variants which reached the genomewide significant threshold were also located on chromosome 7.[14,22,24] The results reveal that the common SNPs that affect TAC concentrations are mainly located within or around the *CYP3A* loci. The SNPs located outside of *CYP3A* loci on chromosome 7 that are significantly associated with TAC concentrations may be caused by partial and complete LD with functional variants in *CYP3A* loci.

In our study cohort, we further analyzed the 23 SNPs with $p < 5\times10^{-8}$ on chromosome 7 and found that rs75125371 T > C had a relatively low LD value ($R^2 = 0.30$) with *CYP3A5*3*. In addition, rs75125371 T > C had a low p value (9.66×10^{-9}) with TAC log(C_0/D). As a result, rs75125371 deviated from the linear regression line, as shown in Figure 2b. According to our previous study, age, sex, and HCT were significantly associated with TAC C_0.[27] Therefore, we used the linear regression model to analyze the association among the 23 SNPs and TAC log(C_0/D) by including age, sex, BMI, HCT, and *CYP3A5*3* as covariates. The results revealed that rs75125371 was the only SNP that showed significance ($p = 0.0265$) with TAC log(C_0/D) among the 23 SNPs (Table 2). Region association plot also showed that rs75125371 may not belong to the same set of functional variants as the other variants analyzed (Figure S3A). We investigated the SNPs in LD with rs75125371 in a 500 kb window upstream and downstream and found that rs917153 and rs141579238 had high LD ($R^2 > 0.7$) with rs75125371 (Figure S3B). The rs917153 and rs141579238 were not included in the GWAS chip, thus we genotyped the two SNPs in our cohort. The result showed that the rs917153 and rs141579238 also had high

LD with rs75125371 ($R^2 = 0.68$ for rs917153; $R^2 = 0.92$ for rs141579238) in our cohort. However, WashU Epigenome Browser shows that only rs75125371 locates in the histone marks' regions in the adult liver tissue. These reveal that rs75125371 may be a functional variant independent of *CYP3A5*3* that affects TAC $\log(C_0/D)$. Dual luciferase reporter gene assay showed that the construct containing the rs75125371 minor allele (C) had significantly higher luciferase activity than the major allele (T) in the Huh7 cells ($p = 1.2\times10^{-5}$) and HepaRG cells ($p = 0.0097$; Figure 4a,b). The luciferase reporter gene assay in HEK-293 T cells showed that the luciferase activity of rs75125371 C was higher with marginal significance ($p = 0.059$) than the major allele (T) (Figure 4C). In addition, rs75125371 C was associated with lower TAC $\log(C_0/D)$ (Figure 3b). These results reveal that rs75125371 C can increase the *CYP3A* gene expression. Taking into consideration that Huh7 cells and HepaRG cells are derived from hepatocyte cellular carcinoma and HEK-293 T cells are derived from human embryonic kidneys, the effect of rs75125371 C increasing *CYP3A* gene expression may be tissue-specific.

We conducted a multivariate regression analysis to evaluate the contribution of clinical factors and genetic variants to TAC C_0 variation. In our cohort, a model with clinical factors, *CYP3A5*3*, and rs75125371 explained 37.34% variance in TAC C_0/D (Table 3). Oetting et al. analyzed TAC trough concentrations in 1345 adult European American recipients and found that a model inclusive of clinical covariates, *CYP3A5*3*, and *CYP3A4*22* (rs35599367) explained 40.1% of the variance in TAC C_0.[22] The reason why our model explains a lesser degree of variation may be due to the differences in race and sample size between the two cohorts. In the study of Oetting et al.,[22] *CYP3A5*3* and *CYP3A4*22* together explained 16% of the total variance, and *CYP3A4*22* explained 3% variance of TAC C_0. In our study, *CYP3A5*3* and rs75125371 together explained 19.45% of the total variance, and rs75125371 explained 2.22% variance of TAC C_0.

In recent years, GWASs have identified *CYP3A* variants affecting TAC blood trough concentration, such as *CYP3A5*3* (rs776746), *CYP3A5*6* (rs10264272), *CYP3A5*7* (rs41303343), and *CYP3A5*22* (rs35599367).[22,24] However, studies and gnomAD version 3.1.2 data set reveal that except for *CYP3A5*3*, the MAF of the other three variants is quite low (<0.001) in east Asian population.[15,32,37] In our study cohort, the MAF of rs75125371 was 0.112. In East Asian, Latino/Admixed American, South Asian, African/African American, and European (non-Finnish), the MAF of rs75125371 is 0.12, 0.060, 0.0019, 0.0011, and 0.00025, respectively (data from gnomAD version 3.1.2 data set). In European (Finnish), Amish, Middle Eastern, and Ashkenazi Jewish, the MAF of rs75125371 is 0.000 (data from gnomAD version 3.1.2 data set). The results reveal that there are different common *CYP3A* variants in different populations that affect TAC C_0.

Our study had several limitations. First, GWASs using SNP arrays are restricted to common SNPs in the CHB population, some rare variants in the CHB population are likely to be missed.[38] Second, although the reporter gene assay indicates rs75125371 can affect gene expression, we are not sure which *CYP3A* expression is affected by rs75125371. Third, in our cohort, the clinical factors and genetic variants explained 37.34% variance in TAC C_0, further investigation is needed to analyze the unknown factors contributing to the unexplained portion of interindividual TAC PK variability.

In conclusion, our study analyzed the association between 512,440 common SNPs and TAC C_0 in 251 Chinese renal transplant recipients and found that rs75125371 was a population-specific variant affecting inter-individual variability in TAC C_0/D in the Chinese population. A model inclusive of clinical factors, *CYP3A5*3*, and rs75125371 explained 37.34% of the TAC PK variability. Further studies to discover and evaluate population-specific, common, and rare variants should be considered in order to incorporate these models and improve personalized TAC dosing regimen in clinical practice.

AUTHOR CONTRIBUTIONS

S.Y.Y., L.L., and N.M. wrote the manuscript. L.L. designed the research. S.Y.Y., C.C.L., and H.J.L. analyzed the data. S.Y.Y., C.C.L., H.J.L., H.X.J., D.M.Y., H.N.Q., W.B.X., X.J.B., S.Q.Z., and R.F.S. performed the research. L.L., H.X.J., and C.J.L. contributed new reagents/analytical tools.

ACKNOWLEDGMENTS

This study was approved by the Ethical Committee of Nanfang Hospital and registered at www.ClinicalTrials.gov (NCT03083769). The authors would like to give special thanks to all the renal transplant recipients involved in this study.

FUNDING INFORMATION

This study was supported by Guangdong Basic and Applied Basic Research Foundation (2019A1515010100, China), Science and Technology Projects in Guangzhou (202201011752, China), and Southern Medical University Innovation Training Program for Undergraduate Students (S202112121123 and S202212121153, China).

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

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REFERENCES

1. Fung JJ, Abu-Elmagd K, Todo S, et al. FK506 in clinical organ transplantation. *Clin Chem*. 1993;39(11 Pt 1):2219-2228.
2. Dumont FJ. FK506, an immunosuppressant targeting calcineurin function. *Curr Med Chem*. 2000;7(7):731-748.
3. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. *Clin Pharmacokinet*. 2004;43(10):623-653.
4. Webster A, Woodroffe RC, Taylor RS, Chapman JR, Craig JC. Tacrolimus versus cyclosporin as primary immunosuppression for kidney transplant recipients. *Cochrane Database Syst Rev*. 2005;4:CD003961.
5. Shrestha BM. Two decades of tacrolimus in renal transplant: basic science and clinical evidences. *Exp Clin Transplant*. 2017;15(1):1-9.
6. Yano I, Masuda S, Egawa H, et al. Significance of trough monitoring for tacrolimus blood concentration and calcineurin activity in adult patients undergoing primary living-donor liver transplantation. *Eur J Clin Pharmacol*. 2012;68(3):259-266.
7. Lancia P, Jacqz-Aigrain E, Zhao W. Choosing the right dose of tacrolimus. *Arch Dis Child*. 2015;100(4):406-413.
8. Vanhove T, Annaert P, Kuypers DR. Clinical determinants of calcineurin inhibitor disposition: a mechanistic review. *Drug Metab Rev*. 2016;48(1):88-112.
9. Danielson PB. The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. *Curr Drug Metab*. 2002;3(6):561-597.
10. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Therap*. 2013;138(1):103-141.
11. Kuehl P, Zhang J, Lin Y, et al. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet*. 2001;27(4):383-391.
12. Hesselink DA, Bouamar R, Elens L, van Schaik RH, van Gelder T. The role of pharmacogenetics in the disposition of and response to tacrolimus in solid organ transplantation. *Clin Pharmacokinet*. 2014;53(2):123-139.
13. Birdwell KA, Decker B, Barbarino JM, et al. Clinical pharmacogenetics implementation consortium (CPIC) guidelines for CYP3A5 genotype and tacrolimus dosing. *Clin Pharmacol Ther*. 2015;98(1):19-24.
14. Yoon JG, Song SH, Choi S, et al. Unraveling the genomic architecture of the CYP3A locus and ADME genes for personalized tacrolimus dosing. *Transplantation*. 2021;105(10):2213-2225.
15. Li CJ, Li L, Lin L, et al. Impact of the CYP3A5, CYP3A4, COMT, IL-10 and POR genetic polymorphisms on tacrolimus metabolism in Chinese renal transplant recipients. *PLoS One*. 2014;9(1):e86206.
16. Hesselink DA, van Schaik RH, van der Heiden IP, et al. Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. *Clin Pharmacol Ther*. 2003;74(3):245-254.
17. Miura M, Satoh S, Kagaya H, et al. Impact of the CYP3A4*1G polymorphism and its combination with CYP3A5 genotypes on tacrolimus pharmacokinetics in renal transplant patients. *Pharmacogenomics*. 2011;12(7):977-984.
18. Tamashiro EY, Felipe CR, Genvigir F, et al. Influence of CYP3A4 and CYP3A5 polymorphisms on tacrolimus and sirolimus exposure in stable kidney transplant recipients. *Drug Metab Pers Ther*. 2017;32(2):89-95.
19. Wang D, Guo Y, Wrighton SA, Cooke GE, Sadee W. Intronic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics J*. 2011;11(4):274-286.
20. Elens L, Bouamar R, Hesselink DA, et al. A new functional CYP3A4 intron 6 polymorphism significantly affects tacrolimus pharmacokinetics in kidney transplant recipients. *Clin Chem*. 2011;57(11):1574-1583.
21. Wang D, Sadee W. CYP3A4 intronic SNP rs35599367 (CYP3A4*22) alters RNA splicing. *Pharmacogenet Genomics*. 2016;26(1):40-43.
22. Oetting WS, Wu B, Schladt DP, et al. Genome-wide association study identifies the common variants in CYP3A4 and CYP3A5 responsible for variation in tacrolimus trough concentration in Caucasian kidney transplant recipients. *Pharmacogenomics J*. 2018;18(3):501-505.
23. Dehghan A. Genome-wide association studies. *Methods Mol Biol*. 2018;1793:37-49.
24. Oetting WS, Schladt DP, Guan W, et al. Genomewide association study of tacrolimus concentrations in African American kidney transplant recipients identifies multiple CYP3A5 alleles. *Am J Transplant*. 2016;16(2):574-582.
25. Min S, Papaz T, Lambert AN, et al. An integrated clinical and genetic prediction model for tacrolimus levels in pediatric solid organ transplant recipients. *Transplantation*. 2022;106(3):597-606.
26. Liu Y, Zhang C, Li L, et al. Genome-wide association study of tacrolimus pharmacokinetics identifies novel single nucleotide polymorphisms in the convalescence and stabilization periods of post-transplant liver function. *Front Genet*. 2019;10:528.
27. Chen D, Lu H, Sui W, et al. Functional CYP3A variants affecting tacrolimus trough blood concentrations in Chinese renal transplant recipients. *Pharmacogenomics J*. 2021;21(3):376-389.
28. Liu J, Ouyang Y, Chen D, et al. Donor and recipient P450 gene polymorphisms influence individual pharmacological effects of tacrolimus in Chinese liver transplantation patients. *Int Immunopharmacol*. 2018;57:18-24.
29. Yu M, Liu M, Zhang W, Ming Y. Pharmacokinetics, pharmacodynamics and pharmacogenetics of tacrolimus in kidney transplantation. *Curr Drug Metab*. 2018;19(6):513-522.
30. Wang P, Zhang Q, Tian X, Yang J, Zhang X. Tacrolimus starting dose prediction based on genetic polymorphisms and clinical factors in Chinese renal transplant recipients. *Genet Test Mol Biomarkers*. 2020;24(10):665-673.
31. Staatz CE, Goodman LK, Tett SE. Effect of CYP3A and ABCB1 single nucleotide polymorphisms on the pharmacokinetics

and pharmacodynamics of calcineurin inhibitors: part II. *Clin Pharmacokinet*. 2010;49(4):207-221.
32. Mohamed ME, Schladt DP, Guan W, et al. Tacrolimus troughs and genetic determinants of metabolism in kidney transplant recipients: a comparison of four ancestry groups. *Am J Transplant*. 2019;19(10):2795-2804.
33. Staatz CE, Goodman LK, Tett SE. Effect of CYP3A and ABCB1 single nucleotide polymorphisms on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors: part I. *Clin Pharmacokinet*. 2010;49(3):141-175.
34. Min SI, Kim SY, Ahn SH, et al. CYP3A5 *1 allele: impacts on early acute rejection and graft function in tacrolimus-based renal transplant recipients. *Transplantation*. 2010;90(12):1394-1400.
35. Metalidis C, Lerut E, Naesens M, Kuypers DR. Expression of CYP3A5 and P-glycoprotein in renal allografts with histological signs of calcineurin inhibitor nephrotoxicity. *Transplantation*. 2011;91(10):1098-1102.
36. Klein K, Thomas M, Winter S, et al. PPARA: a novel genetic determinant of CYP3A4 in vitro and in vivo. *Clin Pharmacol Ther*. 2012;91(6):1044-1052.
37. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581(7809):434-443.
38. Gibson G. Rare and common variants: twenty arguments. *Nat Rev Genet*. 2012;13(2):135-145.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Yang S, Jiang H, Li C, et al. Genomewide association study identifies a novel variant associated with tacrolimus trough concentration in Chinese renal transplant recipients. *Clin Transl Sci*. 2022;15:2640-2651. doi:10.1111/cts.13388