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

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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[C0/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 × 10−8) 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(C0/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 × 10−5) and HepaRG cells (p = 0.0097). A model inclusive of age, sex, hematocrit, CYP3A5*3, and rs75125371 explained 37.34% variance in TAC C0. These results suggest that rs75125371 T>C is a functional and population-specific variant affecting TAC C0 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...
GWAS OF TAC C0 IN CHINESE TRANSPLANT PATIENTS

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 (C0) of TAC-therapeutic drug monitoring (TDM): given an initial dose, then monitor the TAC C0 every 2 or 3 days and adjust the dose according to the TAC C0 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 C0 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 C0 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 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 (C0) were located within or around the CYP3A loci and rs75125371 was a novel functional and population-specific variant affecting TAC C0 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.
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](http://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 \( (C_0/D = \text{C}_0/\text{[dose/body weight]}) \) 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 <1×10\(^{-4}\), 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](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://epigenomemegateway.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 $<5 \times 10^{-8}$. 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/m² | 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/m². 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 (\( \text{CYP3A5*3} \)), rs2740565 and rs2257401 \( (p = 1.169 \times 10^{-15}) \). From our previous study, the rs776746 (\( \text{CYP3A5*3} \)) and rs2257401 (\( \text{CYP3A7*2} \)) variants were 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 \( \text{CYP3A5*3} \) (Figure 2a). In addition, rs75125371 had a low \( p \) value \( (9.664 \times 10^{-9}) \) with TAC \( \log(C_0/D) \) and deviated from the linear regression line in Figure 2b. According to the regional association plot of \( \text{CYP3A} \) locus (Figure S3A), the LD \( R^2 \) value between rs75125371 and \( \text{CYP3A5*3} \) was smaller compared with other SNPs with significant \( p \) values at the genome level. By using age, sex, BMI, HCT, and \( \text{CYP3A5*3} \) 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 \( \text{CYP3A5*3} \) 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 \( \log(C_0/D) \) \( (p < 0.05; \text{Table S3}) \). However, WashU

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**FIGURE 1** Manhattan plot of single-nucleotide polymorphisms associated with TAC \( C_0/D \). All expression quantitative trait locus are shown in order from chromosome 1–22. The horizontal line indicates a \( p \) value of \( 5 \times 10^{-8} \).
| Chr | Position (GRCh38.p13) | SNP    | Gene      | Effect allele | Non-effect allele | MAF    | HWE, p | Call rate, % | Betaa          | p valuea       | Betab         | p valueb     |
|-----|----------------------|--------|-----------|---------------|-------------------|--------|--------|--------------|----------------|---------------|--------------|--------------|
| 7   | 99672916             | rs776746 | CYP3A5    | T             | C                 | 0.2948 | 0.88   | 100          | −0.1278        | 1.169E-15     | NA           | NA           |
| 7   | 99693852             | rs2740565 | CYP3A51P  | A             | T                 | 0.2948 | 0.88   | 100          | −0.1278        | 1.169E-15     | NA           | NA           |
| 7   | 99705062             | 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          | −0.1278        | 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 | 0.88   | 100          | −0.121         | 2.827E-14     | −0.006813   | 0.8864       |
| 7   | 99647457             | rs4646457 | CYP3A5    | C             | A                 | 0.314  | 1      | 99.6016     | −0.1187        | 9.291E-14     | 0.005127    | 0.9144       |
| 7   | 99617051             | rs15435341 | 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 | 0.7699 | 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     | −0.1178        | 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     | −0.01610     | 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             | rs1233983 | 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   | 99434836             | 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   | 99730509             | 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(C₀/D), log-transformed tacrolimus trough blood concentration/dose ratio.

*aBeta and p value of SNPs associated with TAC log(C₀/D) in the linear regression model including age, sex, BMI, and HCT as covariates.

*bBeta and p value of SNPs associated with TAC log(C₀/D) in the linear regression model including age, sex, BMI, HCT, and CYP3A5*3 (rs776746) as covariates.
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 ($p = 1.2 \times 10^{-5}$) 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).

**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 $1.09 \times 10^{-4}$ for age, $9.78 \times 10^{-5}$ for sex, $0.019$ for HCT, $1.57 \times 10^{-8}$ 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. 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. 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. However, clinical factors and CYP3A variants cannot fully explain...
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) | $R^2$ change | Age | Sex | HCT | Sex + HCT | Age + Sex | Age + Sex + HCT | Age + Sex + HCT + CYP3A5*3 | Age + Sex + HCT + CYP3A5*3 + rs776746 | Age + Sex + HCT + CYP3A5*3 + rs75125371(T>C) |
|--------------------------------------|-------------|-----|-----|-----|-----------|----------|----------------|----------------------------|--------------------------------|-------------------------------------|
| $\log(C_0/D)$—Age                     | 0.320*      | 0.330* | 0.330* | 0.318* | 0.224* | 0.209* | 0.209* | 0.209* | 0.290* |
| $\log(C_0/D)$—Age + Sex              | 0.489*      | 0.476* | 0.476* | 0.463* | 0.329* | 0.214* | 0.214* | 0.214* | 0.204* |
| $\log(C_0/D)$—Age + Sex + HCT        | 0.426*      | 0.412* | 0.412* | 0.400* | 0.286* | 0.195* | 0.195* | 0.195* | 0.179* |
| $\log(C_0/D)$—Age + Sex + HCT + CYP3A5*3 | 0.468* | 0.455* | 0.455* | 0.443* | 0.319* | 0.218* | 0.218* | 0.218* | 0.198* |
| $\log(C_0/D)$—Age + Sex + HCT + CYP3A5*3 + rs776746 | 0.475* | 0.463* | 0.463* | 0.452* | 0.330* | 0.222* | 0.222* | 0.222* | 0.205* |
| $\log(C_0/D)$—Age + Sex + HCT + CYP3A5*3 + rs75125371(T>C) | 0.484* | 0.474* | 0.474* | 0.463* | 0.344* | 0.245* | 0.245* | 0.245* | 0.227* |

TAC PK variability. In addition, TAC dose-normalized $C_0$s vary significantly among different ancestry groups. 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 ($p < 5 \times 10^{-8}$; 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), cytochrome p450 oxidoreductase (POR) POR*28 (rs1057868), pregnane X receptor (NR1I2) NR1I2 8055C>T (rs2276707), and peroxisome proliferator activated receptor alpha (PPARA) PPARA SNP rs4253728. 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. 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 $p < 5 \times 10^{-8}$ 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 ($9.66 \times 10^{-9}$) 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 $C_0$. 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 (R² = 0.68 for rs917153; R² = 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(C⁰/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 x 10⁻⁵) 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(C⁰/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 C⁰ variation. In our cohort, a model with clinical factors, CYP3A5*3, and rs75125371 explained 37.34% variance in TAC log(C⁰/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 C⁰. 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., CYP3A5*3 and CYP3A4*22 together explained 16% of the total variance, and CYP3A4*22 explained 3% variance of TAC C⁰. In our study, CYP3A5*3 and rs75125371 together explained 19.45% of the total variance, and rs75125371 explained 2.22% variance of TAC C⁰.

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). 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. 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 C⁰.

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. 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 C⁰, further investigation is needed to analyze the unknown factors contributing to the unexplained portion of inter-individual TAC PK variability.

In conclusion, our study analyzed the association between 512,440 common SNPs and TAC C⁰ in 251 Chinese renal transplant recipients and found that rs75125371 was a population-specific variant affecting inter-individual variability in TAC C⁰/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.

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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.

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**CONFLICT OF INTEREST**

The authors declared no competing interests for this work.
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**SUPPORTING INFORMATION**

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

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