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PHARMACOGENETICS

Genetic and clinical determinants of mizoribine pharmacokinetics in renal transplant recipients

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Abstract

Aim Mizoribine (MZR) is an immunosuppressant for the prevention of allograft rejection in Asian countries, but the great variability in pharmacokinetics (PK) limits its clinical use. This study was to explore genetic and clinical factors that affect the MZR PK process.

Methods Blood samples and clinical data were collected from 60 Chinese renal transplant recipients. MZR plasma concentration was measured at pre-dose (0 h) and 0.5, 1, 2, 3, 4, 5, 6, 8, and 12 h post-dose by high performance liquid chromatography with an ultraviolet detector. PK parameters were calculated by non-compartmental analysis. High-throughput sequenced single nucleotide polymorphism was applied screening possible genetic factors.

Results Extensive inter-individual MZR PK differences were reflected in the process of elimination (k_e, CL/F, MRT and $t_{1/2}$) and intestinal absorption (C_{max} and T_{max}), as well as in the dose-normalized exposure (AUC_{0-12h}/D). From 146 SNPs within 39 genes screened, AUC_{0-12h}/D was found higher in recipients with *CREB1* rs11904814 TT than with G allele carriers (3.135 ± 0.928 versus 2.084 ± 0.379 μg h ml^{-1} mg^{-1}, $p = 0.007$). Recipients with *SLC28A3* rs10868138 TT had lower $t_{1/2}$ as compared to C allele carriers (0.728 ± 0.189 versus 0.951 ± 0.196 h, $p = 0.001$). Serum creatinine (SCr) explained 35.5% of C_0/D variability ($p < 0.001$). Pure effects of genotypes CREB1 and SLC28A3 were 13.7% ($p = 0.004$) and 17.5% ($p = 0.001$) for AUC_{0-12h}/D and $t_{1/2}$, respectively. When additionally taking SCr into models, *CREB1* and *SLC28A3* genotypes explained 20.0% ($p = 0.038$) and 46.5% ($p < 0.001$) of AUC_{0-12h}/D and $t_{1/2}$ variability, respectively.

Conclusion *CREB1* and *SLC28A3* genotypes, as well as SCr, are identified as determinants in predicting inter-individual MZR PK differences in renal transplant recipients.

Keywords Mizoribine · Pharmacokinetics · Gene polymorphism · Renal transplantation

Rui Dai and Jingjie Li contributed equally to this work.

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Introduction

Mizoribine (MZR) is an anti-metabolite immunosuppressant approved for the indication of preventing allograft rejection post-renal transplantation in China and some other Asian countries [1]. As compared to mycophenolic acid (MPA), recipients receiving high dose of MZR were demonstrated with no significant differences in 2-year graft survival rate and acute rejection rate after transplantation, while the incidence of cytomegalovirus (CMV) infection, leucopenia, and gastrointestinal disorders was significantly lower in the MZR treatment of recipients [2–4]. Thus, MZR has been recommended as an alternative immunosuppressant of MPA. MZR is commonly combined with cyclosporine A (CsA) or tacrolimus (TAC) to synergistically exert immunosuppressive effect by inhibiting different immune targets [5–7], and to our knowledge, there are no studies revealing the significant PK interaction between MZR and CsA or TAC.

After absorbed rapidly from intestine, MZR is not metabolized by the liver enzymes such as cytochrome P450s [8], and almost 85% of MZR dose is excreted into the urine in an unchanged form (data from rat) [9]. MZR has to be phosphorylated into an active form MZR 5′P by adenosine kinase (ADK) within the immune cells to exert immunosuppressive effect [1]. Moreover, the serum protein–binding rate is relatively low (approximately 2.3%) in human [10, 11]. Pharmacokinetics (PK) studies showed a highly inter-individual variability in MZR exposure. Our previous study enrolling 40 renal transplant recipients revealed that trough concentration (C_0) and area under the concentration–time curve (AUC) values of MZR varied in almost 10-fold range [12, 13]. Also this PK variability was found in other populations such as healthy volunteers and patients with kidney diseases [14–16].

Multiple factors affected MZR disposition in vivo. Among them, renal function plays an essential role. The creatinine clearance rate (CCr) and serum creatinine (SCr) was proved negatively correlated with apparent terminal half-life ($t_{1/2}$), peak time (T_{max}), peak concentration (C_{max}), and AUC of MZR in our previous study [12]. Besides, a population PK study suggested that intestinal absorption reflected by bioavailability was also responsible for the inter-individual PK differences of MZR [17]. Ihara and his colleagues reported that the bioavailability calculated from 24-h cumulative urinary excretion of MZR varied from 12 to 81% in renal transplant recipients [18] and 60.3 to 99.4% in healthy male volunteers [14, 16]. Drinking more water was demonstrated to increase the BA because of more dispersion of the hydrophilic drug from the tablet in the intestinal and the subsequent increased absorption by the intestinal epithelial cells [16]. Besides, BA of MZR was reported suppressed by the intake of food in humans and rats [19].

Pharmacogenetic studies of MZR only focused on the gene polymorphisms of nucleoside transporters [16, 20], as MZR is a purine nucleoside derivative that requires specific influx transport systems [21]. A study including 34 Japanese renal transplant recipients reported that the bioavailability was significantly lower in recipients with concentrative nucleoside transporters (CNTs) gene *SLC28A1 565-G/A* and *−A/A* alleles than those with *565-G/G* allele [20], and this phenomenon was confirmed in healthy Japanese males [14, 16]. But the *ABCG2 C421A* and *ABCC4 G2269A* polymorphisms did not significantly affect the inter-individual variability in bioavailability [14]. Additionally, P-gp is a well-known drug transporter, but there were no studies reporting the connection between P-gp and MZR concentration.

To improve the understanding of the influences of pharmacogenetic factors on MZR PK process in vivo, we took advantage of a high-throughput sequenced technology in renal transplant recipients to identify the possible single nucleotide polymorphisms (SNPs). A total of 146 SNPs within 39 genes covering MZR-related metabolism enzymes, transporters, and upstream regulators were included for the analysis in our current study. Furthermore, genetic factors were combined with clinical factors to explain more of the PK variability of MZR.

Methods

Patients and study design

This study enrolled a total of 60 Chinese adult inpatient recipients who underwent renal transplantation for the first time at Organ Transplantation Center, the First Affiliated Hospital, Sun Yat-sen University from March 1, 2016 to June 1, 2019. The inclusion criteria were as follows: (1) age between 18 and 60 years old, male or female; (2) primary disease is chronic glomerulonephritis; (3) receiving anti-thymocyte globulin or anti-CD25 monoclonal antibody as immune induction therapy; (4) those whose immunosuppressive therapy was initiated with calcineurin inhibitors (TAC or CsA) and steroid. The following exclusion criteria were implemented: (1) receiving multi-organ transplantation; (2) with concurrent active infection; (3) with history of malignant tumors over 5 years; (4) with other diseases such as mental illness, cardiac dysfunction, diarrhea or other severe gastrointestinal disorders prior to study initiation. MZR (Bredinin® 50 mg/Tablet, Asahi Kasei Pharma, Tokyo, Japan) was orally administered at 1.1–8.9 mg kg^{-1} day^{-1} (actual body weight, twice daily) to recipients on an empty stomach (2 h after meal), and the dose was adjusted according to serum concentration to reach the recommended therapeutic range (1–3 μg mL^{-1}) [10, 14] When MZR concentration achieved steady state (at least 7 days after MZR initiation), blood samples were collected at pre-dose (hour 0) and 0.5, 1, 2, 3, 4, 5, 6, 8, and 12 h post-dose, respectively. The demographic and biochemistry data were collected from a hospital database.

Determination of plasma concentration

Plasma samples were extracted from whole blood by being centrifuged at 800×*g* for 10 min and then frozen at − 20 °C until analysis. The calibration standards (0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 μg/mL) were prepared by separately spiking 10 μL of prepared working standard solution into 100 μL of blank plasma. Quality control (QC) samples were prepared using blank human plasma at concentrations of 0.075, 0.75, and 7.5 μg/mL. A total of 0.4 mL of acetonitrile was used for protein precipitation of spiked samples. The serum MZR concentration was determined using a validated high-performance liquid chromatographic with an ultraviolet detector described in our previous study [12, 22]. Waters™ HPLC system (Milford, MA, USA), equipped with a 600 pump, and a 486 UV-Vis absorbance detector were applied. Mizoribine (friendly obtained from Asahi Kasei Corp, Tokyo, Japan, purity ≥ 99%) and cytarabine (Hua Lian Pharmaceutical Corp, Shanghai, China, purity ≥ 99%) were used as standard and internal standard, respectively. The chromatographic separation was performed using Hypersil BDS C18 column (250 mm × 4.6 mm, 5 μm, Elite Scientific Instruments Co. Ltd., Dalian, China). The mobile phase was a mixture of 10 mM KH_2PO_4 buffer solution (pH 6.3) and 10 mM perchloric acid, at a flow rate of 1.5 mL min^{-1}. The ultraviolet detector was set at a measurement wavelength of 280 nm for MZR. The linear range was 0.02–10.0 μg mL^{-1}, and the lower limit of quantification was 0.02 μg mL^{-1} for MZR in serum.

Identification of genotypes

Genomic DNA was extracted from 200 μL whole blood by Baypure™ automatable magnetic bead extraction Kit (Bay Bio tech Corp, Guangzhou, China). The quantity and quality of genomic DNA were verified with a Multiskan™ FC microplate photometer (Thermo Fisher Scientific, Bremen, Germany) before being genotyped by the Sequenom MassARRAY™ system (Agena Bioscience, Inc., San Diego, CA, USA). All 158 detected SNPs conformed to MAF (mutant allele frequency) > 5% in Hap-Map HCB (Han Chinese in Beijing) (http://www.ncbi.nlm.nih.gov/SNP). Among the SNPs detected, two SNP loci (*CREB1* rs11904814 and *SLC28A3* rs10868138) showed significant influences on MZR PK parameters. The sequences of the forward and reverse primers were designed by Assay Design Suite™ online system (Agena Bioscience, Inc., San Diego, CA, USA). These PCR reactions were carried out 5 μL of solution consisting of 1 μL 0.5 μM Primer Mix, forward primer 5′-ACGTTGGATGGATAGTGTTGTGCATG TAAAG-3′ and reverse primer 5′-ACGTTGGATGGGCA AAGGACTATTGCTCAG-3′ for *CREB1* rs11904814, forward primer 5′-ACGTTGGATGTCAGCACACA GGCCGAAATC-3′ and reverse primer 5′-ACGT TGGATGCCCAGTGGTTAGTGATGTTC-3′ for *SLC28A3* rs10868138, 0.1 μL 25 mM dNTPs, 0.4 μL 25 mM $MgCl_2$, 1 U PCR enzyme, and 1 μL 10 ng of genomic DNA. PCR amplification starts with an initial denaturation at 95 °C for 2 min, continued with 45 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s; finally, extension was performed at 72 °C for 5 min in ABI GeneAmp™ PCR System 2700 (Applied Biosystems, Inc., San Francisco, Foster City, USA). Purified extension reaction products by iPLEX™ Gold Reagent Kit were spotted onto a 384-well Spectro CHIP™ and measured by using the platform MALDI-TOF mass spectrometry within the Sequenom MassARRAY™ genotyping system (all of the from Agena Bioscience, Inc., San Diego, CA, USA). Genotype calling was performed and analyzed by using the MassARRAY Typer software version 4.0.

Statistical analysis

Characteristic data were presented as median and range unless noted otherwise. CCr was calculated by the Cockcroft-Gault formula from body weight, age, sex, and SCr. The MZR PK parameters were computed basing on ten time points of blood concentration by non-compartmental analysis using Phoenix WinNonlin™ tool (version 7.0, Certara L.P Pharsight, St. Louis, MO, USA). The PK parameters included peak concentration (C_{max}), peak time (T_{max}), terminal half-life ($t_{1/2}$), first-order terminal elimination rate constant (k_e), area under the concentration–time curve from 0 to 12 h ($AUC_{0–12h}$), apparent total body clearance (CL/F), apparent volume of distribution (V/F, calculated as $CL/F/k_e$), and mean residence time (MRT). AUC was estimated using linear trapezoidal rule method. Ultimately, 146 candidate SNPs were identified conforming with Hardy–Weinberg equilibrium ($p > 0.05$) via SNP state (https://www.snpstats.net/). Comparisons of pharmacokinetic parameters between two or among more genotypic groups were performed using the non-parametric models, the Mann–Whitney *U* test, and the Kruskal–Wallis test, respectively. The non-parametric Spearman's correlation coefficient was used to test for significant correlation between clinical covariance and PK parameters. Normality was tested using the Shapiro-Wilk test. C_0/D, $AUC_{0–12h}/D$, and $t_{1/2}$ were not normally distributed; thus, a logarithmic transformation was used to linearize the data for regression analysis. Multilinear regression analysis was performed by stepwise method to quantify the influences of clinical factors and SNPs. All statistical tests used IBM SPSS software (version 25.0, Armonk, NY) and two tailed; *p* values below 0.05 were regarded as statistical

significance. Prism 8.0 (GraphPad Software, La Jolla, CA) was used for further photographing.

Results

Patient characteristics

Sixty subjects (37 male and 23 female) who had both DNA and PK profiles were enrolled, and their characteristics are shown in Table 1. All patients received triple immunosuppressive regimen including TAC (46 subjects)/CsA (14 subjects), glucocorticoid, and MZR under conditions of either BK virus or CMV infection or transformed from MPA because of adverse drug reactions such as diarrhea, hepatic injury, or pneumonia. MZR was administrated based on actual body weight, and median dosage was 3.0 mg kg^{-1} day^{-1} (interquartile range 2.1–3.7 mg kg^{-1} day^{-1}). C_0 were observed from 0.17 to 4.29 μg mL^{-1}, and among them, 27 individuals (45.0%) located in 1.0 to 3.0 μg mL^{-1} of therapeutic range, 13 individuals (21.7%) lower than 0.5 μg mL^{-1}, 17 individuals (28.3%) in 0.5 to 1.0 μg mL^{-1}, and 3 individuals (5.0%) beyond the up limitation (Fig. 1). Dose-corrected trough concentration (C_0/D) also exhibited variability (0.11 to 3.60 μg kg mL^{-1} mg^{-1}). Clinical factors used for the following MZR PK determinants analysis are also listed in Table 1.

Table 1 Demographics of subjects

Clinical characteristics	Median (range)
Gender (M/F)	37/23
Age (years)	37 (20–66)
Body weight (kg)	52.5 (39.0–95.0)
Height (cm)	165.0 (150.0–185.0)
Post-renal transplant day (days)	305 (8–3258)
Tacrolimus/cyclosporine A	46/14
Mizoribine daily dosage (mg kg^{-1} day^{-1})	3.0 (1.1–8.9)
Serum creatinine (μmol L^{-1})	157.0 (83.0–580.0)
Creatinine clearance rate (mL min^{-1})	43.36(12.75–92.76)
Uric acid (mmol L^{-1})	361.0 (124.0–742.0)
Alanine aminotransferase (U L^{-1})	11.0 (5.0–62.0)
Aspartate aminotransferase (U L^{-1})	18.0 (7.0–56.0)
Alkaline phosphatase (g L^{-1})	75.0 (44.0–554.0)
Total bilirubin (μmol L^{-1})	8.8 (3.7–19.9)
Serum total protein (g L^{-1})	66.1 (50.2–83.1)
Serum albumin (g L^{-1})	39.3 (28.6–49.9)
Plasma globulin (g L^{-1})	24.4 (15.8–45.8)
Hematocrit (%)	0.34 (0.23–0.51)

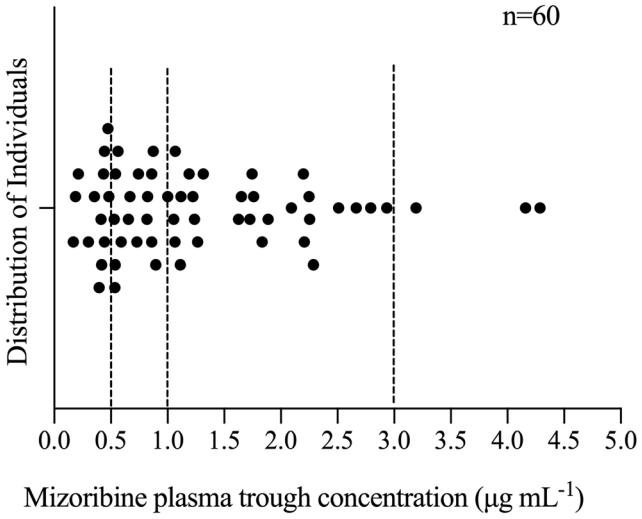

Fig. 1 Distribution of mizoribine plasma trough concentration in 60 renal transplant recipients

Pharmacokinetics characteristics

Extensive inter-individual differences of PK parameters are shown in Table 2. There were considerable variabilities not only in elimination (k_e, CL/F, MRT and $t_{1/2}$), but also in intestinal absorption (C_{max} and T_{max}), as well as in the integral dose-normalized exposure (AUC_{0-12h}/D).

Identification of genetic factors

A total of 146 SNPs within 39 genes that are potentially related to PK and PD characteristics of MZR were screened for the identification of genetic factors. PK variables of MZR are mainly influenced in the steps of absorption and excretion. So, the transporters related to MZR absorption, such as CNTs, ENTs, P-gp, BCRP2, and MRP2 (encoded by *SLC28A*, *SLC29A*, *ABCB1*, *ABCG2*, and *ABCC2*), and the transporters related to excretion, such as OATs and OATPs (encoded by *SLC22A*, *SLCOT*), were selected in our analysis. Besides, the upstream nuclear transcription factors that regulate the genes above, such as HNF4α, CREB1, EGF, LXRα/β, Nedd4–2, were also enrolled in our extensive screen assays. For PD, MZR is phosphorylated into an active form of MZR 5′P by adenosine kinase (ADK) and then the MZR 5′P competitively inhibits two vital enzymes required in GMP synthesis pathway, inosine monophosphate dehydrogenase (IMPDH), and guanosine monophosphate synthetase (GMP-synthetase) [1]. Thus, the metabolic enzymes including ADK, IMPDH1, and GMPS were enrolled. All SNPs comfort with mutant allele frequency (MAF) > 5% in Hap-Map HCB. Additionally, the exome region (coding sequences) and missense mutation were selected in priority.

The enrolled 146 SNPs were included to investigate the association with MZR C_0/D, AUC_{0-12h}/D, $t_{1/2}$, CL/F, and V/F. Each

Table 2 Pharmacokinetic parameters of mizoribine in renal transplant recipients

Pharmacokinetic parameters	Median (range)	Mean (95%Confidence Interval)
C_0 (μg mL^{-1})	1.03 (0.17–4.29)	1.28 (1.03–1.52)
C_0/D (μg kg mL^{-1} mg^{-1})	0.30 (0.11–3.60)	0.50 (0.36–0.66)
AUC_{0-12} (μg h ml^{-1})	18.28 (4.61–75.26)	21.93 (18.43–25.42)
AUC_{0-12}/D (μg h ml^{-1} mg^{-1} kg^{-1})	0.776(0.115–9.053)	1.236(0.858–1.613)
$t_{1/2}$ (h)	5.1 (2.9–21.1)	6.7 (5.7–7.6)
CL/F (L h^{-1})	3.58 (0.33–14.29)	4.91 (4.05–5.76)
V/F (L)	37.26 (7.21–151.88)	44.72 (36.99–52.46)
T_{max} (h)	4.0 (2.0–6.0)	3.7 (3.4–3.9)
C_{max} (μg mL^{-1})	2.51 (0.63–7.05)	2.64 (2.28–3.01)
MRT(h)	8.7 (4.8–51.5)	11.5 (9.4–13.7)
Actual k_e (h^{-1})	0.13 (0.02–0.24)	0.12 (0.11–0.14)

candidate SNP was analyzed using optimal genetic models. SNP screening outcome (frequency distribution and Hardy–Weinberg equilibrium p value) is listed in Table S1. Finally, two SNPs, *CREB1* rs11904814 and *SLC28A3* rs10868138, were displayed significantly correlated with AUC_{0-12h}/D and $t_{1/2}$, respectively (Figs. 2 and 3). Their frequency distributions are shown in Table 3. $AUC_{0-12\ h}$/D in rs11904814 TT allele group was significantly higher than those in the TG and GG group (3.135 ± 0.928 versus 2.084 ± 0.379 μg h ml^{-1} mg^{-1}, $p = 0.007$). $t_{1/2}$ in the rs10868138 TT allele group was significantly lower than those in TC and CC group (0.728 ± 0.189 versus 0.951 ± 0.196 h, $p = 0.001$). And pure effects of genotypes *CREB1* rs11904814 and *SLC28A3* rs10868138 can explain 13.7% ($p = 0.004$) and 17.5% ($p = 0.001$) for AUC_{0-12h}/D and $t_{1/2}$ in univariate analysis, respectively (Table S2).

Determinants of mizoribine pharmacokinetics

Univariate analysis was carried out to evaluate the possible variables significantly affecting MZR PK parameters from 17 clinical factors as listed in Table 1, and we demonstrated that 8 variables including age, height, SCr, CCr, alanine aminotransferase (ALT), alkaline phosphatase (ALP), hematocrit (Ht), and uric acid (UA) were significantly correlated with both C_0/D and AUC_{0-12h}/D ($p < 0.05$), while only SCr and CCr were significantly correlated with $t_{1/2}$ ($rs = 0.380$, $p = 0.003$ and $rs = -0.321$, $p = 0.012$). The results are shown in Table S3.

In the following, a mixed-effect model was established by the combination of the abovementioned genetic and clinical factors to investigate the determinants of MZR PK (Table 4). The estimated effect indicates the change in dependent variable that is expected to occur with a 1-unit increase in (or presence of) the predictor variable, when all other predictors are held constant. Standardized coefficient is indicated to relatively compare the impacts between independent variables. With respect to MZR $logC_0$/D, only SCr explained 35.5% of inter-individual variability ($p < 0.001$), while no significant impact was found in other variables. Per 100 μmol L^{-1} increase in SCr, the increased magnitude of $logC_0$/D was 20.0% (1.58-fold increase in C_0/D). The final model of $logAUC_{0-12h}$/

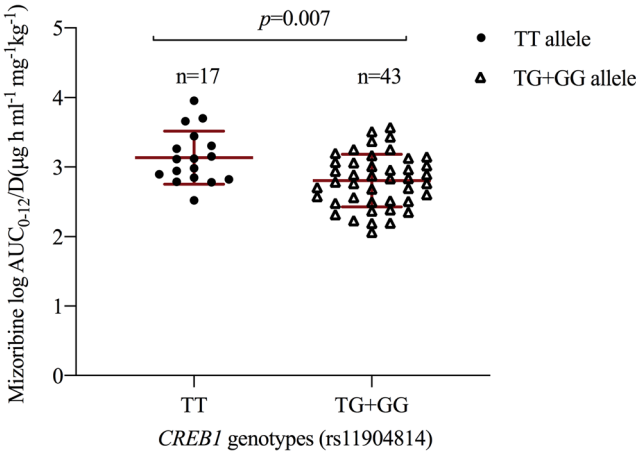

Fig. 2 Comparison of mizoribine log-transformed does-corrected area under the concentration–time curve between recipients with *CREB1* rs11904814 genotypes. The internal solid line represents the mean value and error line represents standard deviation

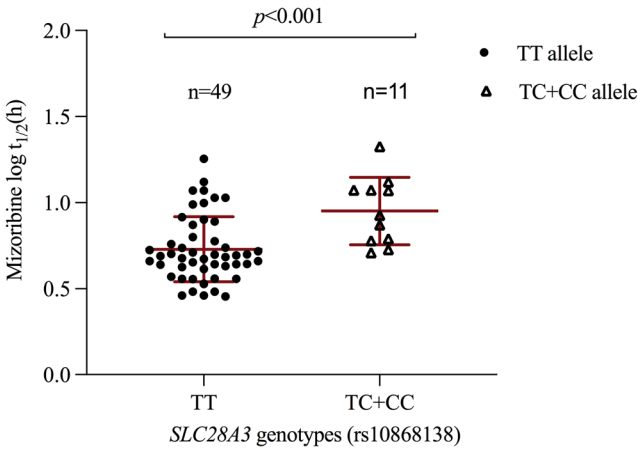

Fig. 3 Comparison of log-transformed mizoribine terminal half-life between recipients with *SLC28A3* rs10868138 genotypes. The internal solid line represents the mean value and error line represents standard deviation

Table 3 Genotype frequencies of *CREB1*-rs11904814 and *SLC28A3*-rs10868138

Gene	SNP	Location	Genotype	Number	Frequency (%)
CREB1	rs11904814	Intronic	TT	17	28
			TG	34	57
			GG	9	15
SLC28A3	rs10868138	non-synonymous	TT	49	82
			TC	10	17
			CC	1	2

D involving SCr and *CREB1* rs11904814 genotype explained 20.0% of inter-individual variability ($p = 0.038$). Per 100 μmol L^{-1} of SCr increase was predicted to result in a 10% increase in $\log AUC_{0-12h}/D$ (1.26-fold increase in AUC_{0-12}/D). Simultaneously, $\log AUC_{0-12h}/D$ in G allele carriers of rs11904814 decreased by 33.1% ($p = 0.004$) as compared to that in T homozygote individuals, indicating AUC_{0-12}/D of TT allele patients decreased by 2.14-fold. The conjunction of SCr with *SLC28A3* rs10868138 genotype can elucidate 46.5% of variability in $\log t_{1/2}$ ($p < 0.001$). Ten percent of increase in $\log t_{1/2}$ was also found when SCr changed, while C allele carriers of rs10868138 increased by 18.5% ($p = 0.003$) in $\log t_{1/2}$ compared to T homozygote subjects, indicating 1.53-fold decrease of $t_{1/2}$ in TT allele patients. Model fits in $\log AUC_{0-12h}/D$ and $\log t_{1/2}$ between predicted and observed values are shown in Fig. 4.

Discussion

In the present study, we demonstrated a large PK variance of MZR in Chinese renal transplant recipients, and only 45% of patients with C_0 located in the recommended therapeutic range (1–3 μg mL^{-1}) [10, 14]. By high-throughput sequenced method, *CREB1* rs11904814 and *SLC28A3* rs10868138 polymorphisms were identified significantly correlated with AUC_{0-12h}/D and $t_{1/2}$, respectively. When taking both genetic and clinical factors into MZR PK parameter models, SCr alone explained 35.5% of MZR C_0/D variance, while the models including rs11904814 and SCr explained 20% of AUC_{0-12h}/D variance, rs10868138 and SCr explained 46.5% of $t_{1/2}$ variant. To our knowledge, this is the first study to systematically evaluate the impacts of the genetic and clinical factors on MZR PK characteristics in renal transplant recipients.

For MZR, AUC has been reported well correlated with C_0 [12]; thus, C_0 is a suitable index for the MZR concentration monitoring in clinical practice. But in our study, 50% of patients had C_0 below 1 μg mL^{-1} (recommended therapeutic range is 1–3 μg mL^{-1} [10, 14]), indicating that the MZR dose 2–3 mg kg^{-1} day^{-1} according to current package insert in China may not provide sufficient drug exposure. Actually, the reported therapeutic range of C_0 was established based on higher dose of MZR (6–8 mg kg^{-1} day^{-1}). Multicenter studies in Japanese renal transplant recipients demonstrated that MZR above 6 mg kg^{-1} day^{-1} is necessary for providing effective immunosuppression while lowering the rate of CMV infection [23, 24]. Thus, whether current immunosuppressive regimen including low dose of MZR in China would guarantee the MZR efficacy and safety needs to be further confirmed.

To fully explain MZR PK variance, we detected 146 SNPs within 39 genes and found that only *CREB1*-rs11904814 T/C could alter the MZR AUC_{0-12}/D. CREB1 is a member of the cyclic adenosine monophosphate (cAMP) response element

Table 4 Predictors of mizoribine pharmacokinetics

Pharmacokinetic parameters	Predictors	Estimated effect	Fold[b]	Standardized coefficients	95% CI	*p* value	R square
C_0/D	SCr (μmol L^{-1})	20.0%[a]	1.58		20.0–30%	< 0.001	0.355
AUC_{0-12}/D	SCr (μmol L^{-1})	10.0%[a]	1.26	0.251	1–20.0%	0.038	0.200
	CREB1-rs11904814 (TT = 1,TG + GG = 2)	− 33.1%	2.14	− 0.371	− 54.3 to (− 11.9%)	0.003	
$t_{1/2}$	SCr (μmol L^{-1})	10.0%[a]	1.26	0.544	10.0–20.0%	< 0.001	0.465
	SLC28A3-rs10868138 (TT = 1,TC + CC = 2)	18.5%	1.53	0.346	8–28.9%	0.001	

[a] The change magnitude of log-transformed dependent variables per 100 μmol L^{-1} increase in SCr

[b] The change folds of dependent variables without log-transformed per 100 μmol L^{-1} increase in SCr or with/without TT genotype in rs11904814 and rs10868138

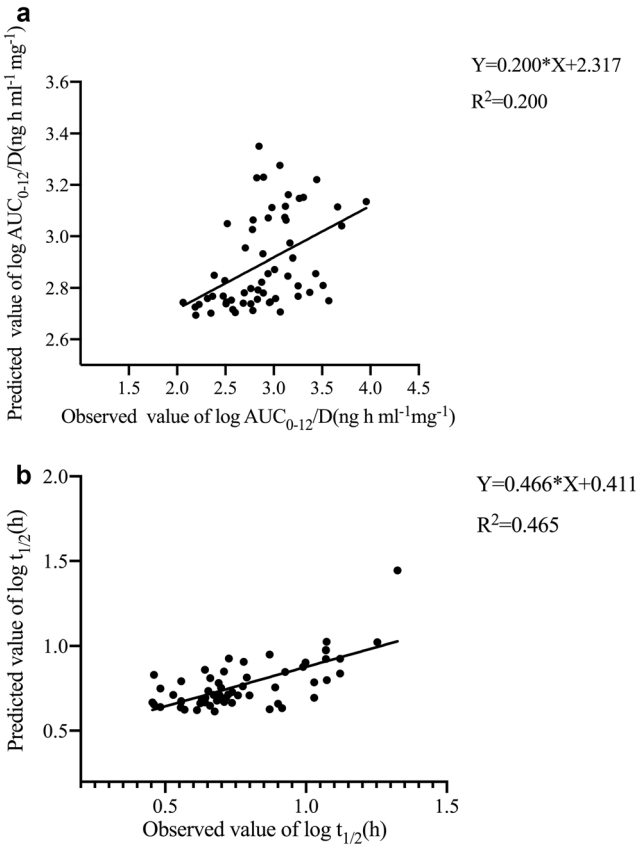

Fig. 4 Overall fit of linear mixed model for mizoribine log-transformed dose-corrected area under the concentration–time curve (**A**) and terminal half-life (**B**)

binding–activating transcription factor (CREB–ATF) family, which binds as a homodimer to the cAMP-responsive element (CRE) in the cAMP-AMPponsive element (CRE) in the cAMPcAMPscription factor (CREThe protein is phosphorylated by several protein kinases and then stimulates downstream cellular gene expression) [25–28]. *CREB1* functional polymorphism plays a critical role in many diseases including major depression, kidney injury of diabetes, and hypertension [26–29]. It was presumed that *CREB1* affected MZR absorption by inducing downstream signals including nucleoside transporters (NTs) [30–32]. NT family predominantly mediates intestinal cellular uptake of natural nucleosides and derivative drugs used in cancer chemotherapy and treatment of viral infections. NTs consist of two evolutionarily unrelated human protein families: concentrative nucleoside transporters (CNTs) encoded by *SLC28A*, with three subtypes hCNT1, hCNT2, and hCNT3, and equilibrative nucleoside transporters (ENTs) encoded by *SLC29A*, also with three subtypes hENT1, hENT2, and hENT3 [33]. Both CNTs and ENTs are distributed in the intestine, kidney, lung, and some other tissues with different affinity and variant in substrate selectivity [34]. It was reported that CNT3 activity was upregulated by CREB at the luminal side of cholangiocytes [30], and CREB might also regulate protein expression of ENT1 as binding sites of CREB found in the 5′-flanking region of ENT1 [31]. MZR is a highly hydrophilic nucleoside analog absorbed rapidly depending on the specific membrane nucleoside transporters [21]. Although no direct studies demonstrated that CNT3 and ENT1 mediated the intestinal transport of MZR, CNT3 was reported pharmacologically relevant to translocate 6-mercaptopurine (6-MP), which has analogous structure with MZR [35]. Meanwhile, the intestinal absorption of MZR could be significantly suppressed by gemcitabine in the mechanism of competitively binding ENT1 [19, 36]. Thus, it is reasonable to assume that *CREB1* polymorphism could have responsibility for the absorption of MZR and further affect the drug exposure in the body.

Interestingly, no impact of *CREB1* rs11904814 polymorphism was found on MZR C_0/D. We infer that C_0 of MZR depends more on renal function, since it is a drug predominantly eliminated via renal excretion. Although CREB1 influenced the MZR absorption process, the effect might be overwhelmed by the variance of MZR renal clearance. In our study, CL/F of MZR was 332.2–14,289.8 mL h^{-1}, showing great inter-individual variance. Actually, MZR bioavailability has been reported to be affected by *SLC28A1 G565A* in both renal transplant recipients and healthy male volunteers, but also, no significant difference was observed in C_0 between the *G565A* genotypes [16, 20], which was consistent with our result.

As mentioned above, MZR may be the substrate of CNTs and ENTs, and these transporters were also distributed in kidney tissue [19, 36, 37]. For example, ENT1 and CNT3 are located in apical membranes of proximal tubule cells to reabsorb drug from urine to blood [33]. In addition, elimination of MZR might also be manipulated by organic anion transporter (OAT) 1 and OAT3 [38, 39], which specifically locate in proximal tubular basolateral membrane in kidney [40]. There was a pharmacokinetic interaction between bezafibrate and MZR in a way of competitive inhibition of OAT1/OAT3 in hOAT1/3-HEK293 cells [38], suggesting that MZR would also be a substrate of OAT1 and OAT3. Thus, it was assumed that the gene polymorphisms of the above transporters might have impacts on renal excretion of MZR. In our study, SNPs from CNTs, ENTs, and OATs were all screened; however, only *SLC28A3* rs10868138 T/G was found in significant correlation with MZR $t_{1/2}$. One of the main reasons was that genomic DNA used for sequencing was derived from recipients, which was different from that from donors. Thus, the influences of transporter gene polymorphisms from donor kidney on MZR behavior has to be further determined.

Our previous study has established models to predict C_0 and $t_{1/2}$ by SCr [12], but for AUC, SCr alone explained less of variance, even taking *CREB1* rs11904814 genotypes into the model, and only 20% of variance was explained. AUC represents the whole drug exposure in the body and may be affected by intestinal absorption, drug metabolism, and disposition;

thus, more precise clinical factors reflecting gastrointestinal function, plasma disposition, and renal clearance, as well as more potential genetic factors, need to be combined to better elucidate the AUC variance. Besides, SCr, together with *SLC28A3* rs10868138 genotypes, explained 46.5% of variance in $t_{1/2}$, confirming that renal function plays a critical role in MZR elimination.

In conclusion, we firstly used high-throughput sequenced single nucleotide polymorphism to comprehensively screen possible SNPs relative to MZR PK process and identified that polymorphisms of *CREB1* and *SLC28A3* were associated with MZR AUC_{0-12h}/D and $t_{1/2}$, respectively. *CREB1* and *SLC28A3*, in combination with SCr, may be applied as genetic and clinical factors in the prediction of MZR PK behavior in renal transplant recipients.

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Critical review: All authors.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

This study was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (approved no: 2015118), and informed consent was obtained from each enrolled patient.

Conflict of interest The authors declare that they have no conflict of interest.

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