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ORIGINAL ARTICLE

Effects of *ABCB1* DNA methylation in donors on tacrolimus blood concentrations in recipients following liver transplantation

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Aims: To investigate the effects of *ABCB1* DNA methylation in donors on individual differences in tacrolimus blood concentrations following liver transplantation.

Methods: Twenty-three donor liver samples carrying the *CYP3A5*3/*3* genotype were classified into 2 groups based on their initial tacrolimus blood concentrations (C_0 >10 μg/L or <5 μg/L) following liver transplantation. *ABCB1* mRNA levels in liver tissues and HepG2 cells were determined by quantitative reverse transcriptase polymerase chain reaction. DNA methylation status in liver tissues and HepG2 cells was determined using Illumina 850 methylation chip sequencing technology and pyrosequencing. 5-Aza-2dC was used to reverse methylation in HepG2 cells. Intracellular tacrolimus concentrations were determined by liquid mass spectrometry.

Results: Genome-wide methylation sequencing and pyrosequencing analyses showed that the methylation levels of 3 *ABCB1* CpG sites (cg12501229, cg00634941 and cg05496710) were significantly different between groups with different tacrolimus concentration/dose (C_0/D) ratios. *ABCB1* mRNA expression in donor livers was found to be positively correlated with tacrolimus C_0/D ratio ($R = .458$, $P < .05$). After treatment with 5-Aza-2-Dc, the methylation levels of the *ABCB1* CpG sites in HepG2 cells significantly decreased, and this was confirmed by pyrosequencing; there was also a significant increase in *ABCB1* transcription, which induced a decrease in intracellular tacrolimus concentrations.

Conclusion: *ABCB1* CpG site methylation affects tacrolimus metabolism in humans by regulating *ABCB1* expression. Therefore, *ABCB1* DNA methylation in donor livers might be an important epigenetic factor that affects tacrolimus blood concentrations following liver transplantation.

KEYWORDS
ABCB1, DNA methylation, donor, liver transplantation, tacrolimus blood concentration

The authors confirm that the Principal Investigator for this paper is Chengcheng Shi and that she had direct clinical responsibility for patients.

1 | INTRODUCTION

The P-glycoprotein (P-gp) transporter, which is encoded by the *ABCB1* transporter gene (also known as the *MDR1* gene), is widely distributed in intestinal epithelial cells, liver cells and renal proximal tubule epithelial cells.[1] Previous studies have shown that *ABCB1* may affect tacrolimus absorption, distribution and excretion.[2,3] Thus, some studies have focused on evaluating the relationship between *ABCB1* gene polymorphism (1236C > T, rs1128503, Gly412Gly; 677G > T/A, rs2032582, Ala893DSer/Thr; 3435C > T, rs1045642, Ile1145Ile of exons 12, 21 and 26) and tacrolimus pharmacokinetics.[3,4] However, the findings of these studies are inconsistent as concerns both single nucleotide polymorphisms and haplotypes, and systematic mechanistic studies to support their conclusions are still lacking. In addition, recent studies carried out on ABCB1 have shown that it functions mainly in prolonging drug retention time in intestinal cells by pumping drugs into the intestinal lumen i.e. on the surface of the gastrointestinal tract, and this also affects tacrolimus absorption.[5–7] However, the effects of *ABCB1* on tacrolimus metabolism in liver cells have not been clearly elucidated.

Although *CYP450* and transporter (e.g. *ABCB1*) gene polymorphisms are important for individual variations in drug metabolism and pharmacodynamics, they do not fully explain such individual differences.[8,9] In a previous study (in press), according to the therapeutic window concentration of tacrolimus in liver transplantation, we recruited 78 patients with significantly different initial tacrolimus blood concentrations (C_0 >10 or <5 μg/L) following liver transplantation at the First Affiliated Hospital of Zhengzhou University. Then corresponding donor liver samples were collected for the genotyping of *CYP3A5* and other genes including *ABCB1*, which have been reported in the literature to be possibly related to tacrolimus metabolism (Tables S1, S2). We found that the polymorphisms of *ABCB1* had no association with initial tacrolimus blood concentration. We also found that significant individual differences still existed in tacrolimus blood concentrations in *CYP3A5* nonexpressors (*CYP3A5*3/*3*), indicating that other key factors may also affect tacrolimus metabolism (Figures S1, S2). In recent times, the influence of epigenetic factors on drug metabolism has received increasing research attention; among these factors, DNA methylation has become a new research hotspot. DNA methylation can alter gene expression through the external regulatory pathway without altering the primary structure of DNA, thereby affecting the metabolism of drugs and endogenous substances.[10] More specifically, cytosine on the CpG island combines with a methyl group transferred by DNA methyltransferase to produce methyl cytosine, which then inhibits DNA transcription.[11] Previous studies have shown that DNA methylation is an important epigenetic factor that affects CYP450 gene expression.[12,13] In a previous study, we also found DNA methylation to play an important role in *CYP3A4* transcriptional regulation.[14] In addition, antitumour drugs, such as daunorubicin, activate *ABCB1* transcription by hypomethylating its promoter region and this possibly results in multidrug resistance.[15,16]

Therefore, for this study, we selected 23 donor liver samples carrying the *CYP3A5*3/*3* genotype but exhibiting varied initial tacrolimus blood concentrations in recipients after liver transplantation and divided them into 2 groups (C_0 >10 or <5 μg/L). DNA methylation sequencing was performed to screen for the different methylation sites of drug metabolism enzymes or drug transporters (such as *ABCB1*), and then we evaluated the relationship between the different methylation sites and the tacrolimus C_0/D ratio. In addition, by treating HepG2 cells with methylase inhibitors, we further verified whether DNA methylation is a key epigenetic factor that affects tacrolimus metabolism.

What is already known about this subject

- P-glycoprotein (P-gp) transporter encoded by the *ABCB1* transporter gene may affect the metabolism of tacrolimus.
- Gene polymorphisms of drug metabolising enzymes or transporters in donor livers do not completely explain the individual differences in tacrolimus metabolism following liver transplantation.
- DNA methylation is an important epigenetic factor affecting gene expression through the external regulatory pathway without change the primary structure of DNA, thus affecting the metabolism of drugs.

What this study adds

- DNA methylation of 3 CpG sites (cg12501229, cg00634941 and cg05496710) on *ABCB1* promoter could affect the expression of *ABCB1*.
- The methylation levels of *ABCB1* CpG sites in donors are associated with individual differences in tacrolimus blood concentrations in recipients following liver transplantation.
- The findings will help provide data to fine tune the modalities of tacrolimus administrations in liver transplant recipients in order to improve patient outcomes.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

All the liver transplantation recipients included in this study met the following criteria: age >18 years; postoperative survival time ≥3 months; normal liver function within 1 month following surgery; and patients having received treatment with tacrolimus in combination with steroids and mycophenolate mofelil after transplantation. The corresponding donors met the following criteria: hepatitis or other infectious disease free; normal liver and kidney function; and no treatment with any drug known to interact with tacrolimus within the 2 previous weeks.

Tacrolimus was administered orally at a dose of 0.05 mg/kg/d from the first day following transplantation. Routine therapeutic drug monitoring for tacrolimus was initiated 48 hours after the administration of its first dose. C_0 in whole blood was determined through an enzyme amplification immunoassay using the EMIT 2000 tacrolimus assay kit (Siemens Healthcare Diagnostics, DE, USA) on the day of blood collection. The tacrolimus C_0/D ratio was calculated by dividing the tacrolimus concentration by the corresponding weight-adjusted dose. Liver samples of the included donors were collected during liver trimming at the time of transplantation, cut into small pieces and immediately stored in liquid nitrogen.

2.2 | DNA methylation microarray screening

The DNA of the 15 donor livers (G1: low tacrolimus C_0/D ratio group, $n = 7$; G2: high tacrolimus C_0/D ratio group, $n = 8$) carrying the *CYP3A5*3/*3* genotype was isolated using DNeasy Blood and Tissue Kit (Qiagen). The purity and concentration of the DNA was estimated using NanoDrop 2000 (Thermo Fisher Scientific). Approximately 500 ng of genomic DNA from each sample was sent to Shanghai Jingzhou Genomics Technology Co., Ltd. for sodium bisulfite conversion using the EZ DNA methylation–Gold Kit (Zymo Research, USA), the standard protocol of which was followed. Genome-wide DNA methylation was assessed using the Illumina Infinium Human Methylation 850 K BeadChip (Illumina Inc., USA) according to the manufacturer's instructions. Array data (.IDAT files) were analysed using the ChAMP function in R for the determination of methylation levels. The methylation status of all probes was denoted as the β value, which is the ratio of the methylated probe intensity to the overall probe intensity (the sum of the methylated and unmethylated probe intensities plus the constant, α, where $\alpha = 100$). CpG sites with $|\Delta\beta| \geq 0.20$ (in test *vs*. control) and adjusted *P* value ≤.05, were considered to be differentially methylated sites. A CpG site was considered to be hypermethylated if its Δβ was ≥0.20, or hypomethylated if its Δβ was ≤−0.20. The average β values for promoters and CpG islands were compared between Group 1 (G1: low tacrolimus C_0/D ratio group) and Group 2 (G2: high tacrolimus C_0/D ratio group). Promoters and CpG islands with $|\Delta\beta| \geq 0.20$ and adjusted *P* value ≤.05, were retained for further analysis.

2.3 | Pyrosequencing analysis

Genomic DNA (500 ng) extracted from the 23 *CYP3A5*3/*3* genotype donor livers was transformed by sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research, Orange County, CA, USA) and further purified using the Wizard DNA Clean-up System (Promega, Madison, WI, USA). The sequences of the primers used for the *ABCB1* DNA methylation analysis are shown in Table 1. Each polymerase chain reaction (PCR) consisted of 100 ng of DNA converted by sodium bisulfite, 100 pM deoxyriboside triphosphate, 10 pM positive/reverse primers and 1 unit of Taq polymerase (Merck KGaA, Darmstadt, Germany), which resulted in a final volume of 25 μL. After initial denaturation at 95°C for 5 minutes, amplification was performed for 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step at 72°C for 5 minutes. PCR products were analysed by nondenatured 6% polyacrylamide gel electrophoresis, following staining with ethidium bromide.

In addition, genomic DNA was extracted from cells treated with the methylation inhibitor, 5-Aza-2-DC and changes in *ABCB1* DNA methylation were also evaluated by pyrosequencing.

2.4 | Cell culture and treatments

The human hepatocellular carcinoma cell line, HepG2, was purchased from the China Centre for Type Culture Collection (Wuhan, China) and confirmed by short tandem repeat analysis provided by the above company. HepG2 was cultured in Dulbecco's modified Eagle medium (DMEM; HyClone/Thermo Fisher Scientific, Beijing, China) supplemented with 10% foetal bovine serum (LONSA SCIENCE S.R.L., Montevideo, Uruguay). To determine the optimum concentration and administration period for 5-Aza-2-DC (Selleck, Shanghai, China) and tacrolimus (Selleck, Shanghai, China), HepG2 cells were seeded into 12-well plates at a density of 4×10^4 cells/well and cultured for 24 hours at 37°C in a 5% CO_2 atmosphere. For treatment with 5-Aza-2-DC, the cells were exposed to 5-Aza-2-DC dissolved in dimethyl sulfoxide (DMSO; 0.1% v/v) at a series of final concentrations (0, 0.1, 0.5, 1.0, 2.5, 5.0, 10 and 50 μM) for 24, 48 and 72 hours. Then, cell viability was evaluated using the CCK-8 kit (Dojindo, Shanghai, China) according to the manufacturer's instructions, and *ABCB1* mRNA and

TABLE 1 Primers used for *ABCB1* DNA methylation analysis

Detected site	Segment	Primer sequence (5′–3′)	Product size (bp)
cg05496710	PCR sense	AAGTAGAATATTTAGGGGAGGTATG	84
	PCR antisense	ACCTTACATTCTCCAATTCTTTAAAA	
	Sequencing	GGAGGTATGGGTTTT	
cg00634941	PCR sense	TGGATTGTTGGATTTGTAGTTTTAATAG	102
	PCR antisense	AAACTCCAATCCCCTTTACTAATA	
	Sequencing	GTTTTTAGGTGATGTTGAT	
cg12501229	PCR sense	GTGAATAGTTGGTGATAATTATTTATTGTG	99
	PCR antisense	AAATAAACATCTCCTTTTTAAACATTTT	
	Sequencing	TGGTGATAATTATTTATTGTGG	

protein expression levels were evaluated by quantitative reverse transcriptase–PCR and western blotting, respectively. For treatment with tacrolimus, the cells were exposed to tacrolimus dissolved in DMSO (0.1% v/v) at a series of final concentrations (0, 0.01, 0.1, 1.0, 10, 50, 100 and 200 μM) for 24, 48 and 72 hours, and cell viability was evaluated using the CCK-8 kit. Based on the results of the optimum time determined by CCK-8 analysis, HepG2 cells were treated with 0, 40, 50, 60, 70, 80, 90 and 100 μM tacrolimus for 24 hours, and cell viability was evaluated using the CCK-8 kit to determine the tacrolimus optimum concentration.

2.5 | 5-Aza-2-DC treatment

HepG2 cells at the logarithmic growth stage (1.2×10^6 cells/well) were spread on 6-well plates. After 24 hours of culturing, the cells were treated with 0 μM (DMSO; 0.1% v/v) or 10 μM 5-Aza-2-DC, and the 5-Aza-2-DC-containing medium was replaced every 24 hours. After treatment with 5-Aza-2-DC for 72 hours, the medium was discarded and replaced with a tacrolimus-containing medium (60 μM), and the cells were cultured for another 48 hours. After 48 hours, the medium was replaced with tacrolimus-free DMEM and the cells were further cultured. Samples of the medium were taken at 0, 4, 6 and 12 hours following its replacement. Next, the cells were digested with trypsin for 2 minutes, and then digestion was terminated by adding more medium. The cells were centrifuged at $300 \times g$ for 3 minutes and washed thrice with phosphate-buffered saline. The supernatant of cell culture and cells were stored at −80°C for further evaluation.

2.6 | Total RNA extraction and RT-qPCR analysis

Total RNA was extracted using the TRIzol Reagent (CoWin Biosciences, Jiangsu, China) according to the manufacturer's instructions. The extracted RNA was reverse transcribed into cDNA using Super Script III Reverse Transcriptase (Vazyme Biotech Co., Ltd., Nanjing, China). ABCB1 mRNA expression was evaluated through a relative quantitative method. The formula, $2^{-\Delta\Delta}$CT, was used to calculate the quantity of DNA amplified. The sequencing primers used for amplification, which were provided by Tsingke Biotechnology Co., Ltd., were:

ABCB1-F: 5′-TTGCTGCTTACATTCAGGTTTCA-3′,
ABCB1-R: 5′-AGCCTATCTCCTGTCGCATTA-3′,
GAPDH-F: 5′-GCACCGTCAAGGCTGAGAAC-3′,
GAPDH-R: 5′-TGGTGAAGACGCCAGTGGA-3′.

2.7 | Western blot analysis

Lysates of HepG2 cells (which were treated with 5-Aza-2-DC and tacrolimus) were run on 8% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with an antibody against P-glycoprotein (Abcam Inc. Cambridge, MA, USA). Antibody binding was detected using enhanced chemiluminescence ECL Plus western blotting detection reagents (Merck Millipore Ltd. Tullagreen, Carrigtwohill, Co. Cork, Ireland).

2.8 | Determination of tacrolimus intracellular concentrations by liquid-mass spectrometry

The quantification of tacrolimus was performed by ultra-high performance liquid chromatography–tandem mass-spectrometry (UHPLC–MS/MS) according to previously described methods with minor modifications.[17,18] Specifically, the collected cells previously treated by 5-Aza-2-DC were resuspended in 100 μL of phosphate-buffered saline or medium and mixed with 50 μL of internal standard (ascomycin, 800 ng/mL, dissolved in methanol), 50 μL of methanol and 500 μL of methyl-tert-butyl ether. After vortexing and centrifugation ($14\,000 \times g$, 5 min, 4°C), 450 μL of the organic layer was dried on a nitrogen blow-dry apparatus. The extract was redissolved in 50 μL of a complex solution (2 mM ammonium acetate and 0.1% formic acid). After vortexing and centrifugation ($12\,000 \times g$, 10 min, 4°C), the supernatant (45 μL) was placed in a liquid injection flask. The mobile phase for UHPLC–MS/MS detection was (acetonitrile [containing 0.1% formic acid]: 2 mmol/L ammonium acetate [containing 0.1% formic acid] = 9:1). An ACQUITY UPLC BEH C18 column (2.1 × 50 mm; 1.7 μm; Waters Chromatography Ireland Ltd., Ireland) was used, and the column temperature was 55°C. The specific product ions were m/z 821.5 and m/z 809.5 for tacrolimus and the internal standard, respectively. Ionisation was carried out in positive ion mode with a capillary voltage of 3.5 kV, a cone voltage of 22/29 (tacrolimus/internal standard), an ion source temperature of 120°C, a desolvation temperature of 350°C, a nitrogen flow rate of 600 L/h, a collision gas pressure of 5×10^{-3} bar and a collision energy of 17/21 (tacrolimus/internal standard).

2.9 | Statistical analysis

SPSS software (Armonk, New York, NY, USA) was used for data analysis, and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) was used to plot graphs. All data are expressed as mean ± standard deviation. An independent sample *t*-test was used to compare and analyse the data between the 2 groups, with the homogenous variance of a bivariate normal distribution. Pearson correlation test was used to analyse the correlation of *ABCB1* mRNA expression with tacrolimus C_0/D ratio. *P* values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Whole genome methylation microarray sequencing of CYP3A5*3/*3 genotype donor livers

Through principal component analysis, approximately 800 000 methylation sites were observed in the 2-dimensional diagram after probe

filtering and normalisation (Figure 1A). Based on the methylation β value between the 5 kb upstream and downstream transcription start sites, significant differences in methylation levels were observed between groups G1 and G2 (Figure 1B). In addition, heat and scatter maps showed that there was a large number of methylation sites, with different levels of methylation, between groups G1 and G2 (Figure 1C and D).

3.2 | Methylation levels and mRNA expression of ABCB1 in relation to tacrolimus blood concentration

Through methylation chip screening, the methylation levels of 3 *ABCB1* sites (cg12501229, cg00634941 and cg05496710) located on chromosome 7 were found to be significantly different between the high and low tacrolimus blood concentration groups (Figure 2A). Using the pyrophosphorylation assay, the methylation levels of these 3 sites were further evaluated in the 23 donor liver specimens carrying the *CYP3A5*3/*3* genotype. We found that the methylation levels of these sites in the high C_0/D group were all significantly lower than those in the low C_0/D group (Figure 2B–D).

Furthermore, *ABCB1* mRNA expression levels were determined by RT-qPCR, and correlation analyses revealed that *ABCB1* mRNA expression in donor livers was positively correlated with the tacrolimus C_0/D ratio ($R = .458$, $P < .05$; Figure 3A). An *ABCB1* mRNA expression level of 0.15 was used as the cut-off value to analyse the tacrolimus C_0/D ratio and differences between the low-and high-expression groups (Figure 3B and C). Meanwhile, the methylation level of the 3 CpG sites was significantly negatively correlated with ABCB1 mRNA expression ($R = -.394$, $P = .063$; $R = -.358$, $P = .094$; $R = -.307$, $P = .154$; Figure 3D). Our results showed that the greater proportion of patients with high blood tacrolimus concentrations were found in the high *ABCB1* expression group.

3.3 | Effects of 5-Aza-2-DC on ABCB1 expression in HepG2 cells

First, the optimal concentration and administration time for 5-Aza-2-DC were determined (Figure 4A–C). Then, based on findings in literature, 10 μM 5-Aza-2-DC was used to investigate the effects of *ABCB1* methylation status on its expression in HepG2 cells. We found

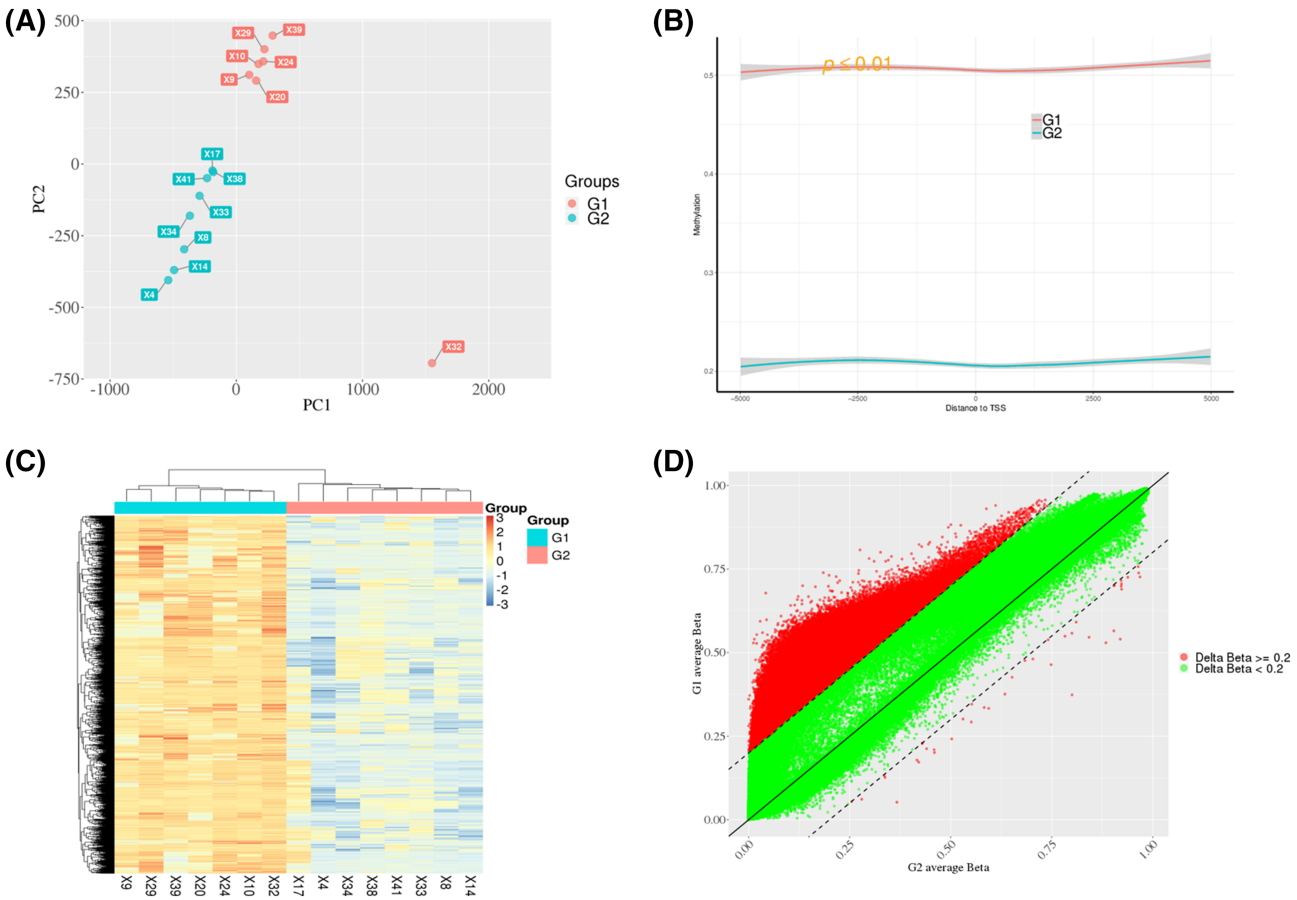

FIGURE 1 Whole genome methylation microarray sequencing of CYP3A5*3/*3 genotype donor liver. (A) Principal component (PC) analysis diagram; (B) distribution of methylation degree between the 5 kb upstream and downstream transcription start sites (TSS); (C) heat map of differential methylation sites; (D) scatter plot of different methylation sites. G1: low tacrolimus C0/D ratio group, $n = 7$; G2: high tacrolimus C0/D ratio group, $n = 8$

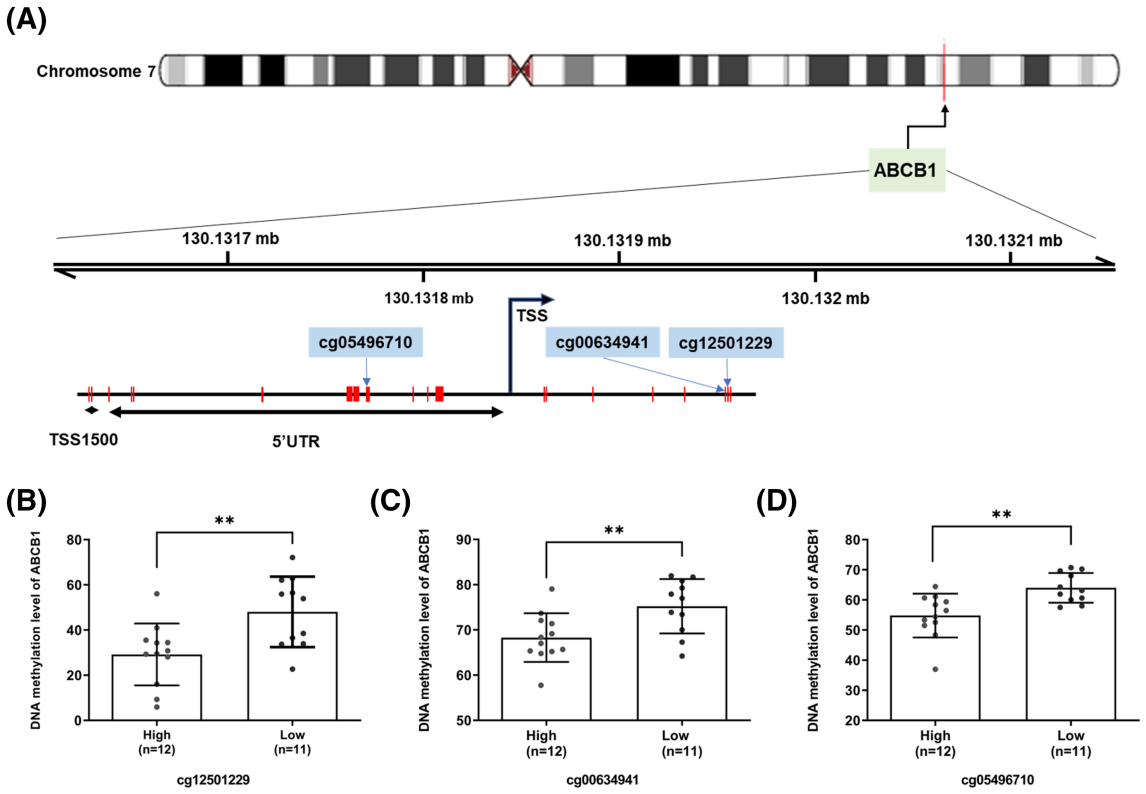

FIGURE 2 The methylation sites (red vertical bars) within the *ABCB1* methylation region located on chromosome 7 (A), and the methylation level at cg12501229 site (B), cg00634941 site (C) and cg05496710 site (D) of *ABCB1* gene in donor livers carried with *CYP3A5*3/*3* genotype and different tacrolimus C_0/D ratio (High: $n = 12$, Low: $n = 11$); $^{**}P < .01$

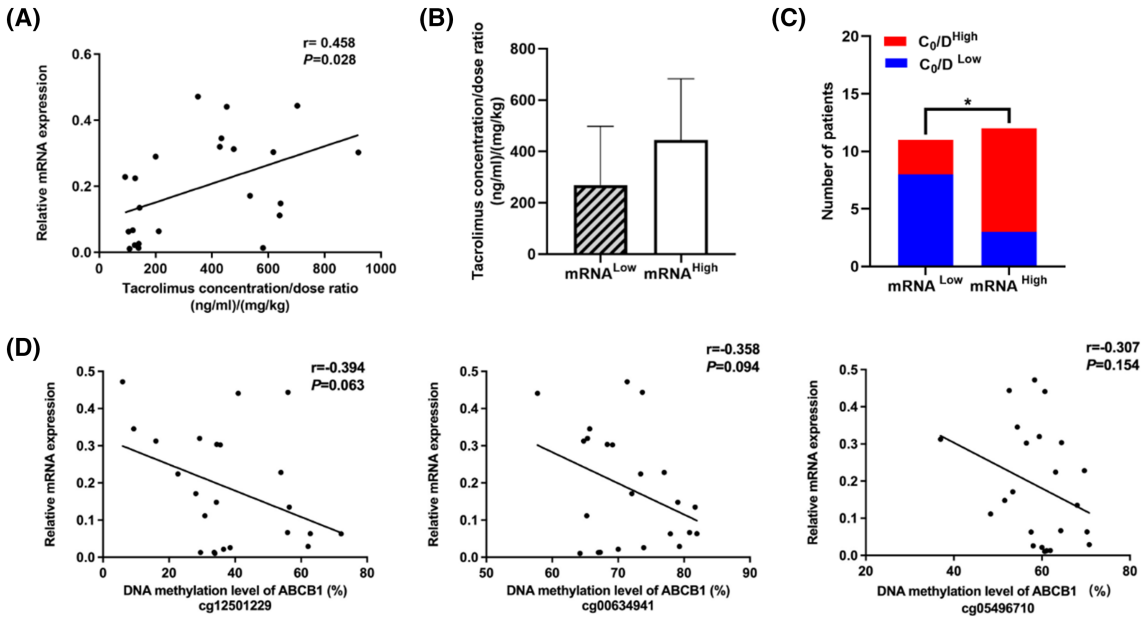

FIGURE 3 Correlation of ABCB1 mRNA expression with tacrolimus C0/D ratio and ABCB1 methylation level of 3 CpG sites in CYP3A5*3/*3 donor livers ($n = 23$). (A) Correlation analyses; (B, C) an ABCB1 mRNA expression level of 0.15 was used as the cut-off value to analyse the tacrolimus C0/D ratio (B) and the tacrolimus C0/D ratio distribution (C); mRNA Low: relative mRNA level of ABCB1 < 0.15; mRNA High: relative mRNA level of ABCB1 > 0.15; $^{*}P < .05$; (D) Correlation between ABCB1 mRNA expression level and methylation level of 3 CpG sites

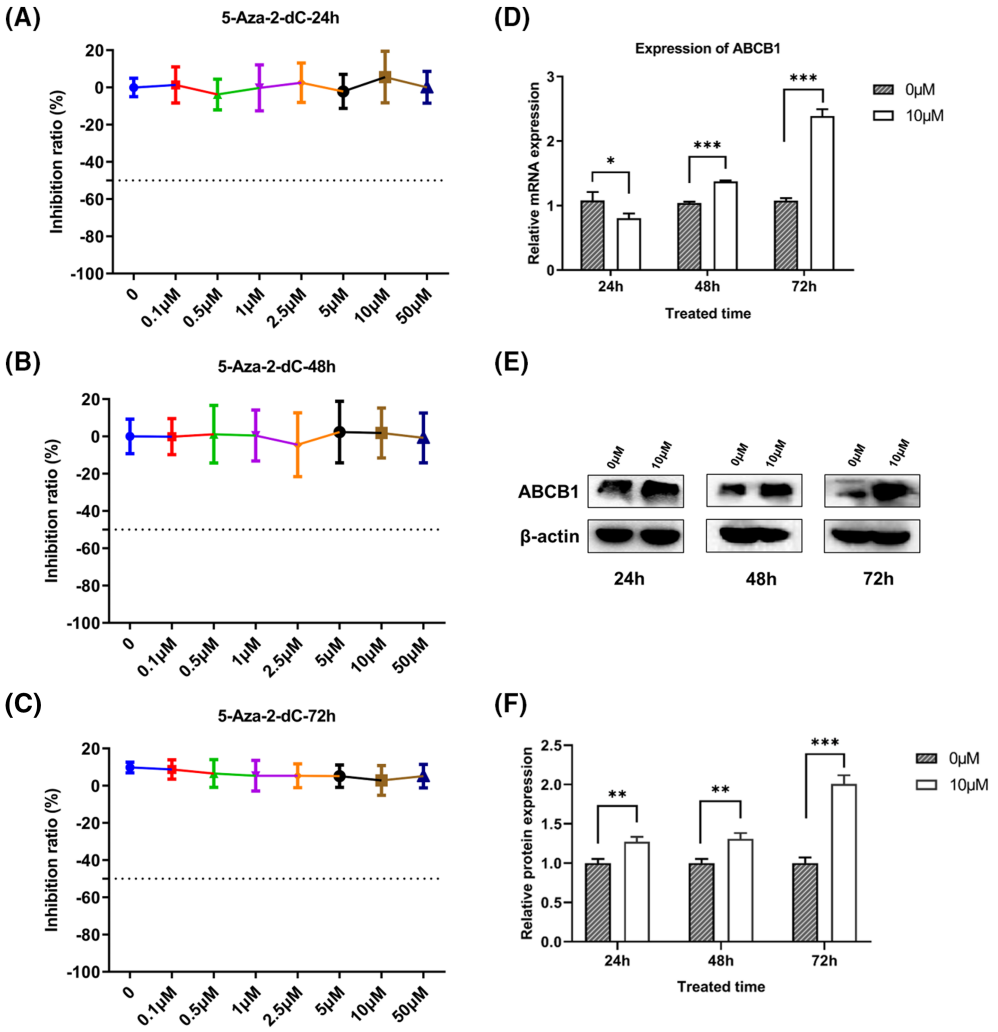

FIGURE 4 Effects of 5-Aza-2-DC on cell viability, *ABCB1* expression in HepG2 cells. (A–C) The effects of 5-Aza-2-DC on cell viability after treated at indicated concentrations of 5-Aza-2-DC for 24 (A), 48 (B) and 72 hours (C); (D–F) *ABCB1* mRNA expression (D) and protein expression (E, F) after treatment with 5-Aza-2-DC (10 μM) for 24, 48 or 72 hours; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ *vs.* 0 μM 5-Aza-2-DC (DMSO; 0.1% v/v)

that after treatment with 10 μM 5-Aza-2-DC for 24, 48 and 72 hours, *ABCB1* mRNA and protein expression levels significantly increased at 72 hours (Figure 4D–F). Therefore, 10 μM 5-Aza-2-DC and 72 hours were selected as the optimal concentration and treatment duration, respectively, for the determination of the effects of 5-Aza-2-DC on the methylation levels of the 3 *ABCB1* CpG sites (cg12501229, cg00634941 and cg05496710).

3.4 | Effects of 5-Aza-2-DC on ABCB1 methylation levels and tacrolimus metabolism in HepG2 cells

A pyrophosphorylation assay was performed to investigate the effects of 5-Aza-2-DC on *ABCB1* methylation. As shown in Figure 5, we found that the methylation levels of the *ABCB1* sites in the 5-Aza-2-DC-treated group (10 μM) were significantly lower ($P < 0.001$) than those in the untreated group (0 μM). To determine the effects of 5-Aza-2-DC on tacrolimus efflux, intracellular tacrolimus concentrations were determined by UHPLC–MS/MS. We found the tacrolimus contents of 5-Aza-2-DC-treated cells to be significantly lower than

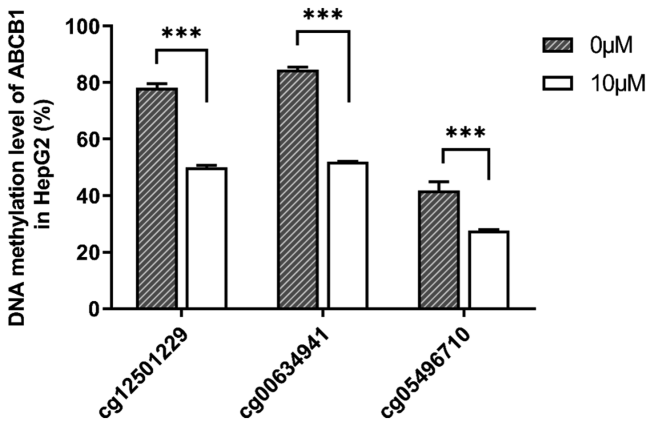

FIGURE 5 Effects of 5-Aza-2-DC (10 μM) on DNA methylation at 3 CpG sites located on *ABCB1* promoter region in HepG2 cells; $^{***}P < .001$ *vs.* 0 μM 5-Aza-2-DC (DMSO; 0.1% v/v)

those of untreated cells at 0, 4 and 6 hours following the removal of tacrolimus-containing medium ($P < .05$). At 12 hours, although there was a slight increase in intracellular tacrolimus concentrations, its

levels in the 5-Aza-2-DC-treated group were still significantly lower than those in the untreated group (0 μM), and this may be due to its intracellular metabolism in a dynamic equilibrium scenario (Figure 6).

4 | DISCUSSION

Several studies have reported that individual differences in drug response cannot be fully explained by polymorphisms in genes encoding drug-metabolizing enzymes or transporters.[19,20] Recently, epigenetic modifications, which regulate the expression of several enzymes and transporters involved in drug metabolism, have been recognised as important factors that affect individual differences in clinical drug response.[21] DNA methylation affects the expression of *CYP450* (*CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2C19*, *CYP2D6*, *CYP2E1* and *CYP2W1*), thus leading to significant individual differences in enzyme expression.[22–25] In addition, DNA methylation regulates the expression of *ABCG2* and *ABCB1*, which play a crucial role in determining the success or failure of cancer chemotherapy by mediating multidrug resistance and individual differences in drug transport.[26,27] As most studies on the methylation of genes encoding drug transporters have been carried out in the field of oncology, we investigated, for the first time, whether *ABCB1* DNA methylation in donor livers affects tacrolimus blood concentrations in liver transplant recipients by regulating its expression.

In this study, we analysed 15 donor liver samples carrying the *CYP3A5*3/*3* genotype using DNA methylation microarray technology and found *ABCB1* methylation levels to be correlated with tacrolimus blood concentrations in liver transplantation patients. At present, the method we used for determining tacrolimus concentration is the most commonly used method despite its crossreactivity with tacrolimus metabolites.[28] Based on the findings of previous studies on *ABCB1* methylation and tacrolimus metabolism[2,29,30] and *ABCB1* mRNA expression level, which is regulated by DNA methylation[27] and inversely correlated with the tacrolimus concentration–oral dose ratio in living-donor liver transplant patients,[31] we speculated that *ABCB1* DNA methylation might be another key factor that affects tacrolimus metabolism by regulating *ABCB1* expression.

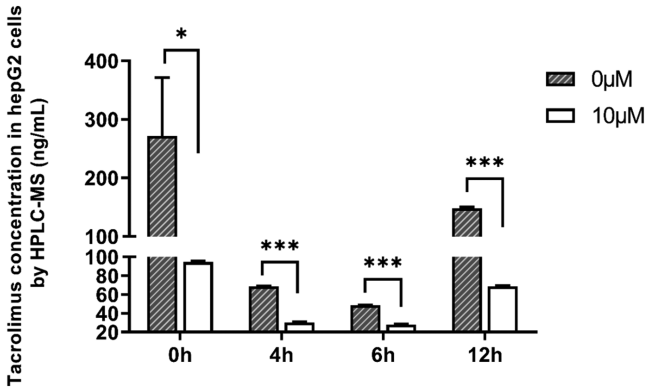

FIGURE 6 Changes of tacrolimus concentration in HepG2 cells over time replaced with tacrolimus-free DMEM after treated with 5-Aza-2-DC (0 μM or 10 μM) 72 h and then incubated with 60 μM tacrolimus for 48 hours; **P* < .05, ****P* < .001 *vs.* 0 μM 5-Aza-2-DC (DMSO; 0.1% v/v)

Previous studies have demonstrated that there exists no correlation between the frequency of *ABCB1* gene polymorphisms and tacrolimus plasma concentrations following renal transplantation,[4,32,33] and this is consistent with the findings of 1 of our previous studies (in press). However, our studies found that there exist significant individual differences in tacrolimus blood concentrations in liver transplant recipients who receive donor livers with the *CYP3A5*3/*3* genotype; thus, for the first time, we evaluated the methylation status of 23 liver samples carrying the *CYP3A5*3/*3* genotype using a methylation microarray assay validated by pyrosequencing. Our findings showed that DNA methylation levels at 3 *ABCB1* CpG sites (cg12501229, cg00634941 and cg05496710) in donor livers were significantly different between the high and low tacrolimus C_0/D ratio groups following liver transplantation. In addition, in consonance with the findings of previous studies,[30,34,35] *ABCB1* mRNA levels in donor livers were found to be negatively correlated with its methylation levels.

To the best of our knowledge, no studies have been carried out on the effects of *ABCB1* methylation, especially of its 3 CpG sites (cg12501229, cg00634941 and cg05496710), on tacrolimus metabolism. Therefore, in this study, the effects of *ABCB1* methylation on tacrolimus efflux from target cells were first evaluated using the methylation inhibitor, 5-Aza-2-DC. It was shown that in HepG2 cells, *ABCB1* methylation levels at its 3 methylation sites significantly decreased following treatment with 5-Aza-2-DC. Moreover, tacrolimus intracellular concentrations significantly decreased following treatment with 5-Aza-2-DC. In addition, *ABCB1* mRNA and protein levels significantly increased following treatment with 5-Aza-2-DC. For clinical samples, the methylation levels of the cg12501229, cg00634941 and cg05496710 sites in the high C_0/D group were all significantly lower than those in the low C_0/D group, and *ABCB1* mRNA expression was found to be positively correlated with tacrolimus C_0/D ratio. These findings indicate that a decrease in DNA methylation could result in an increase in *ABCB1* expression in donor livers, which would lead to an increase in tacrolimus efflux from liver cells and a consequent increase in tacrolimus blood concentrations of the recipient.

This study had limitations, namely, we did not construct a methylation-specific expression plasmid to determine which of the 3 *ABCB1* methylation sites plays a leading role in the regulation of gene expression. Moreover, many factors, such as age, sex, drug exposure, diet and environment, could cause varying DNA methylation states in individuals. All these mechanisms should be further investigated in our future study. By contrast, although multiple studies[36–38] have shown that influence factors, such as genotype, are more important for tacrolimus metabolism post-liver transplant in donors than in recipients, the DNA methylation states of *ABCB1* in recipients should be considered in the future investigation.

5 | CONCLUSION

Our study demonstrated that DNA methylation of *ABCB1* CpG sites (cg12501229, cg00634941 and cg05496710) could regulate its expression in donor livers, thus inducing individual differences in initial tacrolimus concentrations following liver transplantation.

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CONTRIBUTORS

C.C.S.: conception, design, data collection, analysis of data, drafting and revision of manuscript. L.Y.: conception, interpretation of results and critical revision of manuscript. S.T.C.: analysis of data, interpretation of results. L.R.Z.: design and revision of manuscript.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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