DEVELOPMENT OF AN LC-MS/MS METHOD TO DETERMINE TACROLIMUS AND EVEROLIMUS IN KIDNEY TISSUES AND ITS APPLICATION TO KIDNEY TRANSPLANT RECIPIENTS

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CONTENTS

ABBREVIATION LIST

ABCB1	ATP binding cassette subfamily B member 1
AR	acute rejection
C_{blood}	whole blood trough concentration
CNI	calcineurin inhibitor
C_{tissue}	allograft kidney concentration
CYP	cytochrome P450
C_{blood}/D	dose-adjusted whole blood trough concentration
C_{tissue}/D	dose-adjusted allograft kidney concentration
FDA	food drug administration
IF/TA	interstitial fibrosis and tubular atrophy
IS	internal standard
IS-norm MF	internal standard normalized matrix factor
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLOQ	lower limit of quantitation
MF	matrix factor
MRM	multiple reaction monitoring
mTOR	mammalian target of rapamycin
P-gp	P−glycoprotein
PBMC	peripheral blood mononuclear cells
QC	quality control
RSD	relative standard deviation
SubAR	subclinical acute rejection
TDM	therapeutic drug monitoring

INTRODUCTION

Immunosuppressive agents have been continuously evolving over the past several decades since azathioprine was utilized in the first kidney transplantation in 1960s.[1] The introduction of calcineurin inhibitors (CNIs), especially tacrolimus, revolutionized short-term outcomes after kidney transplantation.[2]

Tacrolimus was discovered in 1984 from the fermentation broth of a Japanese soil sample that contained the bacteria *Streptomyces tsukubaensis*.[3] Tacrolimus binds to an immunophilin, FK506 binding protein, creating a complex that inhibits the action of calcineurin phosphatase, associated with T-lymphocyte signal transduction and IL-2 production. The inhibition of calcineurin phosphatase leads to a reduction in immune system activity and hence the risk of organ rejection in transplant recipients.[4] Although tacrolimus has greatly improved short-term allograft survival rates, its long-term use causes considerable nephrotoxicity, which can adversely affect kidney functions and result in allograft loss in kidney transplant recipients.[5-7] The prevention of CNI toxicity is a critical challenge in immunosuppressive regimens after transplantation.[7, 8] There are numerous potential factors that could cause tacrolimus-related toxicity, including tacrolimus systemic levels, local kidney exposure to tacrolimus or tacrolimus metabolites, donor age, and genetic variations in drug transporters and metabolic enzymes, such as P−glycoprotein (P-gp) and cytochrome P450 (CYP)3A.[2, 9-11]

The recent availability of a new class of immunosuppressive agents known as mammalian target of rapamycin (mTOR) inhibitors, provides more options for immunosuppressive therapy.[12] Everolimus is a potent mTOR inhibitor, which inhibits the action of T cells by anti-proliferative and anti-migratory effects by blocking the vascular endothelial growth factor to exert an immunosuppressive effect; its major feature compared to CNI is the lower risk of nephrotoxicity.[12, 13] Several studies have

demonstrated that transplant recipients treated or co-treated with everolimus have an improved kidney function compared to those treated with CNIs.[14-17] However, everolimus-based immunosuppressive therapy is also associated with a high risk of adverse events, including gastrointestinal disorders, hyperlipidemia, leukopenia, proteinuria, and wound healing impairment, which can require the cessation of everolimus treatment.[18, 19] Therefore, a combination of immunosuppressive agents with different mechanisms is commonly recommended to reduce drug-specific side effects.[20] Tacrolimus and everolimus both have a narrow therapeutic window and large individual variability in pharmacokinetics and their co-administration should be closely monitored to ensure the efficacy and safety.[19, 21, 22] Although therapeutic drug monitoring (TDM) of tacrolimus whole blood trough concentration (C_{blood}) has been widely performed, it has only resulted in modest improvements in clinical outcomes. Moreover, the clinical relevance of tacrolimus C_{blood} remains controversial.[23, 24] Even when the concentrations are within the target therapeutic ranges, adverse events are frequently observed, implying that whole blood levels do not necessarily correlate with pharmacological effects.[23-25] In recent years, new approaches to optimizing monitoring strategies have been developed, including directly measuring the concentration of immunosuppressive drugs in the target location where they exert its pharmacologic effects or toxicity.[23, 25, 26] Capron et al. found that tacrolimus peripheral blood mononuclear cells (PBMC) concentrations, but not whole blood concentrations, could predict acute rejection (AR) in liver transplant recipients.[27] Furthermore, a low hepatic tacrolimus concentration was associated with AR after liver transplantation.[28, 29] Theoretically, only unbound drugs are available for uptake into the target organs to exert efficacy and/or toxicities; both tacrolimus and everolimus extensively bind to red blood cells and blood proteins, as such it is reasonable to expect that local concentrations in allograft kidney (C_{tissue}) might better reflect clinical outcomes than C_{blood} in kidney transplant recipients. **Table 1**

summarizes the findings of published studies on the determination of tacrolimus and everolimus concentrations in different matrices.

Table 1. Summary of studies on the concentrations of tacrolimus and everolimus in various biological matrices

Drugs	Recipients	Matrices	Findings	References
Tacrolimus	Liver transplant	PBMC	No significant correlation was observed between tacrolimus blood levels and PBMC concentrations; tacrolimus PBMC levels were significantly associated to the liver Banff rejection score.	**27**
Tacrolimus	Kidney transplant	PBMC	A poor correlation was found between tacrolimus blood concentrations and PBMC concentrations.	**30**
Tacrolimus	Liver transplant	Liver tissues	Tacrolimus hepatic concentrations were significantly correlated with the severity of the organ rejection than blood levels.	**28**
Tacrolimus	Kidney transplant	Kidney tissues	Significant association was observed between tacrolimus blood concentrations and kidney concentrations	**31**
Tacrolimus & everolimus	Liver transplant	PBMC	PBMC concentration was 19.23 and 218.61 times higher than the blood concentration for tacrolimus and everolimus, respectively.	**32**
Everolimus	Kidney transplant	PBMC	A significant association was found between everolimus whole blood and PBMC concentrations.	**33**

Although several studies have developed a bioanalysis method to determine tacrolimus concentrations in kidney tissues, the association between clinical outcome and drug concentration in the kidney remains unclear.[34, 35] In addition, no previous study has determined everolimus concentrations in human allograft kidneys. There might be a potential clinical value for measuring tacrolimus and everolimus C_{tissue} in kidney transplant recipients.

Tacrolimus is predominantly metabolized by intestinal and hepatic CYP3A4 and CYP3A5.[36] The loss-of-function allele *CYP3A5*3* (rs776746, g.6986A.G) has been demonstrated to be the key genetic factor affecting tacrolimus metabolism and pharmacokinetics.[37-39] In Asian populations, the *CYP3A5*3* allele frequency is higher than 70%, and approximately 50% of people are homozygous *CYP3A5*3/*3* carriers (defined as CYP3A5 non-expressors).[40] Compared to *CYP3A5*3/*3* carriers, *CYP3A5*1* carriers (defined as CYP3A5 expressors) require higher tacrolimus doses to achieve target blood concentrations.[41-43] The influences of gene polymorphisms on tacrolimus pharmacokinetics are summarized in **Table 2**. With the exception of *CYP3A5*, other tacrolimus metabolism-related alleles are less common in Asian population, and their impact on tacrolimus pharmacokinetics is still debated. Most of these studies have focused on evaluating the impact of recipient *CYP3A5* genotypes on tacrolimus pharmacokinetics, such as doses and blood, in order to optimize clinical outcomes after kidney transplantation. However, adjusting tacrolimus doses based on the recipient *CYP3A5* genotype did not significantly improve clinical outcomes.[58, 62, 63] It has been reported that the CYP3A5 protein is also expressed in renal tubular epithelial cells[64], thus, it is reasonable to assume that the donor *CYP3A5* genotype might have a closer relationship with clinical outcomes by affecting the local tacrolimus concentration in the allograft kidney.

Table 2. The influences of major polymorphisms on tacrolimus.

Drugs	Allele	Frequency in Asian Population	Findings	References
Tacrolimus	*CYP3A5*3*	0.6-0.74	*CYP3A5*1* carriers had a lower C_{blood} (or C_{blood}/D ratio) and required higher doses than those with the *CYP3A5*3/*3* genotype.	**44-51**
	*CYP3A4*22*	0-0.043	Reduced tacrolimus clearance, dose requirement, and higher exposure compared with those associated with the wild-type allele.	**52-54**
			No significant relationship between the gene polymorphisms and tacrolimus pharmacokinetics	**44, 55-57**
	*CYP3A4*1B*	0	Higher dose requirements and clearance than those in *CYP3A4*1* carriers.	**46, 55, 58**
	*CYP3A4*1 G*	0.2	Lower tacrolimus exposure.	**59-61**

Therefore, the aims of this study were to develop the first liquid chromatography tandem mass spectrometry (LC-MS/MS) method for simultaneously quantifying tacrolimus and everolimus in allograft kidneys, and to investigate the clinical value of tacrolimus and everolimus C_{tissue} in kidney transplant recipients.

CHAPTER 1

DEVELOPMENT OF AN LC-MS/MS METHOD FOR THE DETERMINATION OF TACROLIMUS AND EVEROLIMUS IN KIDNEY BIOPSY SAMPLES

1. INTRODUCTION

Over the past three decades, tacrolimus has been utilized as the first-line immunosuppressive agent, and the short-term clinical outcomes after kidney transplantation have been greatly improved.[65, 66] However, to date, avoiding CNI-induced nephrotoxicity, which can result in kidney graft failure, has remained an unsolved challenge.[2, 67] It has been reported that the development of tacrolimus-induced nephrotoxicity might be related to the overexposure of tacrolimus in the kidney.[10, 68, 69] Everolimus is a potent mTOR inhibitor with a non-nephrotoxic immunosuppressive effect and has shown promise in preventing chronic allograft dysfunction after transplantation.[70, 71] In recent years, co-administration of tacrolimus and everolimus reportedly showed sufficient immunosuppressive efficacy and improved kidney function in transplant recipients.[17, 72, 73]

Both tacrolimus and everolimus have narrow therapeutic ranges and large individual variabilities in pharmacokinetics.[74, 75] Although TDM of tacrolimus and everolimus has been widely performed in clinical practice, the incidence of adverse events remains difficult to predict and prevent.[23-25] Thus, choosing an appropriate matrix for monitoring drug exposure could be a more effective approach to reflect clinical outcomes and improve medication administration.[21, 23] Several studies have reported that low hepatic tacrolimus concentration correlates with rejection after liver transplantation.[28, 29]

Previous studies have developed methods for measuring tacrolimus in human kidney tissues.[34, 35] However, to date, no study has simultaneously determined the C_{tissue} of everolimus and tacrolimus in human kidney allografts. Accordingly, this chapter aimed to develop and validate an LC-MS/MS method to determine the C_{tissue} of tacrolimus and everolimus in clinical kidney biopsy samples.

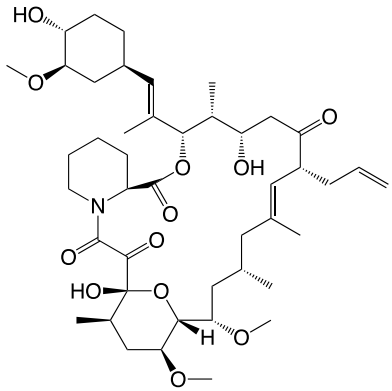
2. MATERIALS AND METHODS

2-1 Regents and animals

Tacrolimus, everolimus, and ascomycin (internal standard [IS]) were purchased from Sigma-Aldrich (Tokyo, Japan) (**Figure 1**). Ammonium acetate was purchased from Nacalai Tesque (Kyoto, Japan). Formic acid, zinc sulfate heptahydrate, and HPLC-grade methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan).

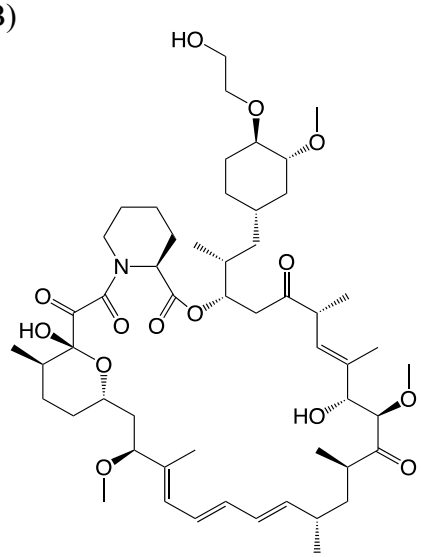
In lieu of blank human kidney tissue, calibration and quality control (QC) samples were prepared using blank kidney tissues from drug-free rats. Male Wistar rats aged 7–9 weeks were purchased from SLC (Hamamatsu, Shizuoka, Japan), and ethical approval (approval number: A30-029-1) was obtained from the Animal Experimentation Committee of the Cantonal Veterinary Service (Kyushu University, Japan). Rats were fasted for 12 h before kidney harvesting, and kidney samples were immediately placed on ice and stored at -80℃ until the day of the assay.

(A)



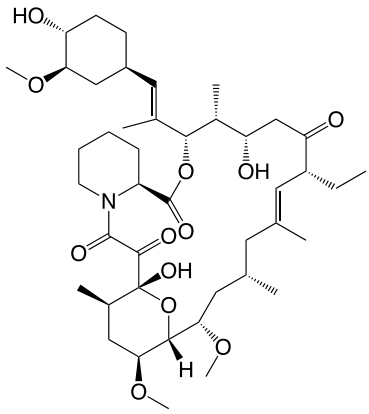
Tacrolimus

(B)



Everolimus

(C)



Ascomycin (IS)

Figure 1. Chemical structure of (A) tacrolimus, (B) everolimus, and (C) ascomycin (IS).

2-2 LC-MS/MS conditions

Quantitation of tacrolimus and everolimus was performed using an LC-MS/MS system (SHIMADZU LCMS-8050, Japan) in positive-ion multiple reaction monitoring (MRM) mode. Chromatographic separation was carried out on a GL Sciences Inertsil-ODS-3 column (3 μm; 2.1 mm × 150 mm) (Tokyo, Japan) maintained at 60 °C. Mobile phase A consisted of 2 mmol/L ammonium acetate with 0.1% formic acid (v/v) in water, and mobile phase B consisted of 2 mmol/L ammonium acetate with 0.1% formic acid (v/v) in methanol. The gradient program was started at 60% B and then increased to 85% B at 3 min, followed by a change to 95% B at 6 min and to 100% B at 6.5 min, switched back to the starting conditions at 60% B from 6.5 min to 6.6 min, then the column was allowed to equilibrate for 1.4 min. The flow rate of the mobile phase was set at 0.25 mL/min and the total analysis time was 8.0 min. LabSolutions software (SHIMADZU, Japan) was used for data acquisition and analysis.

The MRM transitions, collision energies, and retention times of tacrolimus, everolimus, and IS are presented in **Table 3**. The C_{tissue} of tacrolimus and everolimus were converted from ng/mL in the extracted samples to pg/mg tissue in the analytes in biopsy samples.

Table 3. Monitored transitions, collision energies and retention times of tacrolimus, everolimus, and IS.

Compounds	MRM transition	Collision energy	Retention time (min)
tacrolimus	821.4 > 768.35	-22	6.34
everolimus	980.1 > 389.2	-55	6.66
IS	809.3 > 756.35	-23	6.26

IS, internal standard; MRM, multiple reaction monitoring.

2-3 Preparation of samples

Frozen kidney biopsy samples (0.5–1 mg) were thawed and dried on filter paper at room temperature for 90 min. After drying, the sample was weighed and placed in a 1.5 mL empty Eppendorf tube. For each sample, ultrapure water (100 μL) was added, and the sample was first shredded with scissors and then homogenized by passing the tissue fragments 10 times through a 20-gauge needle and 20 times through a 24-gauge needle using a 1.0 mL syringe until there was no obvious fragment in the homogenate. The tissue homogenate (50 μL) was transferred to a new 1.5 mL tube, followed by the addition of methanol (20 μL) and vortexing for 10 s. Subsequently, protein precipitation solution (80 μL) (1 ng/mL IS in 70/30 methanol/zinc sulfate solution 0.1 M) was added to the sample, vortexed for 15 min, and centrifuged for 10 min (9400 g, 4°C). The supernatant was transferred to a filter vial (0.2 μm) for injection into the LC-MS/MS system.

2-4 Preparation of calibration and quality control samples

Rat blank kidney tissue was homogenized with ultrapure water to a concentration of 1 mg of tissue per mL. Working solutions were prepared by mixing and diluting stock solutions (20 μg/mL in 100% methanol) of tacrolimus and everolimus (stored at −80 °C). Six calibration standards were prepared by spiking 20 μL of the working solution into 50 μL of blank homogenates. These calibration standards were then extracted as described above to yield the following final concentrations: 0.02, 0.06, 0.30, 0.60, 1.2, and 2.0 ng/mL for tacrolimus; and 0.04, 0.12, 0.60, 1.2, 2.4, and 4.0 ng/mL for everolimus, respectively. Similarly, the lower limit of quantification (LLOQ) and three concentrations of quality control (QC) samples (low, medium, and high QC) were prepared with independent working solutions to yield the following final

concentrations: 0.02, 0.05, 0.50, and 1.5 ng/mL for tacrolimus; and 0.04, 0.10, 1.0, and 3.0 ng/mL for everolimus, respectively.

2-5 Validation of method

Selectivity, LLOQ, linearity, accuracy, precision, carry-over, recovery, matrix effects, and stability were evaluated according to the principles of the Food and Drug Administration (FDA) guidelines for bioanalytical methods.

2-5-1 Selectivity, LLOQ, and linearity

Six lots of rat blank samples were analyzed to assess the interference from endogenous compounds. The interfering signals at the retention times should be < 20% of the LLOQ for tacrolimus and everolimus and < 5% for the IS. Cross-talk was evaluated by spiking blank kidney samples with a single compound at high QC concentrations to detect interference between tacrolimus and everolimus. The LLOQ was defined as the concentration that yielded a signal-to-noise ratio > 5 for tacrolimus and everolimus.

Method linearity was evaluated by analyzing calibration samples at six concentration levels over three consecutive days. Calibration curves were constructed by plotting the peak area ratio of the analytes to the IS versus the nominal concentrations and calculated using the least squares method. Linearity was acceptable if the coefficient (r^2) of the calibration curves was greater than 0.99 and calibration standards concentrations were within ± 15 % (or ± 20% for the LLOQ) deviation of nominal concentrations.

2-5-2 Accuracy and precision

Intra-day accuracy and precision were determined by analyzing QC samples (LLOQ, low, medium, and high QC) in replicates (n = 5) in a single analytical run. Inter-day accuracy and precision were obtained by repeating the analysis of five replicates over three different days. Inaccuracy was assessed by calculating the bias to nominal concentrations, and imprecision was expressed as the relative standard deviation (RSD %). The acceptance criteria for inaccuracy and imprecision were within the ranges of ± 15 % and 15% (± 20% and 20% for LLOQ), respectively.

2-5-3 Carry-over

Carry-over was evaluated by analyzing blank samples immediately following the highest calibration standard. Carry-over was considered acceptable if the response area in the blank sample was < 20% of the LLOQ and < 5% of the IS.

2-5-4 Recovery and matrix effect

Recovery and matrix effect were assessed at low and high QC levels using a post-extraction addition approach. QC samples were prepared by spiking blank kidney homogenates from six different sources and were extracted as described above (pre-spiked, sample A). For each source, blank kidney samples were first extracted and then spiked with analytes and IS to have the same concentrations as sample A (post-spiked, sample B). Neat solutions containing analytes and IS at the same concentrations as in samples A and B were prepared in methanol/water (50/50) (sample C). Recovery, matrix factor (MF), and IS-normalized MF (IS-norm MF) values were calculated as follows (n = 6):

$$Recovery\ (\%) = \frac{peak\ area\ of\ pre-spiked\ sample\ (A) \times 100}{peak\ area\ of\ post-spiked\ sample\ (B)}$$

$$MF\ (\%) = \frac{peak\ area\ of\ post-spiked\ sample\ (B) \times 100}{peak\ area\ of\ neat\ sample\ (C)}$$

$$IS-norm\ MF\ (\%) = \frac{MF\ of\ analyte \times 100}{MF\ of\ IS}$$

The RSD% of the IS-norm MF calculated from the six matrix lots should be less than 15 %.

2-5-5 Stability

The stability of analytes in tissues was investigated by analyzing low and high QC samples in replicates (n = 3). Bench-top stability was determined by keeping the spiked tissue samples at room temperature for 6 h. Long-term stability was assessed using spiked tissue samples stored at −80 °C for 3 months. Freeze-thaw stability was evaluated after three consecutive freeze-thaw cycles (from −80 °C to room temperature). Post-preparative stability (autosampler stability) was assessed by keeping the extracted samples at 20 °C for 20 h in an autosampler. The samples were considered stable if the bias between the tested condition samples and freshly prepared QC samples at the same concentrations were within ±15 %.

2-6 Application to the clinical biopsy samples

Fourteen adult kidney transplant recipients (age: 31-67) were enrolled in this study. All recipients were co-administered with tacrolimus and everolimus. This study was performed in accordance with the Declaration of Helsinki and its amendments and was approved by the Institutional Review Board of the Kyushu University Graduate School

and Faculty of Medicine (approval number: 588–05). All participants provided written informed consent. A 3-month protocol biopsy was performed for every recipient for histological evaluation according to the Banff 2013 classification. Subclinical acute rejection (SubAR) was identified by the presence of tubulointerstitial mononuclear infiltration with a requirement of < 10 % rise in serum creatinine in 2 weeks before the protocol biopsy and no absence of clinical functional deterioration. Borderline changes (BC) were defined as suspicious for acute rejection in Banff classification, and identified by no intimal arteritis is present, but there are foci of tubulitis (t1, t2, or t3) with minor interstitial infiltration (i0, or i1) or interstitial infiltration (i2, i3) with mild (t1) tubulitis. Patients whose biopsy samples showed no obvious evidence of acute rejection were classified as no rejection (NR). The severity of interstitial fibrosis and tubular atrophy (IF/TA) was graded by the percentage of renal cortex with IF/TA: grade 0, 0-25% of cortical area; grade 1, >25% of cortical area; grade 2, 26-50% of cortical area; grade 3, >50% of cortical area. All biopsies were evaluated by two experienced nephrologists who reached consensus using a light microscope.

The remainder of the protocol biopsy samples were stored at −80 °C to measure the C_{tissue} of tacrolimus and everolimus. Whole venous blood samples were obtained from recipients before they received the morning doses of tacrolimus and everolimus. The C_{blood} of tacrolimus and everolimus were measured by a chemiluminescent immunoassay (CLIA) and an electrochemiluminescence immunoassay (ECLIA) (Architect; Abbott Park, Illinois, USA), respectively.

2-7 Statistical analysis

Statistical analysis was performed using Prism 8.0 (GraphPad Software, San Diego, CA, USA). The correlation between tacrolimus or everolimus C_{tissue} (or C_{tissue}/D) and C_{blood} (or C_{blood}/D) was analyzed using Spearman's correlation. The Kruskal-Wallis test was

used to compare the differences in tacrolimus or everolimus C_{tissue} among recipients with no rejection, borderline changes, and SubAR. The C_{tissue} of tacrolimus or everolimus in different IF/TA grade groups were compared using the Mann-Whitney U test. Statistical significance was set at $P < 0.05$.

3. RESULTS

3-1 Validation of method

3-1-1 Selectivity, lower limit of quantification, and linearity

There was no interfering peak observed at the tacrolimus, everolimus, and IS retention times of 6.34 min, 6.66 min, and 6.26 min in blank samples, respectively, and crosstalk interference was not found in any analysis. Representative chromatograms of tacrolimus, everolimus, and IS in an LLOQ sample and a blank sample are shown in **Figure 2.** Calibration curves were found to be linear with $r^2 > 0.99$, as calculated by a weighing factor of $1/x^2$ for all analytes (**Table 4**). LLOQ was 0.02 ng/mL and 0.04 ng/mL for tacrolimus and everolimus, respectively.

Table 4. Summary of calibration curve for tacrolimus and everolimus.

Analyte	n = 3		
	Coefficients (r^2) (Mean ± SD)	RSD (%)	Bias (%)
tacrolimus	0.9982 ± 0.000147	0.4 – 5.2	-4.1 – 3.7
everolimus	0.9966 ± 0.000954	1.5 – 7.5	-1.9 – 2.1

$$Bias\ (\%) = \frac{Mean\ of\ measured\ value - theoretical\ value}{theoretical\ value} \times 100$$

RSD, relative standard deviation

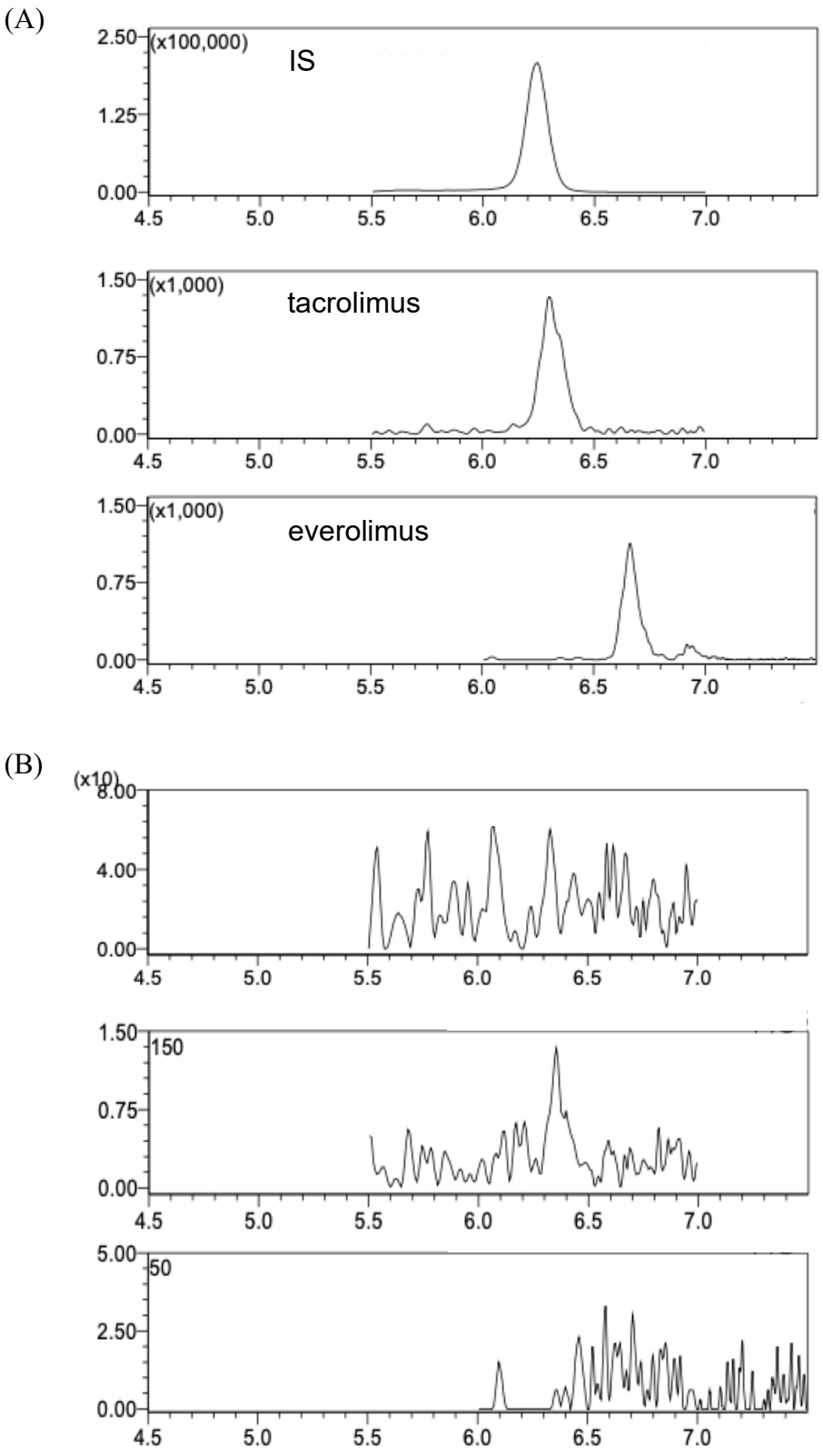


Figure 2. Representative chromatograms of (A) an LLOQ sample and (B) a blank sample. LLOQ, lower limit of quantitation

3-1-2 Accuracy and precision

The intra- and inter-day accuracy and precision of tacrolimus and everolimus are summarized in **Table 5**. Inaccuracy for tacrolimus and everolimus ranged from -9.6 % to -2.2 % at three QC levels (-16.3 % and -9.2 % at LLOQ for tacrolimus and everolimus, respectively). Imprecision was ≤ 12.0% at all validated concentrations of tacrolimus and everolimus. The results demonstrated that the present method for the quantification of tacrolimus and everolimus in kidney tissues was accurate and reproducible.

Table 5. Intra- and inter-day accuracy and precision of tacrolimus and everolimus.

Analyte	Nominal concentration (ng/mL)	Imprecision (RSD%)		Overall bias (%) (n = 15)
		Intra-day (n = 5)	Inter-day (n = 15)	
Tacrolimus	0.02	4.5	1.5	-16.3
	0.05	3.1	6.0	-9.6
	0.5	1.4	7.8	-7.0
	1.5	1.3	8.3	-4.0
Everolimus	0.04	6.7	5.1	-9.2
	0.1	5.0	7.1	-4.1
	1	2.1	12.0	-2.2
	3	3.0	4.2	-6.6

$$Bias\ (\%) = \frac{Mean\ of\ measured\ value - theoretical\ value}{theoretical\ value \times 100}$$

RSD, relative standard deviation

3-1-3 Carry-over

No carry-over was observed in the blank samples analyzed directly after the highest calibration standard samples.

3-1-4 Recovery and matrix effect

As shown in **Table 6**, the recovery for tacrolimus and everolimus ranged from 91.4 % to 105.9 % with RSD ≤ 8.0 % at low and high QC levels. The IS-norm MFs evaluated from six different sources ranged from 91.1 % to 112.2 % with RSD ≤ 6.4 %, indicating that there was no significant matrix effect in the method.

Table 6. Recovery and matrix effect evaluated from 6 rat blank kidney sources.

Analyte	Recovery (%) (n = 6)				IS-norm MF (%) (n = 6)			
	Low QC		High QC		Low QC		High QC	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
tacrolimus	105.9	2.3	103.8	2.8	98.6	3.7	93.6	2.7
everolimus	91.4	7.8	104.7	8.0	112.2	6.4	91.1	6.0

QC, quality control; RSD, relative standard deviation.

MF, matrix factor; IS-norm MF, internal standard-normalized matrix factor.

3-1-5 Stability

Bench-top stability

After standing on the bench for 6 h, low QC samples and high QC samples were processed and analyzed along with freshly prepared QC samples (n = 3). The bias (%) from the fresh low QC and high QC samples was -0.8 % and 0.1 % for tacrolimus and 4.3 % and 0.7 % for everolimus, respectively (**Table 7**). The results showed that the analytes were stable in tissues for up to 6 h at room temperature.

Table 7. Summary of bench-top stability for tacrolimus and everolimus in rat kidney tissues.

	Nominal concentration (ng/mL)	Bias (%) (n = 3)
tacrolimus	0.05	-0.8
	1.5	0.1
everolimus	0.1	4.3
	3.0	0.7

$$Bias\ (\%) = \frac{Test\ condition\ QC\ \ value - fresh\ QC\ value}{Fresh\ QC\ value \times 100}$$

Long-term storage stability

To test long-term storage stability, low QC and high QC samples stored at -80 °C for 3 months were processed and analyzed along with the freshly prepared QC samples (n = 3). The bias % at low and high QC levels was 0.0 % and 1.9 % for tacrolimus and -3.9 % and 8.4 % for everolimus, respectively (**Table 8**). The data suggest that tissue samples are stable at -80 °C for at least 3 months.

Table 8. Summary of long-term storage stability for tacrolimus and everolimus in rat kidney tissues.

	Nominal concentration (ng/mL)	Bias (%) (n = 3)
tacrolimus	0.05	0.0
	1.5	1.9
everolimus	0.1	-3.9
	3.0	8.4

$$Bias\ (\%)\ = \frac{Test\ condition\ QC\ \ value\ -\ fresh\ QC\ value}{Fresh\ QC\ value\ \times\ 100}$$

Freeze-thaw stability

Triplicates of low QC and high QC samples after three freeze-thaw cycles were processed and analyzed along with freshly spiked QC samples. The bias (%) from freshly prepared low and high QC samples was -9.1 % and -5.0 % for tacrolimus, and -12.7 % and -8.2 % for everolimus, respectively (**Table 9**). The results suggest that analytes are stable in tissue samples after three freeze-thaw cycles.

Table 9. Summary of freeze-thaw stability for tacrolimus and everolimus in rat kidney tissues

	Nominal concentration (ng/mL)	Bias (%) (n = 3)
tacrolimus	0.05	-9.1
	1.5	-5.0
everolimus	0.1	-12.7
	3.0	-8.2

$$Bias\ (\%) = \frac{Test\ condition\ QC\ \ value - fresh\ QC\ value}{Fresh\ QC\ value \times 100}$$

Autosampler stability

To evaluate autosampler stability, the prepared low QC and high QC samples were left in the autosampler and analyzed with the freshly prepared QC samples after 20 h (n = 3). The bias % at low and high QC levels was 9.1% and 1.7% for tacrolimus and 3.5% and -7.6% for everolimus, respectively (**Table 11**), demonstrating that the processed samples were stable in the autosampler for 20 h.

Table 10. Summary of autosampler stability for tacrolimus and everolimus in rat kidney tissues

	Nominal concentration (ng/mL)	Bias (%) (n = 3)
tacrolimus	0.05	-9.1
	1.5	-1.7
everolimus	0.1	3.5
	3.0	-7.6

$$Bias\ (\%)\ = \frac{Test\ condition\ QC\ \ value\ -\ fresh\ QC\ value}{Fresh\ QC\ value\ \times\ 100}$$

3-2 Clinical application

3-2-1 Patient characteristics and kidney concentrations of tacrolimus and everolimus

Recipient demographic characteristics are presented in **Table 11**. The measured C_{tissue} ranged from 21.0 to 81.7 pg/mg tissue and 33.5 to 105.0 pg/mg tissue for tacrolimus and everolimus in the fourteen collected kidney biopsies, respectively. According to the histological results, six recipients were diagnosed with borderline changes, and three recipients were diagnosed with subAR.

Table 11. Characteristics of patients.

Characteristics	**n = 14**
Age (years)	50.0 ± 11.9
Sex (male/female)	9/5
Body weight (kg)	58.7 ± 12.8
Reasons for kidney transplantation	
IgA nephropathy	2
Diabetic nephropathy	4
Chronic glomerulonephritis	2
Polycystic kidney	2
Others	4
Serum creatinine (mg/dL)	
Pre-transplant	7.69 ± 2.90
3 months after transplantation	1.31 ± 0.32
Blood urea nitrogen (mg/dL)	
Pre-transplant	68.93 ± 26.15
3 months after transplantation	24.69 ± 11.82
Borderline changes (n)	6
Biopsy-proven SubAR (n)	3

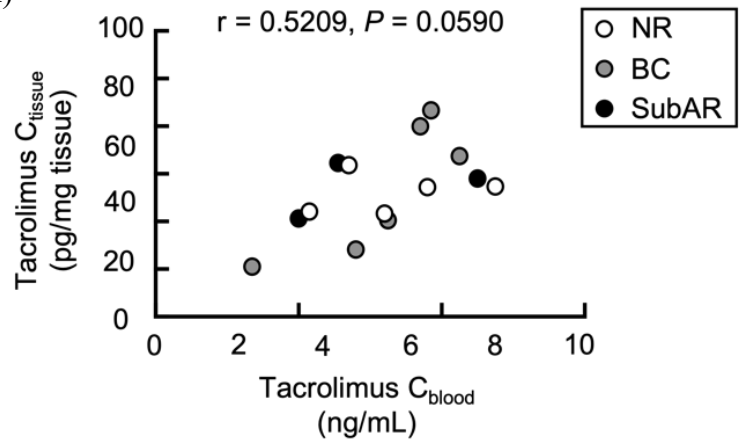
Data are expressed as mean ± SD.

SD, standard deviation; SubAR, subclinical AR

3-2-2 Correlation between the whole blood concentrations and kidney concentrations of tacrolimus and everolimus

No significant relationship was observed between tacrolimus and everolimus C_{tissue} and C_{blood} ($P = 0.0590$ and $P = 0.2272$, respectively) at 3 months after kidney transplantation. However, after normalizing C_{tissue} and C_{blood} of tacrolimus and everolimus by the corresponding doses, significant correlations emerged ($r = 0.9385$, $P < 0.0001$ and $r = 0.6659$, $P = 0.0113$, respectively) (**Figure 3**).

(A)



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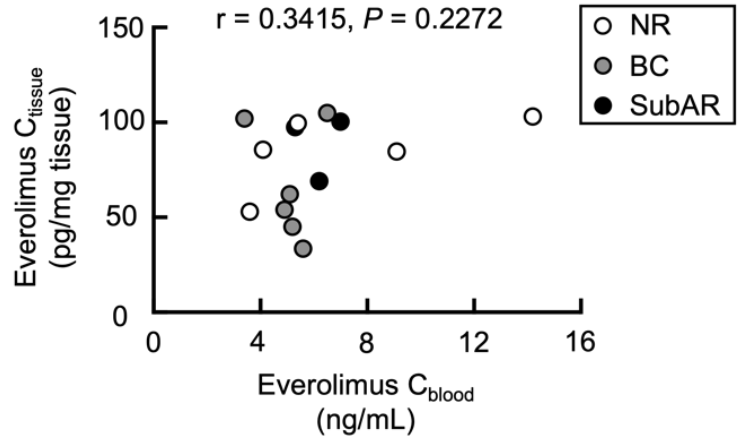

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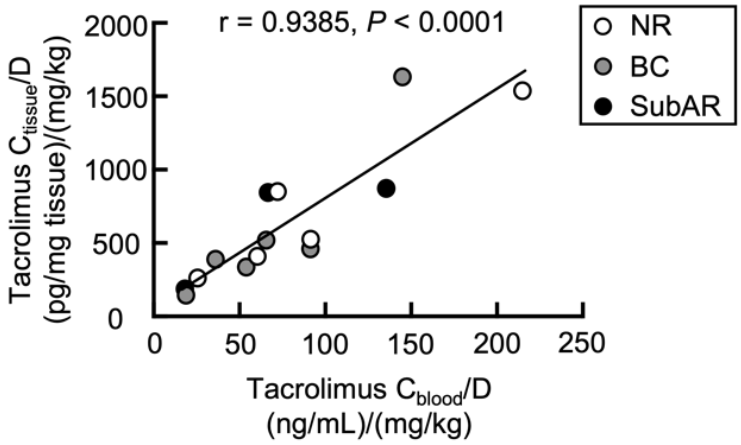

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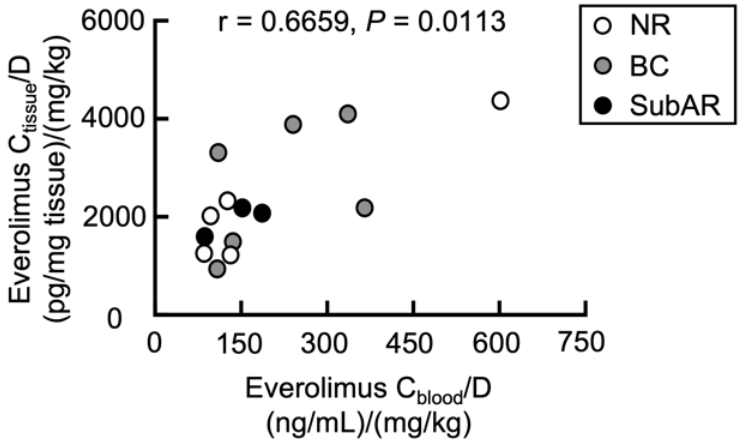
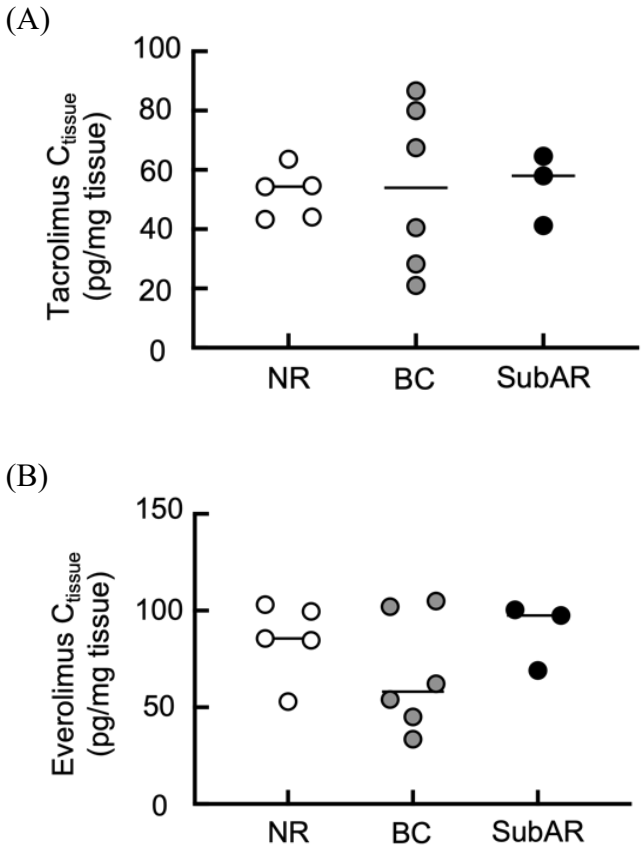


Figure 3. (A) Correlation between tacrolimus C_{blood} and C_{tissue}; (B) Correlation between everolimus C_{blood} and C_{tissue}; (C) Correlation between tacrolimus C_{blood}/D and C_{tissue}/D; (D) Correlation between everolimus C_{blood}/D and C_{tissue}/D. Statistical analyses were performed using the Spearman correlation. NR, no rejection; BC, borderline changes; SubAR, subclinical acute rejection.

3-2-3 Relationships between the histopathological findings and kidney concentrations of tacrolimus and everolimus

There were no significant differences in either tacrolimus or everolimus C_{tissue} among recipients with no rejection (n = 5), borderline changes (n = 6), or SubAR (n = 3) (P = 0.9752 and P = 0.6755, respectively). In addition, there were nine recipients with IF/TA grade 0 and five recipients with grade ≥ 1 according to the Banff 2013 classification. Tacrolimus and everolimus C_{tissue} showed no significant difference between recipients with IF/TA grade 0 (n = 9) and grade ≥ 1 (n = 5) (P = 0.8981 and P = 0.1469, respectively) (**Figure 4**). The C_{tissue}/D of tacrolimus and everolimus were also compared among the recipients with the different histopathological results, respectively; similarly, no significant differences in tacrolimus and everolimus C_{tissue}/D were found (date not shown).



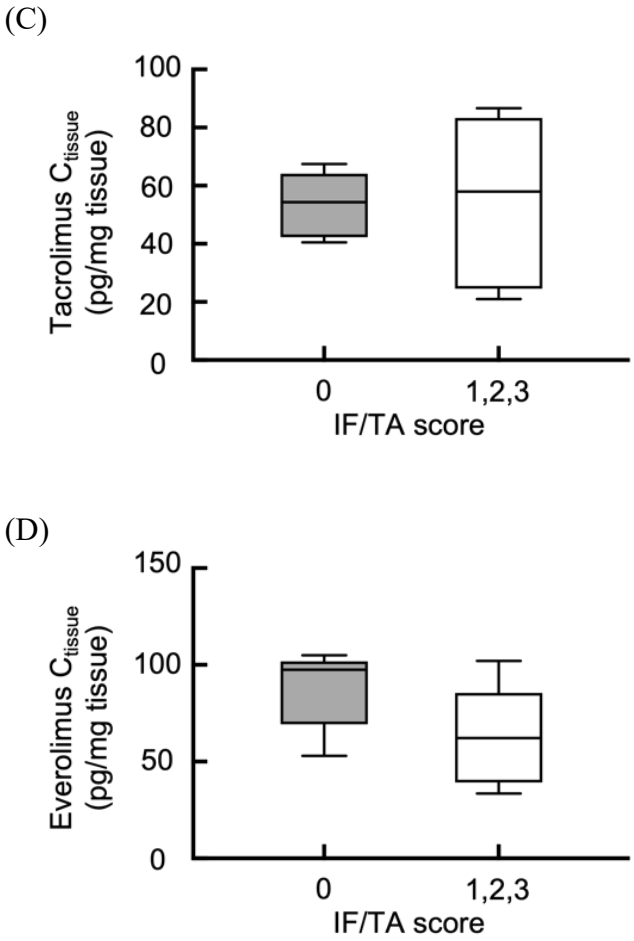


Figure 4. The C_{tissue} of (A) tacrolimus and (B) everolimus in no rejection (n = 5), borderline changes (n = 6), and SubAR groups (n = 3) of recipients at 3-month protocol biopsy. Statistical analyses were performed using the Kruskal–Wallis test. The C_{tissue} of (C) tacrolimus and (D) everolimus between recipients with IF/TA grade 0 (n = 9) and IF/TA grade ≥ 1 (n = 5) at the 3-month protocol biopsy. Statistical analyses were performed using the Mann–Whitney U test. The bars represent median values. NR, no rejection; BC, borderline changes; SubAR, subclinical acute rejection; IF/TA, interstitial fibrosis, and tubular atrophy.

4. DISCUSSION

This is the first study to develop a method for determining tacrolimus and everolimus concentrations in clinical kidney biopsies. The selectivity, linearity, precision, accuracy, stability, recovery, and matrix effect of the developed method were compliant with the requirements of the FDA Bioanalytical Method Validation Guideline. The overall design of the method was aimed at measuring the analyte concentrations in very small amounts of kidney biopsy tissues (wet weight: 0.5-1 mg), the homogenization was preformed manually with a syringe and needle to reduce tissue loss. To remove the interfering compounds in the kidney tissues and avoid matrix effects, a mixture of acetonitrile, zinc sulfate, and water was used to precipitate the protein from the homogenate. In previous study, the sample preparation included incubation with digest buffers at 55°C for 90 min followed by liquid extraction.[34] The preparation procedure used in this study was simple, time-saving, economical, and minimized contamination of the LC-MS/MS system. The LLOQ of tacrolimus in kidney tissue homogenate has been improved from 0.031 ng/mL in previously published work to 0.02 ng/mL[35], sufficient to achieve a reliable quantification of tacrolimus in biopsy samples.

In the evaluation of the matrix effect, no obvious ion suppression/enhancement was observed. Therefore, the common agent ascomycin was chosen as the IS instead of isotope-labeled tacrolimus or everolimus in light of practical and economic considerations. In this study, it was shown that the tissue samples were stable for up to 6 h at room temperature (bias $\leq$ 4.3 %), covering the time range from the biopsy sampling at the recipients' bedside to the sample transport to the laboratory for storage (about 2 h). The developed method has high sensitivity and reproducibility with a simple sample preparation and could allow the quantification of tacrolimus and everolimus concentrations in biopsy-sized kidney tissue samples.

The measured C_{tissue} of tacrolimus and everolimus in kidney transplant recipients was within the concentration range accessible to the newly developed method. A significant association was observed between tacrolimus C_{tissue}/D and C_{blood}/D in recipients, which is consistent with the study of Sallustio et al.[31] However, the correlation between everolimus C_{tissue}/D and C_{blood}/D was weaker (r = 0.6659, *P* = 0.0113) compared to that of tacrolimus (r = 0.9385, *P* < 0.0001). This might be due to the differences in drug transport, which are influenced by factors such as drug lipophilicity, transporter, red blood cells binding, and tissue affinity, affecting drug distribution and equilibration from blood to the organ.[76, 77] Further studies are needed to describe and compare the pharmacokinetics of tacrolimus and everolimus in kidney allografts. Some outliers are shown in **Figure 3**. These cases showed relatively high tacrolimus or everolimus C_{blood}, but low tacrolimus or everolimus C_{tissue}. Although these recipients have a target C_{blood}, there is a possibility of inadequate immunosuppression in the allograft. It was found that recipients with borderline changes had a lower everolimus C_{tissue} than recipients with no rejection, but the difference was not statistically significant. The non-standardized biopsy sampling time possibly caused fluctuations in C_{tissue} during the different dosing intervals. Additionally, the small sample size might have contributed to the lack of statistical significance.

Chronic kidney allograft injury in kidney transplant recipients is often reflected by IF/TA, which are closely associated with progressive graft deterioration.[78] Activation of the mTOR pathway has been reported to be related to extracellular matrix synthesis and kidney fibrosis, and mTOR inhibitors have the potential to protect the graft from fibrosis by diminishing the number of interstitial fibroblasts and myofibroblasts and decreasing TGF-β1 expression.[79] Several studies have demonstrated that mTOR inhibitors could improve the course of IF/TA in kidney transplant recipients.[80-82] In this study, recipients with IF/TA grade 0 tended to have a higher everolimus C_{tissue} than

those with IF/TA grade ≥ 1 (P = 0.1469). It would be interesting to investigate the relationship between everolimus C_{tissue} and IF/TA in a long-term follow-up study to reveal the protective effect of everolimus on kidney allografts in kidney transplant recipients. This study had some limitations. First, tacrolimus and everolimus blood concentrations were measured by CLIA and ECLIA assays instead of LC-MS/MS; thus, the results could be affected by the metabolite cross-activity derived from the immunoassays. Second, the number of enrolled patients was small; consequently, the results might not precisely reveal the relationship between tacrolimus or everolimus allograft kidney concentration and clinical outcome. Further studies with a larger sample size are needed to confirm the clinical value of tacrolimus and everolimus allograft concentrations in kidney transplantation.

5. BRIEF SUMMARY

The developed LC-MS/MS method was fully validated according to FDA requirements. The concentrations in kidney homogenate could be measured in the range of 0.02-2.0 ng/mL for tacrolimus, and 0.04-4.0 ng/mL for everolimus. This method requires only a simple protein precipitation process and has a run time of 8 min. Tissue samples were stable for at least 6 h at room temperature, 3 months of storage at -80 °C, 3 freeze-thaw cycles, and 20 h at an autosampler. The developed method was successfully used to measure kidney tacrolimus and everolimus concentrations in kidney transplant recipients, and it was revealed that the C_{tissue}/D of tacrolimus and everolimus was significantly associated with their corresponding C_{blood}/D.

CHAPTER 2
EFFECT OF DONOR *CYP3A5* GENE POLYMORPHISM ON TACROLIMUS KIDNEY CONCENTRATION IN KIDNEY TRANSPLANT RECIPIENTS

1. INTRODUCTION

Although TDM of tacrolimus has been widely performed in kidney transplant recipients to reduce the risk of AR and CNI-related nephrotoxicity after transplantation, the clinical outcomes have only improved to a limited extent.[23, 83] The relationship between tacrolimus C_{blood} and the risk of rejection or nephrotoxicity is controversial.[84, 85] Numerous studies have attempted to investigate the factors related to tacrolimus adverse events. It is well known that tacrolimus has a high inter-patient pharmacokinetic variability, which is largely attributable to interindividual differences in the functional activity of CYP3A5 enzyme.[37, 42, 46] The *CYP3A5*3* mutant allele (*6986A>G*) in intron 3 of *CYP3A5* is the major defective allele, and it has a significant impact on tacrolimus metabolism and pharmacokinetics.[38, 42] To date, most studies have focused on thes effect of recipient *CYP3A5* genotype (hepatic or intestinal *CYP3A5*) on tacrolimus blood levels; however, the relationship between recipient *CYP3A5* or tacrolimus blood concentrations and clinical outcomes remains controversial.[86-88]

It has been reported that the CYP3A5 protein is also expressed in renal tubular epithelial cells.[64] The human kidney microsomes with the *CYP3A5*1* allele were associated with a higher metabolic activity compared to those with the *CYP3A5*3/*3* genotype.[89] Tacrolimus is metabolized by the CYP3A5 enzyme to produce three major metabolites **(Figure 5)**, namely, 13-O-desmethyl tacrolimus (M1), 31-O-desmethyl tacrolimus (M2), and 15-O-desmethyl tacrolimus (M3), which also have immunosuppressive activity or potential toxicity.[10, 69, 90, 91] Therefore, some studies

have hypothesized that intrarenal concentrations of tacrolimus or tacrolimus metabolites might be more related to clinical outcomes than blood concentrations, and suggested that allograft kidney *CYP3A5* gene polymorphism (donor genotype) could be a biomarker of AR or CNI-related nephrotoxicity in kidney transplant recipients.[84, 92, 93]

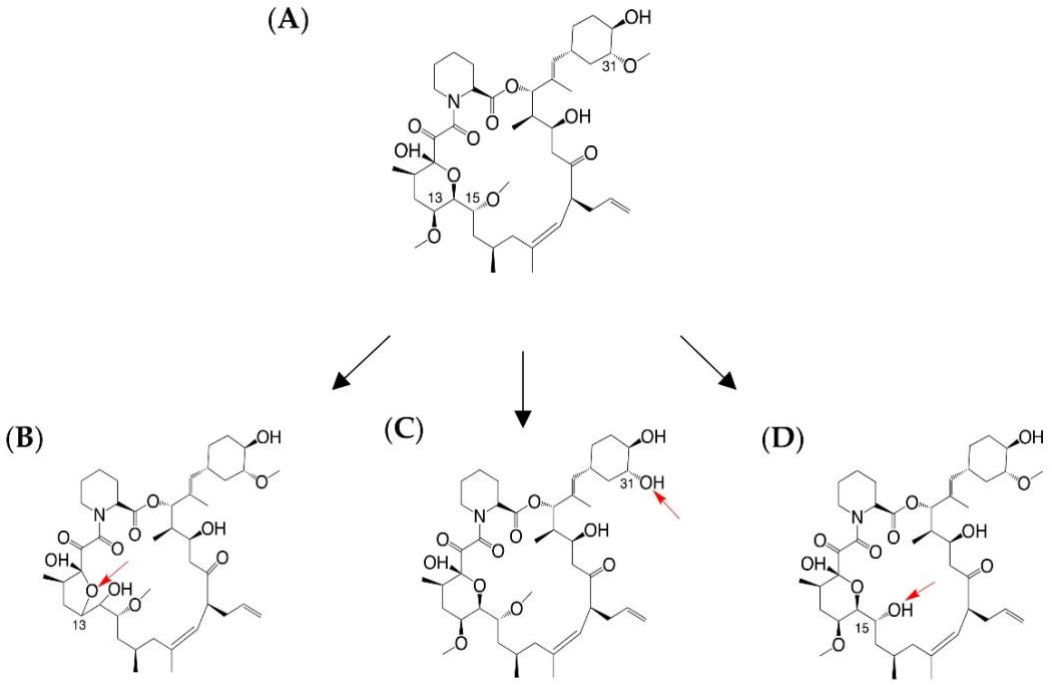


Figure 5. Chemical structures of (A) tacrolimus; (B) 13-O-desmethyl tacrolimus (M1); (C) 31-O-desmethyl tacrolimus (M2); (D) 15-O-desmethyl tacrolimus (M3).

However, to date, little is known about the relationship between donor *CYP3A5* gene polymorphisms and tacrolimus local kidney metabolism. Therefore, this chapter aimed to investigate the potential factors (tacrolimus dose, blood levels, and donor *CYP3A5* gene polymorphism) that affect tacrolimus C_{tissue}, as well as the relationship between tacrolimus C_{tissue} and biopsy-proven SubAR in kidney transplant recipients.

2. MATERIALS AND METHODS

2-1 Patients

A total of 52 Japanese adult kidney transplant recipients (age: 23-69) were enrolled in this study. All patients underwent kidney transplantation between August 2014 and August 2016 at Kyushu University Hospital. All recipients received a triple-drug regimen comprising tacrolimus, mycophenolate mofetil, and prednisolone. This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by the Institutional Review Board of Kyushu University Graduate School and Faculty of Medicine (approval number: 588-00). All patients enrolled in this study provided written informed consent for participation in the study and for the use of their samples.

2-2 Measurements of tacrolimus kidney concentrations

Recipient kidney biopsy samples were collected at 3-months and 1-year protocol biopsy for histological diagnosis, the remaining tissues were immediately deposited in liquid nitrogen, transported to the laboratory within 2 h, and stored at -80 °C until the day of the assay.

A fraction of kidney biopsies (1–3 mg of wet tissue) was used for measuring tacrolimus C_{tissue}. The quantification was performed on a Shimadzu LCMS-8050 liquid triple quadrupole tandem mass spectrometer (Shimadzu, Kyoto, Japan). The frozen kidney biopsy sample was dried on filter paper for 90 min at room temperature. Once dry, the biopsy sample was weighed and homogenized in 100 µL ultrapure water using a syringe and needle. Aliquots of 50 µL of tissue homogenate were transferred to a 1.5 mL microcentrifuge tube, and 20 µL methanol was added and then vortexed for 30 s. Then, 80 µL of protein precipitation solution (1 ng/mL ascomycin as IS in 70/30

methanol/zinc sulphate 0.1 mol/L) was added to the tube and vortexed at 1500 rpm for 15 min. After centrifugation at 9400 × g for 10 min, the supernatant was transferred to vials and injected into an LC-MS/MS system. Quantitation was performed with a GL Sciences Inertsil-ODS-3 (3 μm; 2.1 mm × 150 mm) column. Mobile phase A consisted of 2 mmol/L ammonium acetate with 0.1 % formic acid (v/v) in water, and mobile phase B consisted of 2 mmol/L ammonium acetate with 0.1 % formic acid (v/v) in methanol. The gradient was started at 60 % B, increased to 85% B at 3 min, increased to 95 % B at 6 min, increased to 100 % B at 6.5 min, switched back to the starting conditions at 60 % B from 6.5 min to 6.6 min, and equilibration for 1.4 min. The total analysis time was 8 min. The flow rate was 0.25 mL/min, the column temperature was maintained at 60 °C, and electrospray ionization was performed in positive mode. The analysis was based on MRM of m/z 821.40→768.35 for tacrolimus, 807.20→754.25 for M1/M2/M3, and 809.3→756.3 for IS, respectively.

2-3 Measurement of tacrolimus whole blood trough concentrations

Patient whole blood samples were collected prior to the daily administration of tacrolimus and were measured using a CLIA (Architect; Abbott Park, Illinois, USA). The corresponding tacrolimus C_{blood} values on the biopsy day were obtained from the clinical records at Kyushu University Hospital.

2-4 CYP3A5 genotyping

Donor genomic DNA was extracted from kidney biopsy samples using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany). Recipient genomic DNA was extracted from patients whole venous blood using a Wizard Genomic DNA Purification Kit (Promega, USA). DNA extraction was performed according to the manufacturer's protocols. Donor and recipient *CYP3A5*3 A > G* (rs776746) SNPs were genotyped

using TaqMan probes (Life Technologies, Carlsbad, CA, USA) performed on a LightCycler Nano (Roche, Basel, Switzerland). The polymerase chain reaction process included holding at 90°C for 10 min, followed by 40 cycles of 95°C to 60°C to 72°C, pre-melt holdingat 95°C for 30s and melting at 40°C to 75°C for 0.1°C/s.

2-5 Histological evaluation

All recipients underwent a 3-month protocol kidney biopsy, and 22 of them underwent an additional 1-year protocol biopsy after transplantation. Each biopsy sample was scored according to the Banff 2013 classification to diagnose SubAR as described in Chapter 1, and all biopsies were evaluated by two experienced nephrologists who reached a consensus using a light microscope.

2-6 Statistical analysis

Statistical analysis was performed using Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA). The Mann–Whitney U test was used to compare tacrolimus concentration differences in recipients with different *CYP3A5* genotypes, as well as recipients with and without SubAR. Correlations between tacrolimus dose and tacrolimus C_{tissue} or C_{blood}; tacrolimus C_{blood}/D and C_{tissue}/D; and tacrolimus C_{tissue} and M1 C_{tissue} were analyzed using Spearman's correlation. Statistical significance was set at $P < 0.05$.

3. RESULTS

3-1 Patient characteristics and CYP3A5 polymorphism

A total of 74 kidney biopsy samples were obtained from 52 kidney transplant patients (52 for 3-month protocol biopsy and 22 for 1-year protocol biopsy). The demographic data and genotyping results are presented in **Table 12**. Among the 52 kidney transplant recipients and their corresponding donors, 23 (44.2 %) recipients and 25 (48.1 %) donors exhibited the *CYP3A5*1/*1* or *CYP3A5*1/*3* genotype, while 29 (55.8 %) recipients and 27 (51.9 %) donors carried the *CYP3A5**3/*3 genotype. The allele frequencies for *CYP3A5*3* in donors and recipients were 71.2 % and 74.0 %, respectively. The results were consistent with the allele frequency of *CYP3A5*3* in the Asian population, as summarized in previous studies[21, 94].

Table 12. Characteristics of patients.

Characteristics	**n = 52**
Age (years)	43.9 ± 13.3
Sex (male/female)	31/21
Body weight (kg, range)	58.15 ± 14.48
Reasons for kidney transplantation (n)	
IgA nephropathy	8
Diabetic nephropathy	8
Chronic glomerulonephritis	10
Polycystic kidney	3
Type 1 diabetes	2
Type 2 diabetes	3
Hypertensive nephrosclerosis	3
Others	15
Serum creatinine (mg/dL)	
Pre-transplant	7.85 ± 3.38
3-month after transplantation	1.14 ± 0.28
1-year after transplantation	1.15 ± 0.25
Donor *CYP3A5* genotype (n, %)	
*1/*1 or *1/*3	25 (48.1 %)
*3/*3	27 (51.9 %)
Recipient *CYP3A5* genotype (n, %)	
*1/*1 or *1/*3	23 (44.2 %)
*3/*3	29 (55.8 %)

Data are expressed as mean ± standard deviation.

3-2 The impact of CYP3A5 polymorphism on tacrolimus pharmacokinetics

The influence of the donor and recipient *CYP3A5* polymorphisms on tacrolimus pharmacokinetics was evaluated by comparing the C_{blood} and C_{blood}/D of tacrolimus in kidney transplant recipients. The recipient *CYP3A5*3/*3* group had a significantly higher C_{blood}/D than the recipient *CYP3A5*1* (**1/*1 + *1/*3*) group at 3 months after kidney transplantation (P = 0.0008), which was consisted with the previous studies.[44-51] In contrast, no significant relationship was observed between the donor *CYP3A5* genotype and tacrolimus whole blood levels (**Table 13**).

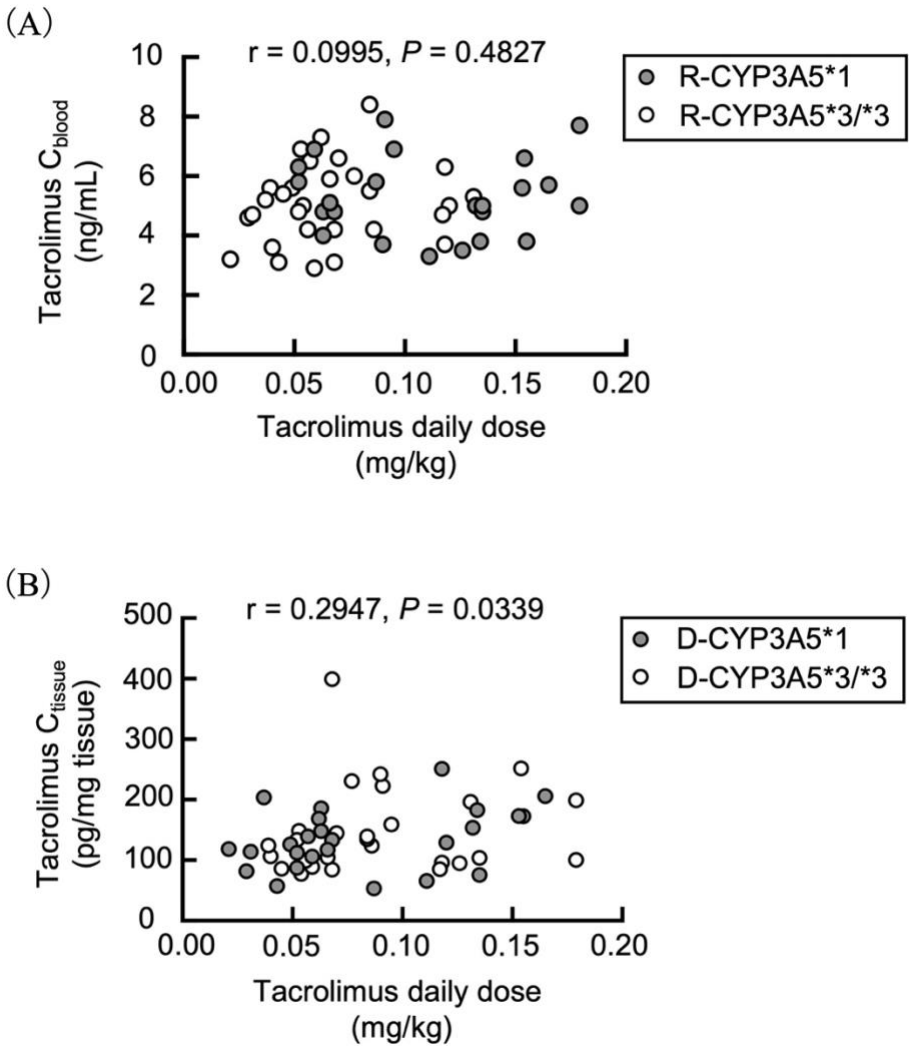
Table 13. Tacrolimus pharmacokinetics parameter according to *CYP3A5* genotype.

PK-Parameter	*CYP3A5* genotype	3-month protocol biopsy (n = 52)			1-year protocol biopsy (n = 22)		
		n	Mean ± SD	*P* value	n	Mean ± SD	*P* value
C_{blood} (ng/mL)	R-**1/*1* + R-**1/*3*	23	5.30 ± 1.32	0.5429	8	5.01 ± 1.45	0.7765
	R-**3/*3*	29	5.09 ± 1.34		14	5.24 ± 0.97	
C_{blood}/D (ng/mL)/(mg/kg)	R-**1/*1* + R-**1/*3*	23	57.08 ± 30.30	**0.0008**	8	57.45 ± 32.08	0.1266
	R-*3/*3	29	89.72 ± 38.55		14	88.30 ± 47.72	
C_{blood} (ng/mL)	D-**1/*1* + D-**1/*3*	25	5.03 ± 1.11	0.5697	10	5.32 ± 1.24	0.4857
	D-**3/*3*	27	5.32 ± 1.50		12	5.02 ± 1.08	
C_{blood}/D (ng/mL)/(mg/kg)	D-**1/*1 +D-*1/*3*	25	80.65 ± 43.09	0.5983	10	84.20 ± 49.45	0.3463
	D-**3/*3*	27	70.32 ± 33.70		12	71.15 ± 41.24	

PK, pharmacokinetics; R, recipient; D, donor; C_{blood}, whole blood trough concentration; C_{blood}/D, dose-adjusted whole blood trough concentration; SD, standard deviation.

3-3 Relationship between tacrolimus dose and tacrolimus concentrations in whole blood and kidney

Tacrolimus C_{tissue} values measured in 74 kidney biopsy samples ranged from 52 to 399 pg/mg tissue. There was a weak but significant positive correlation between tacrolimus daily dose and C_{tissue} (r = 0.2947, *P* = 0.0339) (**Figure 6**) at 3 months after transplantation, but no correlation between tacrolimus daily dose and C_{blood}.



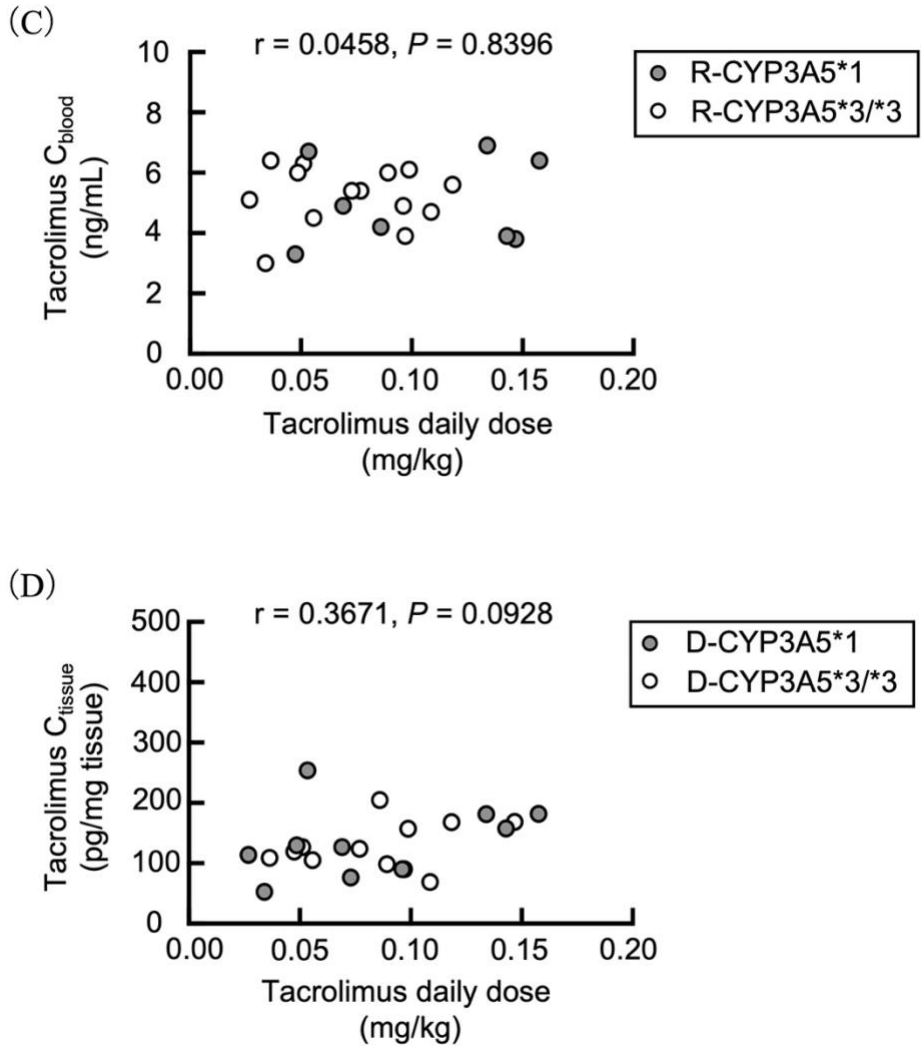


Figure 6. Correlations between tacrolimus daily dose and (A) tacrolimus C_{blood}, and (B) tacrolimus C_{tissue} at 3 months after transplantation (n = 52). Correlations between tacrolimus daily dose and (C) tacrolimus C_{blood}, and (D) tacrolimus C_{tissue} at 1 year after transplantation (n = 22). Statistical analyses were performed using the Spearman correlation. R, recipient; D, donor; C_{blood}, whole blood trough concentration; C_{tissue}, allograft kidney concentration.

3-4 Correlation between tacrolimus kidney concentrations and whole blood concentrations

Significant correlations between tacrolimus C_{tissue}/D and C_{blood}/D were observed both at 3 months and 1 year after transplantation (r = 0.7604, *P* <0.0001, and r = 0.7572, *P* <0.0001, respectively) (**Figure 7**). These findings indicate that tacrolimus dose and blood concentration may be influential factors in tacrolimus kidney exposure.

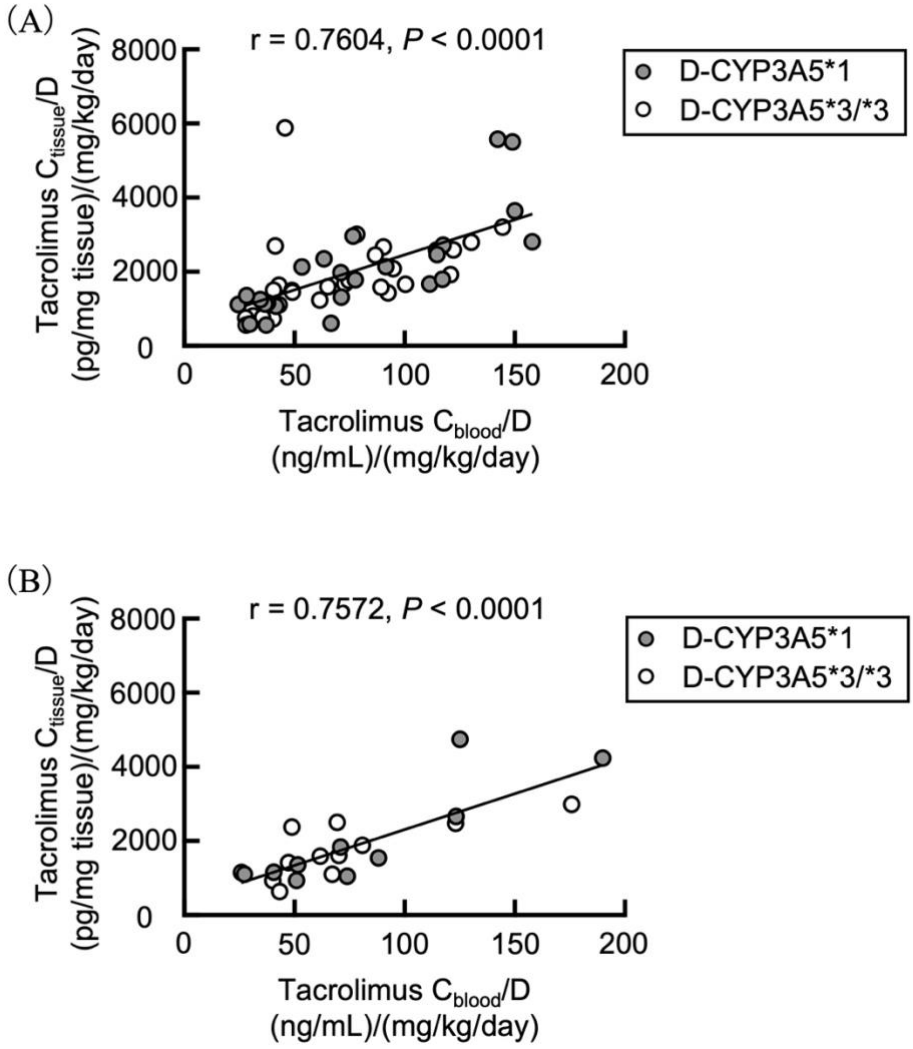


Figure 7. Correlation between tacrolimus C_{blood}/D and C_{tissue}/D at (A) 3 months (n = 52) and (B) 1 year after transplantation (n = 22). Statistical analyses were performed using the Spearman correlation. D, donor; C_{blood}/D, dose-adjusted whole blood trough concentration; C_{tissue}/D, dose-adjusted allograft kidney concentration.

3-5 Influence of recipient and donor *CYP3A5* genotype on tacrolimus kidney concentrations at 3 months and 1 year after transplantation

Tacrolimus C_{tissue}/D were compared in different recipient and donor *CYP3A5* genotype groups, respectively. As shown in **Figure 8**, recipient *CYP3A5* polymorphism has a significant impact on tacrolimus C_{tissue}/D (*CYP3A5*1 vs. CYP3A5*3/*3* = 1503.06 ± 737.12 *vs.* 2371.39 ± 1346.33, P = 0.0096) at 3 months after transplantation, but no significant relationship was observed between the donor *CYP3A5* genotype and tacrolimus kidney exposure.

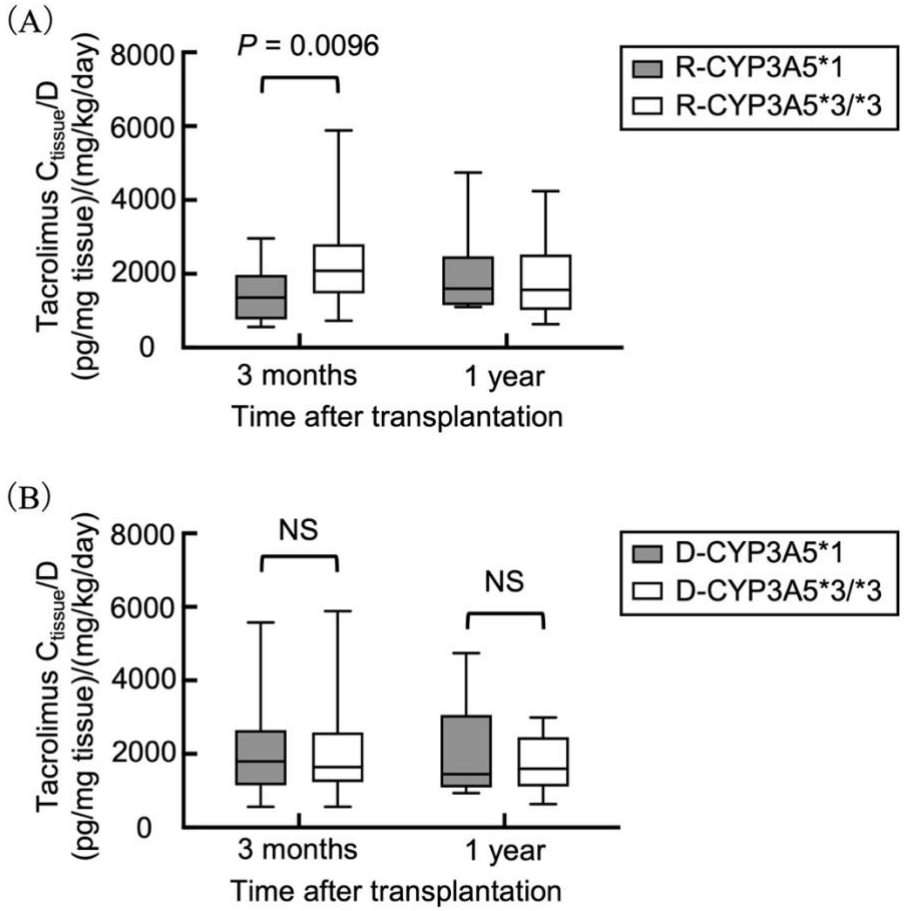
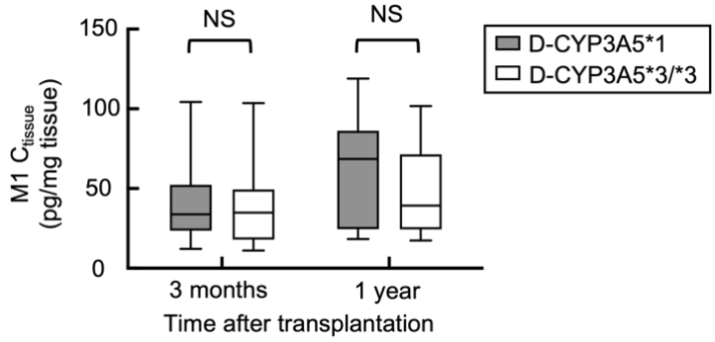


Figure 8. Effects of (A) recipient *CYP3A5* genotype and (B) donor *CYP3A5* genotype on tacrolimus C_{tissue}/D at 3 months (n = 52) and 1 year after transplantation (n = 22). Statistical analyses were performed using Mann–Whitney U test. The bars show the standard deviation in each group. C_{tissue}/D, dose-adjusted allograft kidney concentration.

3-6 Influence of donor *CYP3A5* genotype on tacrolimus metabolite concentrations in kidney at 3 months and 1 year after transplantation

To further investigate the metabolism of tacrolimus in the kidney, the concentrations of three major tacrolimus metabolites (M1, M2, and M3) were measured in 74 biopsy samples, of which 66 (89.2 %), 15 (20.3 %), and 3 (4.1 %) samples had M1, M2, and M3 concentrations above the lower limit of quantification (LLOQ, 0.01 ng/mL), respectively. The mean C_{tissue} of M1, M2, and M3 was 29.1 %, 8.43 %, and 5.18 % of tacrolimus C_{tissue}, respectively. Due to the low number of detections of M2 and M3, only the association between M1 C_{tissue} and the donor *CYP3A5* genotype was investigated. Similarly with tacrolimus C_{tissue}, the donor *CYP3A5* polymorphism had no significant impact on M1 C_{tissue}. However, significantly associations between M1 C_{tissue} and tacrolimus C_{tissue} were observed both at 3 months and 1 year after kidney transplantation ($r = 0.6008$, $P < 0.0001$, and $r = 0.6632$, $P = 0.0014$, respectively) (**Figure 9**).

(A)



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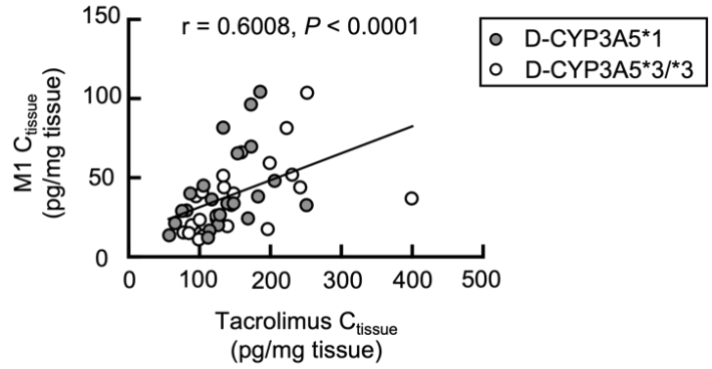

(C)

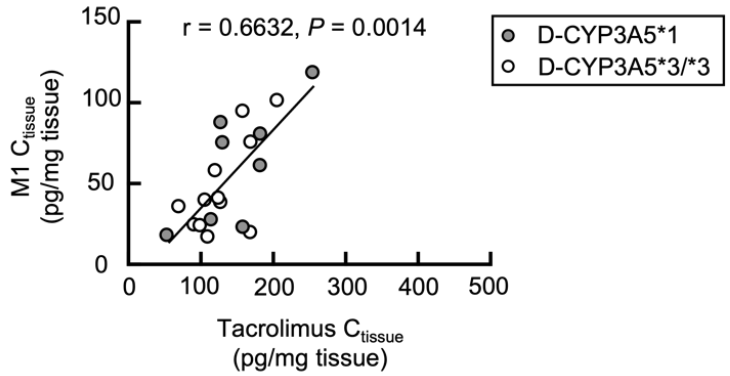

Figure 9. (A) Effect of donor *CYP3A5* genotypes on M1 C_{tissue} at 3 months (n = 46) and 1 year (n = 20) after kidney transplantation. Statistical analyses were performed using Mann–Whitney U test. Correlation between the C_{tissue} of tacrolimus and M1 at (B) 3 months (n = 46) and (C) 1 year after transplantation (n = 20). Statistical analyses were performed using Spearman's correlation Bars show the standard deviation in each group. D, donor; C_{tissue}, allograft kidney concentration.

3-7 Relationship between SubAR and tacrolimus kidney concentrations

Seven (13.5%) and four (18.2%) recipients were diagnosed with biopsy-proven SubAR at 3 months and 1 year after kidney transplantation, respectively. By comparing tacrolimus C_{tissue} and C_{tissue}/D between the no rejection and SubAR groups of recipients, no significant difference was found either at 3 months or 1 year after kidney transplantation (**Figure 10**).

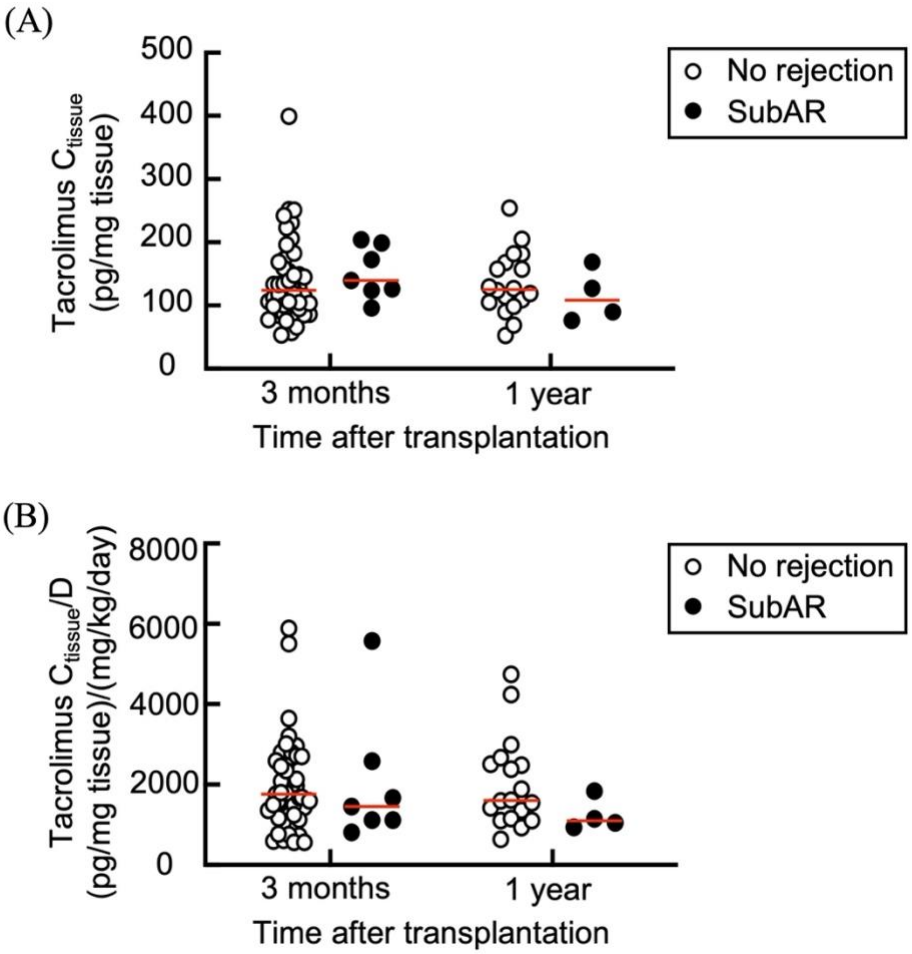

Figure 10. Differences in tacrolimus (A) C_{tissue} and (B) C_{tissue}/D between the no rejection group and SubAR group at 3 months (n = 52) and 1 year after transplantation (n = 22). Statistical analyses were performed using Mann–Whitney U test. Bar shows the median value in each group. C_{tissue}, allograft kidney concentration; C_{tissue}/D, dose-adjusted allograft kidney concentration. SubAR, subclinical acute rejection.

4. DISCUSSION

Most previous studies have focused on investigating the impact of recipient *CYP3A5* genotype on tacrolimus pharmacokinetics; however, the role of donor *CYP3A5* polymorphism in kidney transplant recipients is unclear. The purpose of this chapter was primarily to investigate the effect of donor *CYP3A5* on tacrolimus kidney metabolism and whether it could reflect and predict tacrolimus kidney levels. First, the influence of recipient *CYP3A5* polymorphism on tacrolimus pharmacokinetics has been confirmed and consisted with the previous studies. On the other hand, there was no effect of donor *CYP3A5* polymorphism on tacrolimus C_{blood} or C_{blood}/D, as expected. CYP3A5 metabolic capability in the kidney was substantially lower than that in the liver and thus, the donor *CYP3A5* polymorphism is unlikely to contribute significantly to tacrolimus whole blood exposure.

Next, the association between tacrolimus dose and tacrolimus C_{blood}, and tacrolimus C_{tissue} in kidney transplant recipients was investigated. There was no significant relationship between tacrolimus dose and C_{blood}, which could be attributed to the large inter-patient variability in the binding of tacrolimus with erythrocyte and plasma protein. However, a weak but significant association between tacrolimus C_{tissue} and tacrolimus dose was observed, which consistent with the previous studies[31, 95], suggesting tacrolimus C_{tissue} appeared to reflect tacrolimus dose and hence the unbound tacrolimus. Furthermore, tacrolimus kidney exposure was also associated with tacrolimus blood levels, while donor *CYP3A5* genotype did not appear to have a significant impact on tacrolimus and M1 levels in the kidney, indicating that local metabolism could not be vital in determining tacrolimus kidney exposure. There are several possible reasons for this result. First, in addition to genetic polymorphisms, there are other variabilities in kidney CYP3A5 mRNA and protein expression levels that affect the local metabolism

of tacrolimus in the kidney. Although the *CYP3A5*1* allele was shown to be associated with a higher CYP3A5 mRNA expression compared to *CYP3A5*3/*3*, CYP3A5 protein was also found in kidney sections with the *CYP3A5*3/*3* genotype, and the difference in protein expression levels was limited to the proximal tubule.[96] In this study, it was unable to ensure all biopsies were sampled from the same location of the graft kidney, and the levels of CYP3A5 expression levels and tacrolimus intrarenal distribution were unknown. Second, ischemia and reperfusion injury in kidney transplant surgery may also cause a down-regulation of CYP3A5 expression levels in the allograft kidney.[97, 98] Last but most important, CYP3A5 mRNA expression in the kidney is from 5% to 25% of that in the liver[99], and the rate of tacrolimus metabolism in human kidney microsomes is 10% of that in human liver microsomes.[100] Thus, the effect of the donor *CYP3A5* polymorphism on tacrolimus kidney levels is negligible and likely counterbalanced by tacrolimus hepatic metabolism. Kuypers et al. demonstrated that the recipient *CYP3A5*1* variant is associated with tacrolimus-related nephrotoxicity and suggested that this is possibly due to higher concentrations of toxic metabolites produced by hepatic metabolism.[91] In this study, recipient *CYP3A5* polymorphism showed a significant impact on tacrolimus kidney levels, which could be mediated by the modulation of tacrolimus whole blood concentrations. Consequently, the recipient (liver and intestine) *CYP3A5* polymorphism might play a more important role in the kidney accumulation of tacrolimus compared to the donor (graft kidney) *CYP3A5* polymorphism. It should be noted that the 1-year correlation between tacrolimus C_{tissue} and M1 C_{tissue} was stronger than the of 3-month correlation, which could be due to the recovery of CYP3A activity in transplant recipients. A study found that a gradual increase in CYP3A activity from immediately before to 82 days after kidney transplantation by measuring 4β-Hydroxycholesterol (an exogenous marker of CYP3A enzymes activities) concentrations, implying that CYP3A impairment resulting

from end-stage renal disease is regained subsequent to transplantation.[101] The further study is needed to evaluate the long-term CYP3A enzyme activity in recipients after kidney transplantation.

Tacrolimus is also a substrate of the drug efflux transporter P-gp, a membrane drug efflux transporter encoded by the ATP-binding cassette subfamily B member 1 (*ABCB1*) gene, which may affect tacrolimus accumulation.[43, 86, 93, 102] *ABCB1* polymorphism has been identified as a critical factor in intracellular tacrolimus exposure.[103, 104] It is widely accepted that *ABCB1* polymorphism and expression levels are more likely to be associated with tacrolimus tissue distribution and drug effect or toxicity in the allograft.[9, 21, 105, 106] It was reported that *ABCB1*, but not *CYP3A5*, polymorphisms in the liver could significantly influence tacrolimus hepatic concentrations in liver transplant recipients.[107] The *ABCB1 3435T* allele has been correlated with lower P-gp function and has a significant impact on tacrolimus metabolism *in vitro*.[108] Since the kidney P-gp activity, expression levels, and polymorphisms were not evaluated in this study, the role of P-gp on tacrolimus intrarenal exposure needs to be further investigated.

In this study, several recipients had relatively low tacrolimus C_{tissue} despite having tacrolimus C_{blood} in the therapeutic range, implying that they may be at risk of AR due to insufficient graft immunosuppression. Nevertheless, no significant association was found between tacrolimus C_{tissue} or C_{tissue}/D and the incidence of biopsy-proven SubAR.

The small sample size and unstandardized biopsy sampling time of this study might have resulted in a lack of power to detect the influence of donor *CYP3A5* genotypes on tacrolimus C_{tissue}. Thus, the possible effect of *CYP3A5* polymorphisms on tacrolimus metabolism in the kidney cannot be eliminated. Multivariate analysis involving *CYP3A5* and *ABCB1* gene polymorphisms and protein expression should be combined and assessed in a large cohort study to further investigate the determinants of tacrolimus kidney exposure.

5. BRIEF SUMMARY

The study in this chapter demonstrated a correlation between tacrolimus C_{tissue}/D and C_{blood}/D and that donor *CYP3A5* gene polymorphism alone was not sufficient to predict the kidney concentration of tacrolimus at 3 months and 1 year after kidney transplantation, and tacrolimus C_{tissue} could not reflect the SubAR in kidney transplant recipients.

Further large clinical studies are needed to investigate the clinical relevance of C_{tissue} of tacrolimus or its metabolites. Moreover, new biomarkers and monitoring strategies for intrarenal tacrolimus should be explored to identify recipients who are at high risk of adverse events but with a target tacrolimus concentration in the blood.

SUMMARY

Chapter 1 Development of an LC-MS/MS method for the determination of tacrolimus and everolimus in kidney biopsy samples

TDM is necessary for immunosuppressive therapy with tacrolimus and everolimus after kidney transplantation. Several studies have suggested that the concentrations of immunosuppressive agents in allografts may better reflect the clinical outcomes than whole blood concentrations. This chapter aimed to develop a method for the simultaneous quantification of tacrolimus and everolimus concentrations in clinical biopsy samples and investigate their correlation with histopathological findings in kidney transplant recipients.

Fourteen biopsy samples were obtained from kidney transplant recipients at 3 months after transplantation. Kidney allograft C_{tissue} of tacrolimus and everolimus was measured by LC-MS/MS, and the corresponding C_{blood} was obtained from clinical records. The developed method was validated over a concentration range of 0.02–2.0 ng/mL for tacrolimus and 0.03–3.0 ng/mL for everolimus in kidney tissue homogenate. The C_{tissue} of tacrolimus and everolimus in kidney biopsy tissues ranged from 21.0 to 86.7 pg/mg tissue and 33.5 to 105.0 pg/mg tissue, respectively. C_{tissue}/D of tacrolimus and everolimus was significantly correlated with their corresponding C_{blood}/D ($r = 0.9385$, $P < 0.0001$ and $r = 0.6659$, $P = 0.0113$, respectively). No significant association was observed between tacrolimus and everolimus kidney levels and the histopathologic outcomes at 3 months after transplantation. This method is suitable for measuring tacrolimus and everolimus concentrations in biopsy-sized kidney samples, and it could support further investigation of the clinical relevance of tacrolimus and everolimus allograft concentrations after kidney transplantation.

Chapter 2 Effect of Donor *CYP3A5* Gene Polymorphism on Tacrolimus Kidney Concentration in Kidney Transplant Recipients

Recipient (liver and intestine) *CYP3A5* gene polymorphism plays an important role in tacrolimus pharmacokinetics after kidney transplantation. CYP3A5 protein is also expressed in renal tubular cells; however, little is known about its influence on tacrolimus kidney exposure and hence graft outcome. The aim of this chapter was to investigate how tacrolimus C_{tissue} could be predicted based on donor *CYP3A5* gene polymorphism in kidney transplant recipients.

A total of 52 Japanese kidney transplant recipients receiving tacrolimus were enrolled in this study. Seventy-four kidney biopsy specimens were obtained at 3 months and 1 year after transplantation to determine the donor *CYP3A5* polymorphism and measure tacrolimus C_{tissue} by LC-MS-MS. Tacrolimus C_{tissue} ranged from 52 to 399 pg/mg tissue (n = 74). Tacrolimus C_{tissue}/D was significantly correlated with tacrolimus C_{blood}/D at 3 months and 1 year after transplantation (r = 0.7604, P <0.0001 and r = 0.7572, P <0.0001, respectively). Recipient but not donor CYP3A5 gene polymorphism showed a significant impact on tacrolimus C_{tissue}/D (P = 0.0096). These data implied that tacrolimus kidney accumulation is associated with the systemic tacrolimus levels after kidney transplantation, and donor *CYP3A5* gene polymorphisms alone cannot be used to predict tacrolimus intrarenal exposure.

CONCLUSION

In this study, the first LC-MS/MS method for measuring tacrolimus and everolimus concentrations in kidney tissues was developed, validated, and successfully applied to clinical kidney biopsy samples from kidney transplant recipients. In the future, this method could be valuable for investigating the mechanism of tacrolimus-related nephrotoxicity and to optimize the co-administration strategy of tacrolimus and everolimus after kidney transplantation. Furthermore, this study demonstrated correlations between tacrolimus and everolimus allograft kidney levels and their corresponding whole blood levels. In addition, the study revealed that donor *CYP3A5* gene polymorphism alone was insufficient to predict tacrolimus allograft kidney concentrations at 3 months and 1 year after kidney transplantation.

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