Stepwise Regression Analysis of the Determinants of Blood Tacrolimus Concentrations in Chinese Patients with Liver Transplant

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Abstract: Tacrolimus (FK506) is one of the immunosuppressive drugs used effectively to prevent allograft rejection after liver transplantation. Narrow therapeutic range and individual variance in pharmacokinetics make it difficult to establish a fixed dosage for all patients. Genetic polymorphism in drug metabolizing enzymes and in transporters may influence tacrolimus exposure.

A stepwise regression analysis was used to analyze the relationship between blood concentrations of tacrolimus (54 blood samples at the day of 1 week, 2 week and one month after liver transplantation) and genetic & non-genetic factors in 18 Chinese liver transplant patients. The equation of multiple stepwise regression was: Y (tacrolimus'blood concentration) = 34.534 − 0.247 (age) − 0.510 (weight) + 1.688 (dose) + 6.876 (recipient's CYP3A5 genotype) − 3.097 (donor's CYP3A5 genotype), $P < 0.01$.

The factors impacting patient's tacrolimus blood concentrations in a descending order are weight, recipient's *CYP3A5* genotype, dose, age, donor's *CYP3A5* genotype. Among those, patient's weight and recipient's CYP3A5 genotype could significantly impact the blood concentration of tacrolimus. The influence of recipient's CYP3A5 gene polymorphism is much more obvious than that of donor's. Neither donor's nor recipient's MDR1 genetic polymorphisms were correlated with the blood concentration of tacrolimus.

Key Words: Liver transplantation, tacrolimus, chinese, CYP3A5, MDR1, stepwise regression analysis.

INTRODUCTION

Tacrolimus (FK506), brand name Prograf, is a new type of macrolide immunosuppressant which was separated from actinomycete by Japan Fujisawa pharmaceutical company in 1984. Tacrolimus was first used in clinic by Stazal from Organ Transplantation Centre of Pittsburgh University in 1989. In 1993, tacrolimus was approved to be used in preventing allograft rejection after liver transplantation by FDA [1,2]. Because of its narrow therapeutic range and individual variability in pharmacokinetics, it is difficult to establish a safe and effective dosage regimen of tacrolimus. The blood concentrations of tacrolimus taken orally at the same dose for different patients are extremely different. It is suggested that the variance of genetic background is one of the factors impacting patients' blood concentrations of tacrolimus. Among them, cytochrome P450 (CYP) subfamily *CYP3A5**3 and P-glycoprotein (P-gp) encoding gene *MDR1* polymorphisms are the most influencing factors [3,4]. Besides, some non-genetic factors, such as patient's gender, age, weight, and time course after transplantation, may have effect on the pharmacokinetics of tacrolimus in absorption and metabolism. The purpose of this study was to examine the frequency of *CYP3A5**3 and *MDR1* (C3435T and G2677T/A) genotypes in Chinese liver transplant patients. Then, a stepwise regression analysis was performed to investigate genetic and non-genetic factors such as age, sex, time-course after operation and donor/recipient's genetic polymorphisms impacting tacrolimus's blood concentration.

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EXPERIMENTAL SECTION

Reagents and Equipment

DNA extraction and purifying kits were from Fermentas Company; PCR primers were designed according to the literature or Prime 5 software, and synthesized by Invitrogen Corporation (PAGE-rank). The primer sequences are shown in Table **1**. Taq DNA polymerase was from Fermentas Company; Restriction enzymes were from Invitrogen Company; Agarose and ethidium bromide were products from Sangong Biotechnology Company.

One ml tissue grinder (Sangong); Eppendoff 5840R refrigerated centrifuge; 700-PCR instrument (Bio RAD); DYY-6C electrophoresis instrument; Gel electrophoresis imaging system (BioImaging Systems, UVP Corporation); Electronic balance (Sartorius).

Subjects

A total of 29 subjects of Chinese liver transplant patients were collected from June 2006 to March 2007 in our hospital. 18 subjects, who met inclusion criteria, were enrolled in this study. All cases signed an informed consent. Inclusion criteria: age of 18 to 70 years old; the first time liver allograft transplantation; the conventional FK506 + MMF +

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Table 1. Primer Sequences of *CYP3A53, *MDR1* C3435T and G2677T/A**

Gene	Primer sequence
*CYP3A5**3	
	forward： 5'-CATGACTTAGTAGACAGATGAC-3'
	reverse： 5'-GGTCCAAACAGGGAAGAAATA-3'
MDR1 C3435T	
	forward： 5-TGCTGGTCCTGAAGTTGATCTGTGAAC-3'
	reverse： 5-ACATTAGGCAGTGACTCGATGAAGGCA-3'
MDR1 G2677T/A	
	forward： 5-TACCCATCATTGCAATAGCAG-3'
	reverse1： 5-TTTAGTTTGACTCACCTTTCTAG-3'
	reverse2： 5-TTTAGTTTGACTCACCTTCCC-3'

prednisone triple immunosuppressive oral therapy; regular out-patient visit. Exclusion criteria**:** patients for any reason (such as drug abuse or mental illness) can not guarantee the compliance; patients with serious gastrointestinal disorders affecting the absorption of drugs or active gastrointestinal ulcers; patients for the second time liver transplant or combination organs transplant; patients occurring acute rejection or grave infection during the first month after transplantation.

Patients started to take an initial dose of 2mg / d of tacrolimus at the second day after liver transplantation. Following doses were adjusted based on blood concentrations. A strict measurement was employed to ensure correct dosage of medicine and timing of blood drawing. One ml of whole blood (EDTA anticoagulant) was drawn at 8 am in the morning of 1 week, 2 week and 1 month after liver transplantation.

Genotyping of *CYP3A5* and *MDR1*

One ml of peripheral blood was drown from all patients. 30 mg tissue of spleen was obtained from donors during liver transplantation; DNA was extracted and stored in refrigerator at 4°C.

Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) methods were previously described and employed for genotyping [5]. In the first step, extracted donor and recipients DNA samples as templates were amplified in order to get polymorphic sites that include fragments of genes. In second step, restriction enzymes were used to cut the DNA fragments and then fragments were tested for specific genotyping. In order to reduce pollution, we have done positive control and negative controls in the operation. The positive control was the DNA sample we obtained from previous study. The negative control used sterilized and deionized water in place of DNA added into the reaction system.

In brief, the PCR reaction conditions of *CYP3A5**3 site: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, a total of 35 cycles. The PCR product was digested by restriction enzyme SspI. Digested products were separated on 4% agarose gel (AGE). The PCR reaction conditions of *MDR1* G2677T/A sites: denaturation at 96℃ for 30 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 30 seconds, a total of 35 cycles. The PCR product was digested by restriction enzyme XbaI. Digested products were separated on a 12% native polyacrylamide gel electrophoresis. The PCR reaction conditions of *MDR1* C3435T site: denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 1 min, a total of 35 cycles, the PCR product digested by restriction enzyme MboI , digested products were separated on a 4% agarose gel (AGE).

Determination of Blood Concentration of Tacrolimus

The blood concentrations were determined by enzyme-multiplied immunoassay technique (Emit) in the Viva-E automatic immunoassay analyzer (Dade Behring Inc., USA). The assay was performed according to manufacturer's instruction and result of quality control was within permission.

Statistical Analysis

Clinical data of each case was recorded, including patients' age, gender, weight, tacrolimus blood concentrations and doses at the day of 1 week, 2 week and one month after liver transplantation. *CYP3A5* and *MDR1* genotype data of each case was recorded. All statistics were done by SAS (version 6.12) program.

Tacrolimus blood concentration was set as the dependent variable (Y), while 11 factors such as patient's age, gender, weight, medicine doses, time-course after operation, donor and recipient's CYP3A5 and MDR1 genotypes were set as independent variables (X). A stepwise regression analysis method was used for multi-factor analysis. Statistical significance level impacting factors selected and rejected were taken an average of 0.05. Patient's age, weight, medicine doses, tacrolimus blood concentration are continuous variables; Gender, time-course after operation, donor and recipient's CYP3A5 and MDR1 genotypes are discrete variables. Specific description of the variables is summarized in Table **2**.

RESULT

For 18 cases of Chinese liver transplant patients (17 males and 1 female), the average age was 51.8 ± 8.9 years old (range, 33 to 64 yrs), the average weight was 60.4 ± 13.5 kg, and the average dose of tacrolimus was 3.5 ± 1.4 mg/d.

A stepwise regression equation was obtained as follows: Y=34.534－0.247(age)－0.510(weight) + 1.688(dose) + 6.876(recipient's *CYP3A5* genotyping) － 3.097(donor's *CYP3A5* genotyping). Result of significance test was highly significant (F=10.666, P<0.01). Residual standard deviation=5.78792, R^2=0.5263, correction R^2=0.4769. The Stepwise regression analysis results of factors impacting tacrolimus blood concentration are summarized in Table **3**.

By comparing the value of standard partial regression coefficient, it was found that factors impacting patient's tacrolimus blood concentrations in a descending order are weight, recipient's *CYP3A5* genotype, dose, age, donor's

Table 2. Factors of Stepwise Regression Analysis and the Assignment

Variable	Index	Character and Assignment
X1	time	1week=1,2week=2,1month=3
X2	age	real age
X3	gender	male=1,female=2
X4	recipient's *CYP3A5*	*1/*1=1,*1/*3=2,*3/*3=3
X5	recipient's *MDR1*-3435	CC=1,CT=2,TT=3
X6	recipient's *MDR1*-2677	GG=1,GT/GA=2,TT/AT=3
X7	donor's *CYP3A5*	*1/*1=1,*1/*3=2,*3/*3=3
X8	donor's *MDR1*-3435	CC=1,CT=2,TT=3
X9	donor's *MDR1*-2677	GG=1 ,GT/GA=2,TT/AT=3
X10	dose	real dose (mg/d)
X11	weight	real weight (kg)
Y	blood concentration	real value (ng/ml)

CYP3A5 genotype. Neither donor's nor recipient's MDR1 genetic polymorphisms were correlated with the blood concentrations of tacrolimus.

DISCUSSION

The absorption after oral administration of tacrolimus is mainly located in the jejunum and ileum. Tacrolimus is metabolized by cytochrome P4503A subfamily (CYP3A) enzymes in the intestinal tract and liver. CYP3A5 is an important CYP3A subfamily member, which is related to tacrolimus first-pass elimination [3,6]. A base mutation (A6986G) in *CYP3A5* gene intron 3 is defined as *CYP3A5* *3. It is the most common mutations of CYP3A5 and closely related to the low activity of the enzyme [7]. P-gp, which is encoded by MDR1, has a wide distribution in the special cell surface of organs with secretion and excretion function. It affects absorption of tacrolimus by pumping fat-soluble molecule out of the cell so as to reduce the direct absorption. Therefore, the variance of P-gp activity will impact tacrolimus absorption [8]. MDR1 gene polymorphism and P-gp expression and function are closely related. G2677T / A cause P-gp 893 amino acid replacement of alanine to serine or threonine, resulting in enzyme function change. Although C3435T does not lead to P-gp amino acid changes, it may affect the P-gp expression.

In our previous study, we found that the genetic polymorphism of CYP3A5 in Chinese patients with liver transplant is an important factor impacting tacrolimus blood concentration. CYP3A5 *1 carriers need higher doses of tacrolimus in order to achieve the goal value of blood concentration, which suggests that CYP3A5 genetic polymorphism in early postoperative patients plays an important role in the tacrolimus metabolism *in vivo* [5]. However, the genetic polymorphism could not fully elucidate all the variability of drug effect. Non-genetic factors, such as patient's gender, age, weight, and time course after transplantation, should be considered as well.

In this study, we evaluated the determinants of blood tacrolimus concentrations in Chinese patients with liver transplant. To our knowledge, it is the first attempt to combine genetic and non-genetic factors by a stepwise regression analysis. It was found that factors impacting patient's tacrolimus blood concentrations in a descending order are:

Table 3. Stepwise Regression Analysis Result of Factors Impacting Tacrolimus Blood Concentration

Variable	Partial Regression Coefficient	*t*	*P*	Standard Partial Regression Coefficient
X2 (age)	-0.247	-2.463	0.0174	-0.274
X4 (recipient's *CYP3A5*)	6.876	4.042	0.0002	0.512
X7 (donor's *CYP3A5*)	-3.097	- 2.311	0.0252	-0.254
X10 (dose)	1.688	2.640	0.0112	0.299
X11 (weight)	-0.510	-7.125	0.0001	-0.861

weight, recipient's *CYP3A5* genotype, dose, age, donor's *CYP3A5* genotype. As expected, patient's weight shows the most important effect on tacrolimus blood concentration while tacrolimus patient's age have minimal impact. Interestingly, the partial regression coefficient of donor's CYP3A5 genotype was negative, which may suggest transplanted liver was not functional enough at early time after operation. The recipient's CYP3A5 genotype has a more significant effect on tacrolimus blood concentration than donor's one.

In conclusion, body weight and recipient's CYP3A5 genotype can significantly influence the blood concentrations of tacrolimus in Chinese liver transplant patients. The influence of recipient's CYP3A5 gene polymorphism is much more obvious than that of donor's. Future studies will needed to collect more data of liver transplantation cases to verify whether this equation can be used to predict blood concentration of tacrolimus in clinic setting.

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