

Full length Research Paper

Influence of gene polymorphism on the pharmacokinetics of calcineurin inhibitors: In renal transplant patients from India

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The calcineurin inhibitors (CIs), cyclosporine (CsA) and tacrolimus (Tac), are immunosuppressive drugs used to prevent rejection in patients with organ transplant. Both drugs have a narrow therapeutic range and show highly variable pharmacokinetics. This study will determine the role of gene polymorphisms involved in drug absorption and metabolism, ie ABCB1C3435T and CYP3A5A6986G, with respect to inter-individual variability in CsA/Tac levels, in a cohort of renal transplant recipients. Genotypes were assessed by PCR-RFLP in 201 renal transplant cases on immunosuppressive therapy for more than three months. ABCB1TT exhibited high CsA and Tac levels (1033.97±284.37ng/dl; 8.57±3.86ng/dl) and blood C₂ levels/dose ratio (5.40±1.76; 2.92±1.70) when compared to CC and CT genotypes. Similarly the CYP3A5GG genotype was associated with poor metabolism and showed increased CsA/Tac C₂ levels (1171±319.02ng/dl; 9.85±3.71ng/dl) as well as levels/dose ratio (5.78±1.68; 3.36±1.51). This suggests that CsA/Tac blood levels are regulated by these two genes. When gene-gene interaction was evaluated it was observed that ABCB1TT and CYP3A5GG genotypes showed the highest blood levels of Tac, however, in case of CsA/CYP3A5GG genotype is responsible for higher blood levels, irrespective of the ABCB1 genotype. These individuals would require a lower CsA/Tac dose to maintain therapeutic levels. Determination of these genotypes before renal transplant may alert clinicians and enable them to manage immunosuppression effectively and prevent complications.

Keywords: Calcineurin inhibitors, Cyclosporin, Tacrolimus, ABCB1 polymorphism, CYP3A5, Polymorphisms.

INTRODUCTION

The calcineurin inhibitors, cyclosporine (CsA) and tacrolimus (Tac), are highly effective immunosuppressive drugs used to prevent organ rejection in patients with organ transplant. Both drugs have a narrow therapeutic range and show highly variable pharmacokinetics. Elevated blood levels of these drugs leads to over suppression of the immune system and nephrotoxicity, while lowered blood levels to under suppression and

organ rejection. Hence continuous monitoring of blood drug levels is currently employed for appropriate patient management. CsA and Tac doses are adjusted based on blood concentrations at two hours post dose (C₂) levels (Holt et al., 2002, Oellerich et al., 2002, Jusko et al., 1995). Inter patient variability of blood levels depends on rate of absorption from the intestine and rate of metabolism by cytochrome P450 enzymes of these drugs.

Both CsA and Tac are substrates for intestinal P-glycoprotein (PGP), which is a member of the ATP binding cassette family of membrane transporters (Tejani et al., 2001). In the small intestine, PGP is expressed at

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Table 1. Demographic profile of patients with ESRD-T.

S.No	Demographic details	No of individuals (Range in years)
1	Renal Transplant recipients	201
2	CsA / Tac	82 (41%) / 119 (59%)
3	Males/Females	153/48
4	Age (years) : a) Males (Mean± SD)	40.09 ± 10.7 (13 – 63)
	b) Females (Mean± SD)	40.52 ± 9.59 (21- 63)
5	Weight (Kgs) : a) Males (Mean± SD)	65.1±13.01 (36 – 108)
	b) Females (Mean± SD)	67.06 ± 12.09 (38 – 90)
6	Sample collected after Transplant (months) (Mean± SD)	20.4± 15.3 (03 – 36)

the apical surface of mature enterocytes, where it functions as an efflux pump and prevents the absorption of drugs and xenobiotics from the intestinal lumen by active extrusion from the cell interior (Hall et al., 1999, Zhang et al., 2001). PGP is the product of the multidrug resistance 1 (MDR1) / ATP binding cassette super family 1 (ABCB1) gene (Saeki et al., 1993). The PGP activity and expression is known to be altered by a single base change, C3435T in ABCB1 gene (Balaram et al., 2003).

CYP proteins are heme containing monooxygenases which play an essential role in the phase I metabolism of endogenous compounds (such as steroids, fatty acids, and prostaglandins), exogenous chemicals (including drugs, carcinogens) and environmental pollutants. The CYP system consists of more than 50 isozymes. In humans, the CYP3A isozyme group is most abundantly expressed in the liver and intestine, consisting of approximately 30% and 70% of total CYP content respectively. The CYP3A subfamily consists of homologous proteins encoded by four genes: CYP3A4, CYP3A5, CYP3A7 and the recently identified CYP3A43 (Domanski et al., 2001, Gellner et al., 2001). These are responsible for the phase I metabolism of over 50% of all clinically used drugs, including CsA and Tac (Kuehl et al., 2001). The subfamily, CYP3A5 is the most important enzyme and represents at least 50% of the total hepatic content in people expressing CYP3A5 (Kuehl et al., 2001, Gorski et al., 1994). The single base change, 6986A>G in intron 3 (designated CYP3A5*3) produces a truncated, nonfunctional protein (Gellner et al., 2001, Sharon et al., 2006).

The number of renal transplants in India are increasing every year due to the availability of more transplant centres in our country. All individuals undergoing an organ transplant are required to use immunosuppression, both CsA and Tac are routinely used on a long term basis and their blood levels need to be maintained for appropriate management of the patient. The aim of the study was to determine the role of ABCB1 C3435T and CYP3A5 A6986G gene polymorphisms assessed by PCR-RFLP and their interaction with respect to interindividual variability in CsA and Tac blood levels in

a cohort of renal transplant recipients from India. Results will help in better management of transplant patients by reducing post transplant complications.

MATERIAL AND METHODS

Subjects

Renal transplant (RT) recipients included in the study were on routine follow up at the outpatient clinic of the department of Nephrology, Kamineni Hospitals, Hyderabad, India. Patients with no history of diabetes or hepatic disease according to clinical and biochemical investigations carried out prior to transplant were included in the study. 201 unrelated patients who had a single renal allograft and were on immunosuppressive drugs, either CsA (n=82) or Tac (n=119) for more than 3 months were sampled for genotype analysis and for estimating blood CsA and Tac (C₂) levels. Clinical data, personal and family history were recorded in a well-designed proforma. The demographic profiles of the renal transplant recipients are given in Table 1. The Institutional Ethics Committee approved the study.

Samples

4ml of peripheral blood sample was drawn from each patient for assessing drug levels and genotyping.

Measurement of cyclosporine and tacrolimus blood levels

CsA and Tac (C₂ levels 2 hours post dose) value in whole blood were assayed on the COBAS INTEGRA 400 (Roche Diagnostic Systems, Basel, Switzerland) using reagents based on the Enzyme Multiplied Immunoassay Technique (EMIT) (Dade Behring Inc, Cupertino, CA, USA). Details on the sensitivity of the EMIT assay in our laboratory has been published by our group previously

Table 2. Dose and Blood levels of CsA, Tac in patients with different ABCB1 genotype: The ratio of levels/dose has been calculated for each genotype.

Genotype	N=201		Age (yrs)		Weigh(kg)		Dose (mg)		Drug levels in blood (ng/dl)		Levels/Dose	
			Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	
ABCB1	CsA	Tac	CsA	Tac	CsA	Tac	CsA	Tac	CsA	Tac	CsA	Tac
	N=82	N=119	40.62	39.92	64.77	65.26	204.38	3.21	964.13	8.07	5.00	2.51
			10.24	10.62	10.00	14.39	77.35	1.57	303.09	3.50	1.65	1.38
CC	9.75%	21%	38.5	40.31	67.0	63.73	200.00	3.54	846.37	7.65	4.47	2.16
	16.41%		9.69	11.96	10.23	14.84	78.11	1.19	310.17	3.31	1.70	1.04
CT	45.12%	42%	42.75	37.98	61.34	66.44	205.71	3.18	1012.05	7.98	5.14	2.50
	43.28%		9.39	11.51	9.7	17.15	67.81	1.77	314.73	3.36	1.50	1.73
TT	45.12%	37%	40.61	38.22	65.97	65.62	207.43	2.93	1033.97	8.57	5.40	2.92
	40.29%		11.65	9.8	10.07	11.20	86.13	1.77	284.37	3.86	1.76	1.70
CYP3A5	CsA	Tac	41.72	38.59	63.45	66.22	206.27	3.16	1000.11	8.14	5.27	2.53
	N=82	N=119	10.58	11.38	11.06	14.51	79.34	1.49	301.94	3.46	1.81	1.29
AA	15.85%	29.41%	42.61	40.55	63.38	70.18	202.08	3.73	864.75	7.15	4.80	2.01
	23.88%		9.83	12.56	12.95	13.61	88.20	1.86	242.85	3.17	1.81	1.23
AG	53.65%	47.05%	40.45	39.53	66.81	63.37	201.74	2.86	963.64	8.42	5.23	2.64
	49.75%		8.01	8.94	8.44	15.80	79.13	1.24	343.97	3.39	1.96	1.72
GG	30.48%	23.52%	42.10	35.72	60.17	65.13	215.00	2.90	1171.96	9.85	5.78	3.36
	26.36%		13.91	11.37	11.81	14.12	70.71	1.37	319.02	3.71	1.68	1.51

(Kusumanjali et al., 2005).

Isolation of DNA and Genotype Analysis

Genomic DNA was isolated by the method routinely used in our laboratory (Moova et al., 2007). Genotyping for the ABCB1 and CYP3A5 polymorphism was performed by Polymerase chain reaction (PCR) using specific published primers synthesized from Bioserve Biotechnology Ltd (Hyderabad, India), followed by RFLP analysis (Balaram et al., 2003, Jean-Nicholas et al., 2005). A three step PCR by the method of Movva et al., (2007), was carried out. Briefly there was initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 45 seconds, final extension at 72°C for 7 minutes. Amplified ABCB1 PCR products were digested with *Mbo*I and CYP3A5 with *Ssp*I enzymes (MBI Fermentas, Hannover, MD, USA), in a total volume of 20 μ l for 2 hours at 37°C, and subsequently analyzed on 2% agarose / TBE (Tris-borate-EDTA) gel with ethidium bromide staining, Bands were imaged and analyzed by documentation in UV I Tech gel documentation system (Cambridge, UK).

Statistics

Data are reported as mean \pm standard deviation (SD) and percentage. Statistical comparisons between group

means were carried out by chi-squar test (χ^2) comparing the genotype distributions was carried out by MedCalc version 7.4.3.0 software (Windows 98/NT/Me/2000/XP). Two-tailed P values less than 0.05 were considered significant.

RESULTS

In the present study 201 non-diabetic end stage renal disease (ESRD) patients who received a renal transplant and were on immunosuppressive therapy were included. 76 % of the cases were males and 24% were females, both belonged to a similar age and weight range (Table 1). All cases were maintained on CI, 41% of these were on CsA, while 59% on Tac.

ABCB1 C3435T polymorphism (rs#1045642) showed 16.41% of CC, 43.28% of CT and 40.29% TT genotypes in renal transplant patients. Individuals with TT genotype showed high mean CsA drug level (1033.97 \pm 284.37ng/dl) in blood when compared to CC genotype (846.37 \pm 310.17ng/dl). Blood C₂ levels and dose ratio was also significantly elevated (p<0.05) in patients having TT genotype when compared to CC and CT genotypes (Table 2). Renal transplant patients on Tac which were assessed for the same polymorphism showed that C₂ levels altered with genotype with individuals TT genotype having high C₂ levels / dose ratio (2.92 \pm 1.70ng/dl), when compared to the other genotypes (p<0.05) (Table 2). The data indicates that ABCB1 T allele is inefficient for CI extrusion from intestine in a dose dependent manner,

Table 3. ABCB1 and CYP3A5 genotypes with CsA drug levels in three categories.

CsA drug level range (ng/dl)	n = 82 (%)	ABCB1 Genotype & Allele					CYP3A5 Genotype & Allele				
		CC	CT	TT	C	T	AA	AG	GG	A	G
		>1000 (%)	41 (50)	03 (7.31)	21 (51.21)	17 (41.47)	26 (32.5)	54 (67.5)	04 (9.75)	21 (51.21)	16 (39.04)
500-1000 (%)	37 (45.13)	04 (11.1)	13 (33.3)	20 (55.5)	20 (27.8)	52 (72.2)	08 (19.4)	20 (55.5)	09 (25)	34 (47.2)	38 (52.8)
< 500 (%)	04 (4.87)	02 (50)	02 (50)	00 (0)	06 (75)	02 (25)	02 (50)	02 (50)	00 (0)	06 (75)	2 (25)

Note: Figures in brackets are percentages

Table 4. ABCB1 and CYP3A5 Genotypes with Tac Drug Levels in Three Categories.

Tac drug level range (ng/dl)	n = 119 (%)	ABCB1 Genotype and Allele					CYP3A5 Genotype and Allele				
		CC	CT	TT	C	T	AA	AG	GG	A	G
		>10 (%)	28 (23.5)	05 (17.85)	10 (35.71)	13 (46.42)	20 (35.71)	36 (64.29)	08 (28.57)	10 (35.71)	10 (35.71)
4 – 10 (%)	81 (68.08)	18 (22.22)	37 (45.67)	26 (32.09)	73 (45.06)	89 (54.93)	22 (27.16)	44 (54.32)	15 (18.51)	88 (54.32)	74 (45.68)
<4 (%)	10 (8.4)	04 (40)	04 (40)	02 (20)	10 (50)	10 (50)	05 (50)	03 (30)	02 (20)	13 (65)	07 (35)

Note: Figures in brackets are percentages

resulting in increased blood levels of both CsA and Tac.

CYP3A5 A6986G polymorphism (rs#776746) evaluation in renal transplant patients showed that 23.88% had AA, 49.75% AG and 26.36% GG genotypes. Individuals having GG genotype showed increased CsA C₂ levels in blood (1171±319.02ng/dl) (Table 2), which was significantly different from individuals with AA genotype (p<0.05). Similarly GG genotype had high Tac blood levels (9.85±3.71ng/dl) and C₂ levels/dose ratio (3.36±1.51) compared to AA genotype (Table 2).

When the patients were grouped according to both ABCB1 and CYP3A5 genotypes there was a significant difference in both CsA and Tac drug levels/drug ratio, however, there was no significant difference between mean age, weight and drug dose between three genotypes (Table 2).

However, when the patients were grouped based on CsA blood drug levels into three categories, which are high, intermediate and low. It was observed that 50% of individuals had increased drug levels and 5% decreased, while 45% were in the recommended drug level range (Table 3). The ABCB1 T allele and CYP3A5 G allele appear to be responsible for the high blood levels in 92% of the cases with >1000ng/dl CsA (Table 3), where as none of the cases with low blood levels (<500ng/dl) had the TT and GG genotypes.

Similarly patients on Tac were categorized into 3 gro-

ups based on >10, 4 to 10 and <4ng/dl of the blood drug level (Table 4). It was observed that 23% of individuals had increased drug levels and 8% decreased, while 68% were in recommended drug level range. The ABCB1 T allele and CYP3A5 G allele were present in higher percentage of the cases with >10 ng/dl Tac levels (Table 4) but the striking absence of TT and GG genotype seen with CsA was not observed here.

When gene-gene interaction was evaluated it was observed that ABCB1TT and CYP3A5GG genotypes showed the highest blood levels of Tac, however, in case of CsA CYP3A5GG genotype is responsible for higher blood levels, irrespective of the ABCB1 genotype (Figure 1).

DISCUSSION

The present study highlights the importance of ABCB1 and CYP3A5 polymorphisms in the pharmacokinetics of calcineurin inhibitors and management of renal transplant patients in Indian population. Although therapeutic drug monitoring is routinely performed for this class of drugs, both acute and chronic toxicity occurs commonly in clinical practice. CsA and Tac are oral immunosuppressants which require to be absorb and metabolize to give specific blood levels essential for graft

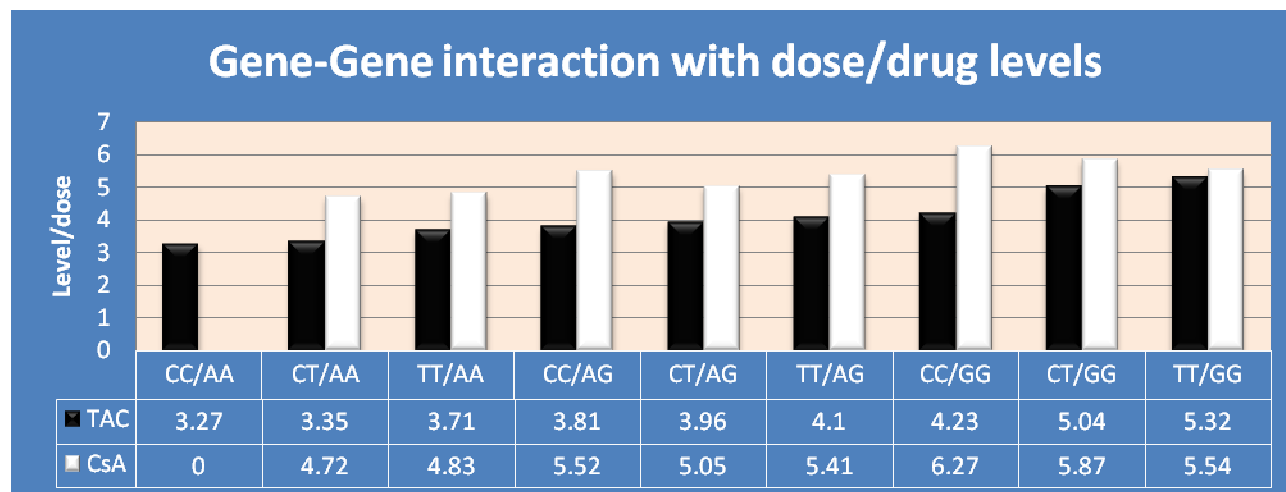


Figure 1. Gene-Gene interaction between ABCB1 and CYP3A5 regulating CsA and Tac drug levels.

maintenance. The polymorphisms C3435T (rs#1045642) of ABCB1 and A6986G (rs#776746) CYP3A5 known to effect blood levels of CI were assessed in the present study (Kusumanjali et al., 2005).

ABCB1 protein acts as a localized drug transporter actively exporting drugs out of the cell. It plays an important role in defines against xenotoxins (Hoffmeyer et al., 2000). The functional C3435T polymorphism of ABCB1 is associated with altered duodenal PGP expression. The percentage of renal transplant patients with TT genotype in this study cohort was 40.29%, indicating that a substantial number of individuals in our population have a genotype favouring high blood levels of CsA, which may be nephrotoxic. Individuals homozygous for TT genotype are considered to have lower PGP expression and hence higher blood concentrations of CsA and Tac, which are both PGP substrates (Tejani et al., 2001).

The CYP3A5 gene produces a niphedipine oxidase, involved in the metabolism of endogenous compounds and xenobiotics. The role of CYP3A5 enzymes in CI pharmacokinetics has been recognized and several polymorphisms have been associated with drug levels, but the A6986G sequence variation has shown a role in the metabolism of immunosuppressive drugs (Rebbeck et al., 1998, Hall et al., 1999, Zhang et al., 2001, Kuehl et al., 2001). The 6986A>G change in intron 3 of CYP3A5 (also designated CYP3A5*1/CYP3A5*3) produces a truncated and nonfunctional protein (Sharon et al., 2006). Individuals with GG genotype are expected to be poor metabolisers and have high CsA and Tac blood levels (Ashavaid et al., 2010). 26.36% of renal transplant patients in our cohort had the GG genotype, which is responsible for increased blood levels of CsA and Tac.

In the present study 201 renal transplant patients were evaluated, 82 of them were being managed on CsA and 119 on Tac, these were assessed for both drug levels

and genotypes. The optimum dose recommended after transplant for CsA is 8mg/kg body wt and blood drug level in the first 6 months of transplant are expected to be approximately 300ng/dl of CsA, which is gradually reduced to 125ng/dl by 3years. All the patients from this study had blood drug levels values >300ng/dl despite taking doses which were below the recommended range (i.e., ~500mg calculated with mean weight of patients, refer to Table 2). Thakur et al (2008) also did not find Indian transplant patients with C2 levels in the recommended range.

Although there is no fixed regime for CsA therapy it is commonly accepted that blood C2 levels should not be less than 500ng/dl or more than >1000ng/dl (Thakur et al., 2008). In our study ~5% of the cases had blood C2 values of CsA <500ng/dl, while 50% had values >1000ng/dl (Table 3), which can be explained by the high percentage of TT genotype in the cohort of renal transplant patients studied. ABCB13435 TT genotypes showed a significantly ($p<0.05$) higher CsA drug levels (1033.97 ± 284.37 ng/dl) and C2 levels/dose ratio (5.40 ± 1.75) in blood when compared to CC genotype (Table 2). Similar to this an earlier study also showed that patients with ABCB1 3435 TT genotype were characterized by higher CsA concentrations after oral administration when compared to patients with homozygous CC genotype (Anglicheau et al., 2004). It was seen that 92.5 % of patients with elevated CsA (>1000ng/dl) levels had a T allele (Table 3). While 100% of the cases with low (<500ng/dl) had a C allele. This suggests that CsA dose should be decided based on the individual's genotype. Ashavaid et al (2010) did not find any association of elevated CsA drug levels and ABCB1 TT and CYP3A5 GG genotypes, which is different from our observations and this, may be due to the variation in drug range categories selected in the two studies.

The recommended dose of Tac is 0.15 gm/kg and

blood levels are 10-15ng/dl, despite taking doses which were below the recommended range (i.e., ~3.21gm calculated with mean weight of patients, refer to (Table 2) 23% of patients in our study had blood levels >10ng/dl, while 8% had less than <4ng/dl. Blood levels of Tac were higher in patients homozygous for ABCB1 3435TT genotype when compared with homozygous 3435CC and this was statistically significant ($p < 0.05$) (Table 2). Earlier studies also found a correlation between the drug levels/dose ratio and ABCB1 C3435T polymorphisms with the ratio being significantly higher in subjects homozygous for TT (Ashavaid et al., 2010, Azarpira et al., 2006).

Genetic variability in CYP3A5 may also influence pharmacokinetics of these two CI and directly modulate their pharmacodynamic features (Ashavaid et al., 2010). Results from our study show that higher CsA doses are required to maintain therapeutic blood levels in patients homozygous for CYP3A5 A allele, who were considered as extensive metabolizers, compared to patients with CYP3A5 G allele. Furthermore G allele was seen in 90% of cases, who had >1000ng/dl of CsA blood levels. All patients with high CsA levels had a G allele, however, none of the cases with low blood levels, (Table 3) had it. A recent meta-analysis showed that CYP3A5*3/*3 (GG) require lower doses of CsA when compared with CYP3A5 *1/*1 and *1/*3 (AA and AG) (Zhu et al., 2010). In this study Tac blood levels were similarly higher in individuals homozygous for 6986GG genotype when compared with 6986AA genotypes (Table 2). An earlier study from India supports our results, however, correlation of genotypes and blood levels were not observed for Tac in a Chinese study (Zhang et al., 2005).

Most studies have evaluated the influence of individual SNPs on the pharmacokinetics of CI, but the combined effect of two or more polymorphisms has been rarely evaluated.

Individuals with high C2 levels of CsA (>1000ng/dl), the ABCB1 3435TT and CYP3A5 6986GG genotypes independently accounted for increased blood level of CsA. While in individuals with elevated C2 levels of Tac ABCB1 3435T allele is more important than the CYP3A5G allele. When the gene-gene interaction was evaluated it was observed that ABCB1TT and CYP3A5GG genotypes showed the highest blood levels of Tac, however, in case of CsA CYP3A5GG genotype is responsible for higher blood levels, irrespective of the ABCB1 genotype (Figure 1). This gene-gene interaction illustrates the complex inter-relationship between genes and CI therapy.

The available pharmacogenetic information does not permit exact pharmacokinetic predictions, however, adverse drug effects due to increased blood levels of CsA/Tac can be predicted when both ABCB1 and CYP3A5 polymorphisms are evaluated. Pharmacogenetic and pharmacogenomic research studying the effects of other genetic polymorphisms on drug absorption,

metabolism, disposition and response may eventually provide information which will account for 100% of the drug levels (Barraclough et al., 2010).

Our findings are of clinical importance because patients carrying the ABCB1 T and CYP3A5 G allele may run the risk of over-immunosuppression and nephrotoxicity, while ABCB1 C and CYP3A5 A patients with the same dose of CsA or Tac may run the risk of under-immunosuppression and rejection. Determination of these genotypes before transplant may alert clinicians and help them to choose appropriate doses of the two calcineurin inhibitors for management of renal transplant patients.

Furthermore, the influence of ethnicity may play a role, as variant genotypes are often more frequent in particular ethnic groups. Suitable set of gene polymorphisms specific for a particular population can be used in the clinical setting for individualizing immunosuppressant therapy for a successful transplant programme.

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