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Study on the association of *UGT1A9* gene c.98T>C polymorphism and mycophenolic acid plasma levels in renal transplant patients

L.R. Ruschel[1], V.M. Schmitt[1,2], A.B. Silva[2], C.S.A. Oliveira[3], K. Flach[2], D.O. d'Avila[3] and F.V. Thiesen[1,2]

[1]Programa de Pós-Graduação em Biotecnologia Farmacêutica, Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil
[2]Departmento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil
[3]Programa de Pós-Graduação em Medicina e Ciências da Saúde, Escola de Medicina, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil

Corresponding author: F.V. Thiesen
E-mail: fvthiesen@pucrs.br

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ABSTRACT. Mycophenolate mofetil (MMF) is a prodrug active only after its hydrolysis to mycophenolic acid (MPA). The UGT1A9 enzyme is of special interest since it is the main enzyme involved in the glucuronidation of MPA. Single nucleotide polymorphisms (SNPs) in the UGT1A9 gene may be responsible for individual differences in the pharmacokinetics of MMF. Expression levels and the activity of UGT1A9 may depend on the presence of some SNPs located in the gene promoter region (-2152C>T and -275T>A), as well as changes in the coding region (c.98T>C). The objective of this study was to evaluate the effect of allelic variants of the UGT1A9 c.98T>C polymorphism (rs72551330; g. 87289T>C) on MMF metabolism in

renal transplant patients. MPA and MPA 7-O glucuronide (MPAG) levels were determined on plasma samples of kidney transplant patients (N = 39) by high-performance liquid chromatography using ultraviolet detection. DNA was isolated from leukocytes and stored at -20°C. The presence of SNPs was investigated using polymerase chain reaction, followed by amplicon sequencing. The analysis of the *UGT1A9* c.98T>C polymorphism revealed that all study patients presented the TT genotype. Diverse MPA and MPAG plasma concentrations were detected, including therapeutic, subtherapeutic, and toxic levels. A standardized molecular method permitted identification of *UGT1A9* c.98T>C polymorphism genotypes in the examined renal transplant patients. All individuals of the study group presented the same genotype (c.98TT) for that polymorphism. Thereby, no association between the c.98T>C polymorphism and MPA and MPAG plasma levels could be evaluated, despite different levels of these compounds being observed.

Key words: Mycophenolate mofetil; Therapeutic drug monitoring; Pharmacogenetics; Renal transplant; UGT1A9

INTRODUCTION

End-stage renal disease (ESRD) has important social and economic consequences, which can be reduced by transplant, substantially improving the quality of life and life expectancy of the affected individuals. In the United States, more than 100,000 patients are on waiting lists for a solid organ transplant (SOT). Significant advances in histocompatibility and immunosuppression have dramatically improved graft and patient survival rates (Thomas et al., 2015).

Transplant success depends on a well-established management, especially in the case of patients with ESRD, to avoid rejection (acute/chronic) or immunosuppressive therapy-related toxicity (Tolou-Ghamari et al., 2015).

Immunosuppression after a transplant requires special attention. Low levels of immunosuppression increase rejection risk, while high levels augment the risk of adverse effects (Murray et al., 2013). Drugs used for SOT have high pharmacokinetics and pharmacodynamics variability and may lead either to lack of efficacy or drug toxicity. Therapeutic drug monitoring is used to guide immunosuppressive therapy because subtherapeutic or toxic concentrations can be easily achieved in these cases (de Jonge and Kuypers, 2008).

Mycophenolate mofetil (MMF) is a widely used immunosuppressant in transplant patients. MMF has been employed for the prevention of acute kidney rejection and has become the first-line drug in SOT. A vast majority of patients undergoing a kidney transplant in the United States and Europe is currently treated with MMF in the post-transplant period (Matas et al., 2014).

Mycophenolic acid (MPA), the main active metabolite of MMF, is a non-competitive and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme in the purine nucleotides of new synthesis pathway (Tang et al., 2015). In subsequent phases of biotransformation, MPA is mainly glucuronidated by uridine diphosphate-glucuronosyltransferase enzymes (UGTs). By sharing the UGT1A9 isoform, MPA is converted to MPA 7-O glucuronide (MPAG), the predominant and inactive metabolite of that drug (Pazik et al., 2014). By action of another enzyme group, UGT2B7, it originates the pharmacologically

active acyl glucuronide metabolite (AcMPAG), which inhibits lymphocyte proliferation (Guo et al., 2013). MPAG is excreted in the bile, being hydrolyzed to MPA by the action of intestinal microbiota, being, subsequently, reabsorbed. Such enterohepatic circulation contributes from 35 to 40% of the area under the curve MPA concentration/time from 0 (zero) to 12 h (van Schaik et al., 2009).

Previous studies assessed the effect of allelic variants of the UGT1A9 gene in drug metabolism showing, for example, that gene promoter polymorphisms can affect its expression level, increasing or decreasing MPA exposure (Rovin et al., 2009; Korprasertthaworn et al., 2012; Pazik et al., 2014).

During an *in vitro* study, Korprasertthaworn et al. (2012) evaluated the effect of *UGT1A9* c.98T>C allele on MPA glucuronidation. The study showed that the most common UGT1A9 c.98T variant is characterized by hyperbolic enzyme kinetics, while the enzyme encoded by the UGT1A9 c.98C allelic variant displays an atypical kinetic activity with partial substrate inhibition. In a study carried out on healthy volunteers, significantly higher levels of MPA were observed after a single administration of MMF in carriers of the UGT1A9 c.98C allele, compared to those with wild-type allele; yet MPAG levels were similar (Lévesque et al., 2007).

Clinical studies have identified many genetic variants influencing drug responses and treatment outcomes (Crews et al., 2012; Salari et al., 2012). Clinical factors, such as kidney function and serum albumin concentration, genetic polymorphisms of some enzymes involved in the metabolism of MPA may contribute to the variability of MPA pharmacokinetics (Guo et al., 2013). Advances in human genome sequencing have generated predictive genetic tests useful for decision-making in health care. Such tests may suggest risk or susceptibility to diseases in asymptomatic individuals, or predict responses to medications (i.e., pharmacogenomics) or environmental factors (nutrigenomics predicting responses to dietary factors) (Jonas and Wines, 2013).

An example of a drug class, which will gain benefit from pharmacogenomics studies, is the class of immunosuppressants, including MMF, with major implications for human health, especially on kidney transplant recipients (van Schaik et al., 2009). There is a known relationship between the effectiveness of MPA-based products and acute drug exposure in the prevention of allograft rejection (Le Meur et al., 2007; van Gelder et al., 2008). The aim of the present study was to investigate whether there is an association of allelic variants or genotypes of *UGT1A9* c.98T>C polymorphism (rs72551330; g.87289T>C) with plasma levels of MPA and MPAG in a group of kidney transplant recipients.

MATERIAL AND METHODS

Sample

The study population comprised kidney transplanted individuals, 18 years or older, attending the Nephrology Outpatient Clinic at São Lucas Hospital and using MMF for at least 4 months. All participants signed an Informed Consent Form before enrollment. Demographic and clinical data were obtained from hospital records. The study protocol was approved by the Institutional Research Ethics Committee (Protocol number 06/02965).

Blood samples were collected by venipuncture before the MMF morning dose (C0), in vacuum tubes containing sodium EDTA. Samples were immediately taken to the laboratory, centrifuged at 2000 rpm; plasma was separated and stored at -20°C for later analysis.

MPA and MPAG dosage

Dosages of MPA and MPAG were performed by high-performance liquid chromatography with an ultraviolet detector (HPLC/UV - Agilent 1100, Santa Clara, CA, USA), using a C18 column. The method had been previously validated in our laboratory, showing a detection limit of 0.001 µg/mL for MPA, and 0.53 ± 0.09 µg/mL for MPAG; quantification limit of 0.0937 ± 0.0077 µg/mL for MPA, and 1.8 ± 0.1 mg/ mL for MPAG; linearity of 0.99989 to MPA and 0.99914 to MPAG; intra-assay precision varying from 0.89 to 1.14% for MPA and from 1.20 to 2.57% for MPAG, and accuracy of 91.5 to 102.3% for MPA and 99.89 to 105.85% for MPAG.

DNA extraction

Leukocyte DNA was extracted following a standard protocol (Lahiri and Nurnberger, 1991), with minor adaptations, and stored at -20°C.

Stored samples DNA viability analysis

The viability of DNA stored samples was conducted using primers GH20 (5'-CCA GAA GAG GGT AGG ACA AC-3') and PC04 (5'-CCA CTT CAT CCA CGT TCA CC-3'), specific for the human beta globin gene. Reaction mix consisted of 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 2 mM $MgCl_2$, 200 mM dNTP mixture, 10 pmol of each primer, 1 U Taq DNA polymerase in a total volume of 25 µL. Electrophoresis on a 1.5% agarose gel was performed, and DNA integrity analyzed.

Analysis of the UGT1A9c.98T>C polymorphism

The analysis of the *UGT1A9* c.98T>C polymorphism was conducted using the polymerase chain reaction (PCR), followed by sequencing.

For the polymorphism analysis, primer forward Mico 98D (5'-GTT CTC TGA TGG CTT GCA CA-3') and primer reverse Mico-98R (5'-ATG CCT CCC GAG GAG AAT TT-3') were used. PCR conditions were: 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 2 mM $MgCl_2$, 200 mM dNTPs mix, 10 pmol of each primer, 1 U Taq DNA polymerase in a total volume of 25 µL. The amplification program used was an initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 35 s, 72°C for 30 s, with a final extension at 72°C for 6 min. An amplicon of 167 bp indicated successful amplification of the target region (Jiao et al., 2008).

Amplicon, purification, and sequencing

Purification was performed using the enzyme purification method with exonuclease I and Fastap (FastAP™ Thermosensitive Alkaline Phosphatase) (Thermo Scientific, Waltham, MA, USA).

Sequencing of PCR products was performed on an ABI 3500 sequencer (Applied Biosystems, Foster City, CA, USA), with the same primers used for amplification. Allele sequence definition was performed with BLAST, using as reference sequence the NG 002601.2 (GenBank).

RESULTS

Fifty patients were enrolled in the study; however, DNA sequencing after a viability test was achievable in 45 samples, yet only 39 of these presented adequate quality electropherogram for analysis.

All patients received at least one immunosuppressive drug besides MMF, by the Nephrology Service post-transplant standard care. No patient experienced an acute graft rejection episode, two patients had leukopenia, and data on diarrhea were not found in patients' records. Some demographic and clinical data of study group are shown in Table 1. The study population presented an equitable gender distribution, aged 40.6 ± 12.5 years, qualified as a young adult study population.

Table 1. Demographic and clinical characteristics of the study participants (N = 39).

Parameter	Value
Female gender	20
Age (years; means ± SD)	40.6 ± 12.5
Race - N (%)	
White	23 (59.0)
Black	7 (17.9)
Uninformed	9 (23.1)
Transplant characteristics	
Post-transplant follow-up (months) - median (IQR)	31 (14.5-36.7)
Biochemical parameters	
MPA plasma level [µg/mL, median (IQR)]	2.4 (1.3-4.4)
Range of MPA plasma level - N (%)	
Therapeutic	18 (46.1)
Toxic	15 (38.5)
Subtherapeutic	6 (15.4)
MPAG plasma level (µg/mL, means ± SD)	
Creatinine blood concentration (mg/dL, means ± SD)	81.5 ± 41.1
Range of creatinine blood concentration [mg/dL, N (%)]	2.0 ± 1.0
Normal	4 (12.9)
Increased	27 (87.1)

According to Shaw et al. (2007), therapeutic levels of MPA should range from ≥1.0 to 3.0 µg/mL. In the current study, 46% of patients had plasma levels in this range, and 38.5% of MPA levels were above the therapeutic range. Analysis of the *UGT1A9* c.98T>C polymorphism showed all patients presenting the TT genotype.

DISCUSSION

The current Brazilian population exceeds 195 million people, being highly miscegenated, made up from European, African, and Native American predecessors (Suarez-Kurtz et al., 2014). According to the Brazilian Institute of Geography and Statistics (IBGE), 48.4% of the Brazilian population report themselves as white and 6.8% as African-Brazilian (Suarez-Kurtz, 2010; Pena et al., 2011). In our study population, there was a predominance of white individuals (59%).

In 38.5% of the evaluated patients, MPA levels were above therapeutic range, signifying adequate immunosuppression, yet increased risk of toxic drug effects. It has been known that the most common side effects of MMF therapy are diarrhea and leukopenia, which may reduce treatment adhesion, and significantly compromises patients' life quality (Shaw

et al., 2007). Moreover, 15.4% of patients had plasma levels below therapeutic levels, which poses a risk of graft rejection. Two patients had leukopenia, but in none of the reports, diarrhea was enlisted.

In studies conducted by Zicheng et al. (2006) and Fu et al. (2014), using 2 g/day as starting dose of MMF, approximately 30-40% of Chinese patients had high levels of MPA. On the contrary, with the same initial dose of MMF, only a minority of Caucasian patients had MPA levels above minimum therapeutic level (Mourad et al., 2001; van Gelder et al., 2008; Gaston et al., 2009; de Winter et al., 2011). In studies with Asian patients, it was found that after an initial MMF dose of 750 mg twice-a-day, instead of 1000 mg, comparable efficacy with a lower incidence of adverse events (leukopenia, gastrointestinal symptoms, and cytomegalovirus infection) was achieved (Jirasiritham et al., 2004; Jiao et al., 2007). Patients from a Mazidi et al. (2013) study showed elevated plasma levels of MPA (104.8 ± 32.3 mg/mL); this may be partially due to other genetic factors, such as simultaneous UGT1A8 and UGT2B7 polymorphisms or SNPs in the MRP-2 gene, or non-genetic factors, such as ethnicity, dietary habits, and drug interactions. The authors conclude that *UGT1A9* gene polymorphisms may be partially responsible for the interindividual differences among stable renal transplant recipients, although most patients had acceptable levels of MPA plasma (Hesselink and van Gelder, 2005; Chen et al., 2007).

UGT1A9 is one of the main enzymes involved in the metabolism of MPA, and its increased activity may reduce MPA plasma concentration and, therefore, the mediated immunosuppression (Picard et al., 2005). The *UGT1A9* c.98T>Cis a functional polymorphism that results in reduced enzyme activity and lower glucuronidation rate of the c.98C variant, compared to the wild form. Thus, the detrimental effect of the allele *UGT1A9* c.98C in graft function and patients' survival rate may be a result of a reduced potential of detoxification of the UGT1A9 enzyme, leading to increased exposure to toxic substances, or, in the case of our study, to MPA (Pazik et al., 2011). This increased exposure to MPA - previously known - may be desirable, once it is below toxic levels, as the treatment target is keeping plasma levels within the therapeutic range.

In a study conducted by van Schaik et al. (2009), examining the relationship between the UGT1A9 c.98T>C polymorphism and exposure to MPA, in 340 kidney transplant recipients, a 50% increase in exposure to MPA was demonstrated for the polymorphic *UGT1A9* c.98C variant in the post-transplant period, in patients receiving MMF.

According to Lévesque et al. (2007), after a single dose of MMF given to healthy volunteers, an increase in exposure to MPA and urine excretion of its metabolite was observed in patients with the variant c.98T>C.

According to Pazik et al. (2014), comparing homozygous c.98TT transplant patients with c.98C allele carriers, those had worse renal graft function, from the first month on, manifested as significantly lower glomerular filtration rates ($P = 0.02$), which persisted 8 years ($P = 0.03$) after the procedure.

Studies by van Schaik et al. (2009) and Bosó et al. (2014), performed in Caucasian populations (with 338 and 570 renal transplant patients, respectively) showed a frequency of the *UGT1A9* allele c.98C between 1 and 4%. A study conducted by Zakerska et al. (2013) with 308 Polish patients showed that 1.6% of the study population carried the c.98C allele. In the study of Pazik et al. (2011), enrolling 103 kidney recipients of Polish origin, it was found a 1.7% frequency of the c.98C allelic variant. In our study, analysis of the *UGT1A9* c.98T>C polymorphism revealed that none of our patients exhibited the allele. In fact, the

chance of finding patients carrying this allele in a small study population would be scant - such observation has been corroborated by our study. As only individuals homozygous for the c.98T allele were found, it was not possible to determine whether there is any association between the *UGT1A9* c.98T>C polymorphism and plasma levels of MPA and MPAG.

The variation in MPA levels, in patients undergoing therapy with MMF, may be not necessarily associated with the polymorphism, as different factors, such as comorbidities and multiple concomitant drugs offered to transplant patients could affect those levels (Mazidi et al., 2013). In our study, different MPA plasma levels were observed, despite the patients' homogeneity about the wild-type allele (TT). Nevertheless, additional polymorphisms of the *UGT1A9* gene could affect MPA levels, apart from gene polymorphisms that encode another phase I and II enzymes, or carriers such as UGT1A8, UGT2B7, and MRP2 (van Schaik et al., 2009).

Among *UGT1A9* gene polymorphisms that could also modify the response to MMF are I399C>T, M33T, -2152C>T, -665, -331/-440, and -275T>A variants (Girard et al., 2004; Hesselink and van Gelder, 2005; Inoue et al., 2007). *In vitro* experiments showed that polymorphisms -275T>A and -2152T>C are related to increased hepatic expression of UGT1A9 and increased MPA glucuronidation activity, compared to individuals with the wild-type allele (Girard et al., 2004).

In a previous study by Mazidi et al. (2013), the T-275A polymorphism occurred in 15% of patients (N = 40), and they had significant lower MPA levels in comparison with non-carriers or wild-type carriers. In the study of Kuypers et al. (2005), there was a similar frequency of the T-275A polymorphism (16.8%), while the frequency of -2152C>T was 12.6%. This SNP was not detected in the population study of Mazidi et al. (2013). The lack of detection of some of these mutations was assessed in Japanese and Chinese populations in which none of these SNPs were observed (Saeki et al., 2006; Kagaya et al., 2007; Jiao et al., 2008).

Considering the possibility that the polymorphisms mentioned above may influence the pharmacokinetics of MMF, the next step of this study will be to evaluate other polymorphisms to investigate the possible association with the diversity of MPA and MPAG levels found in our patients. Also, expanding the size of the study population could be an attractive alternative.

In summary, renal transplant patients genotyped for the *UGT1A9* c.98T>C polymorphism were determined, and all individuals in the investigated study population displayed the same genotype (c.98TT), thus compromising the association analysis. Besides bearing the same genotype, study patients presented a considerable variation in MPA and MPAG plasma levels, suggesting that additional variables, such as other gene polymorphisms, or environmental factors could interfere with MPA and MPAG plasma levels.

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