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Development of De Novo Donor-specific HLA Antibodies and AMR in Renal Transplant Patients Depends on *CYP3A5* Genotype

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Background. The single-nucleotide polymorphism *CYP3A5* rs776746 is related to a reduction in the metabolizing activity of the *CYP3A5* enzyme. People carrying at least one copy of the wild-type allele, defined as *CYP3A5* expressers, exhibit higher clearance and lower trough concentrations of tacrolimus than homozygous nonexpressers, and this difference may affect alloimmunization and allograft function. **Methods.** We retrospectively studied 400 kidney transplant recipients treated with a tacrolimus-based immunosuppression regimen to detect *CYP3A5* genotype, de novo formation of HLA antibodies and donor-specific antibodies (DSAs), and clinical outcome up to 5 y after transplant. **Results.** We found that 69 (17%) of the 400 patients were *CYP3A5* expressers. During the first 3 y after transplant, *CYP3A5* expressers tended to have lower tacrolimus trough levels than nonexpressers, although their tacrolimus dosage was as much as 80% higher. De novo DSAs were found more frequently in *CYP3A5* expressers than in nonexpressers (13/69 [19%] versus 33/331 [10%], $P = 0.02$). De novo DSA-free survival rates ($P = 0.02$) were significantly lower for expressers than for nonexpressers. *CYP3A5* genotype had no effect on allograft failure, but *CYP3A5* expressers exhibited a significantly higher frequency of antibody-mediated rejection. *CYP3A5* expresser status was an independent risk factor for the development of de novo DSAs (relative risk, 2.34, $P = 0.01$). **Conclusions.** Early detection of *CYP3A5* expressers, enabling genotype-based dose adjustment of tacrolimus immediately after renal transplant, may be a useful strategy for reducing the risk of de novo DSA production and antibody-mediated rejection.

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INTRODUCTION

Tacrolimus is the most commonly used calcineurin inhibitor for maintenance immunosuppressive regimens after renal transplant. However, tacrolimus has a narrow therapeutic window and a high degree of interindividual and intraindividual variability in pharmacokinetics.[1,2] Potential overexposure to tacrolimus is associated with adverse effects such as nephrotoxicity, hypertension, tremor, and diabetes, whereas underdosing increases the risk of acute rejection and allograft failure.[3,4] Therefore, the routine use of close therapeutic drug monitoring is necessary for avoiding suboptimal immunosuppression. The concentration-to-dose ratio (C/D ratio) of tacrolimus is used as a surrogate for tacrolimus metabolism to guide tacrolimus therapy and subsequent dose adjustments.[5] Low C/D ratios contribute to reduced renal function, a higher number of acute allograft rejections, and higher mortality rates.[6,7]

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The inter- and intraindividual variability of tacrolimus pharmacokinetics is attributed to multiple factors, such as drug–drug interaction, dietary changes, circadian rhythm, gastrointestinal events, and nonadherence to medication regimens.[8] Genetic variants in tacrolimus-metabolizing enzymes are a nonmodifiable factor accounting for a substantial portion of the variable pharmacokinetics of tacrolimus.[9,10] The *CYP3A5* subfamily are the key enzymes that heavily affect tacrolimus metabolism.[9,11] *CYP3A5* is mainly expressed in the liver and intestine, but it is also present in the kidney and prostate.[12] The best-studied single-nucleotide variant of *CYP3A5* is related to an A-to-G transition located at genomic position 6986 within intron 3 (rs776746).[9,13] The substitution of G for A leads to an alternative splice variant with an early stop codon that generates a nonfunctional protein.[9,14] Consequently, the functional variant leads to a loss of function of the *CYP3A5* enzyme, resulting in 40%–50% of the variability in tacrolimus dose requirements.[15] People carrying one or more copies of the wild-type *1 allele are called *CYP3A5* expressers, whereas those with the homozygous *3/*3 genotype are classified as *CYP3A5* nonexpressers.[9] Compared to nonexpressers, *CYP3A5* expressers exhibit 40%–50% higher tacrolimus clearance and 40%–50% lower tacrolimus trough levels.[16-18] Correspondingly, patients carrying the wild-type *1 allele should be given tacrolimus doses 1.5- to 2-fold higher than usual to achieve target therapeutic concentrations.[9] In addition, 12–24 mo after kidney transplant, patients expressing the *CYP3A5**1/*1 genotype or the *1/*3 genotype exhibit a C/D ratio 1.8–2.5 times lower than that exhibited by *CYP3A5* nonexpressers.[19]

To date, evidence about acute rejection and allograft loss due to differences in *CYP3A5* genotype is conflicting. Several studies found no relationship between *CYP3A5* variant and renal function, biopsy-proven rejection rate, or allograft survival.[19-23] One study found a significantly earlier onset of acute rejection among *CYP3A5* expressers than among nonexpressers.[18] A large meta-analysis of 21 studies, performed by Rojas et al,[24] found an increased risk of acute rejection among *CYP3A5* expressers; the effect disappeared when only studies with biopsy-proven rejection episodes were included. However, all previously published studies investigated the total rate of acute rejection or focused only on the cellular type of rejection. Data about the relationship between *CYP3A5* expresser status and the development of de novo donor-specific anti-HLA antibodies (DSAs) and antibody-mediated rejection (AMR) were missing, and the follow-up duration was short, on average 6–12 mo after transplant.[24] Because the effect of the *CYP3A5* genotype on the occurrence of DSAs and AMR has been poorly explored to date, we evaluated the association of CYP3A5 genotype with alloimmunization and renal transplant outcome and focused primarily on determining the association between *CYP3A5* expresser status and the risk of the development of de novo DSAs and AMR in a large cohort of 400 renal allograft recipients who were followed up for at least 5 y after transplant.

MATERIALS AND METHODS

Study Population

This retrospective single-center study was approved by the institutional ethics board (19-9071-BO) and enrolled a total of 400 adult patients who initiated and maintained tacrolimus therapy.

Clinical and laboratory data were collected for posttransplant follow-up of up to 9 y. For most patients, induction therapy consisted of basiliximab. Patients with panel-reactive antibody levels >25% or previous transplants were treated with thymoglobulin. ABO-incompatible transplant recipients were treated with a single dose of 500 mg intravenous rituximab, immunoadsorption, and intravenous immunoglobulin. Maintenance immunosuppression therapy was applied according to the standard-of-care protocol, with tacrolimus, mycophenolate mofetil, or mycophenolic acid, and steroids. Fifty-three patients were treated with mammalian target of rapamycin inhibitors such as everolimus or sirolimus instead of with mycophenolate mofetil. All patients were treated with low-dose prednisolone/prednisone; steroids were not withdrawn from any patient.

All documented rejection episodes were biopsy-proven. Biopsies were performed for cause only during the study period and were analyzed according to the latest available Banff grading criteria.[25] The estimated glomerular filtration rate was calculated with the Chronic Kidney Disease Epidemiology Collaboration equation.[26] Allograft failure was defined as a return to dialysis and GFR reduction as a reduction in renal function of >50%.

Cytomegalovirus (CMV) infection was determined by CMV viremia >65 IU/mL. BK polyomavirus viremia was characterized by BK polyomavirus DNA >400 copies/mL. Epstein-Barr virus reactivation was suspected when Epstein-Barr virus viremia >1000 IU/mL was detected.

Tacrolimus trough levels were measured weekly to 3 mo after transplant, monthly to 6 mo, and then at least twice annually, with corresponding tacrolimus dosing obtained from the medical record. Tacrolimus trough levels were measured with chemiluminescent microparticle immunoassay (Architect Tacrolimus; Abbott Diagnostics, Lake Forest, IL) and doses adjusted to achieve our target trough levels of 6–8 ng/mL up to 3 mo and 5–7 ng/mL thereafter. We took into account tacrolimus trough measurements and corresponding daily doses obtained within the first 6 mo posttransplant, as well as tacrolimus trough measurements at years 1, 2, and 3. We calculated the ratio of tacrolimus blood concentration to daily dosage of tacrolimus (C/D ratio).

HLA Typing of Recipients and Donors

For HLA typing of recipients and donors, we isolated DNA from peripheral blood samples. HLA class I (HLA-A, -B, -C) and II (HLA-DRB1, -DQB1) typing was performed at the first-field resolution level as described.[27] Second-field typing was performed to type for selected high-resolution HLA alleles and serologic equivalents according to established Eurotransplant procedures.[28] HLA-DP and HLA-DQA typing was not performed, and HLA-DP– and HLA-DQA–specific antibodies were excluded from further analysis with respect to a putative donor specificity of the anti–HLA-DP and -DQA antibodies.

HLA Antibody Detection and Specification

All patients were screened for anti-HLA class I and II antibodies before transplant. The pretransplant patient

sera collected closest to the date of transplant were used for screening. Pretransplant sensitization status was determined for all patients with the standard immunoglobulin G complement-dependent cytotoxicity test with and without the addition of dithiothreitol to exclude antibodies of the IgM isotype. In addition, all patients were tested with a Luminex-based LABScreen Mixed bead assay (One Lambda; Thermo Fisher Scientific, Inc.). In step-by-step analysis,[29] the anti-HLA class I and/or II positive sera with positive were subsequently specified with LABScreen single-antigen bead assays (One Lambda; Thermo Fisher Scientific Inc.). All beads with normalized median fluorescence intensity (MFI) values higher than 1000 were considered to be positive for anti-HLA antibodies. To address the potential effect of interfering antibodies or prozone effects on our MFI analyses, we analyzed the sera after multiple freezing and thawing and ethylenediaminetetraacetic acid treatment.[30]

The results of pretransplant lymphocytotoxic T-cell crossmatches (complement-dependent cytotoxicity crossmatch) were negative for all recipients. Anti-HLA antibody status after transplant was monitored at months 3, 6, and 12 after transplant and annually thereafter. Additional screening was performed in case of allograft dysfunction. For the current study, de novo anti-HLA antibodies were determined earliest 4 wk after renal transplant. We considered samples to be positive for de novo anti-HLA antibodies only when the antibodies were detected at least twice. Nonrecurring evidence of anti-HLA antibodies after transplant was not considered.

CYP3A5 Genotyping

DNA samples were isolated from peripheral whole blood with spin columns (Qiagen) or with an automated system using magnetic separation technology (Chemagic; Chemagen PerkinElmer).

Polymerase chain reaction for *CYP3A5* rs776746 genotyping was performed under the following conditions: 95 °C for 5 min; 38 cycles at 95 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s; and final elongation for 10 min at 72 °C (forward primer, 5′ TGTACCACCCAGCTTAACGA 3′; reverse primer, 3′ TTGTACGACACACAGCAACCT 5′). Genotyping by pyrosequencing was performed with a PyroMark Q96 MD instrument (Qiagen) with the sequencing primer 5′ GCTCTTTTGTCTTTCA 3′ according to the manufacturer's instructions. The Hardy-Weinberg equilibrium (HWE) was calculated with Pearson's χ^2 goodness-of-fit test, and genotypes were considered deviant from the HWE at a significance level of $P < 0.05$. *CYP3A5* rs776746 results were within the HWE ($\chi^2 = 0.89$, $P = 0.34$).

Statistical Analysis

Categorical variables were expressed as numbers and percentages. Comparisons between groups were made with the χ^2 test. Continuous variables were compared with one-way analysis of variance. Kaplan-Meier survival curves were compared using the Gehan-Breslow-Wilcoxon test. To evaluate the independent factors influencing anti-HLA DSA antibody–free, anti-HLA antibody–free, and AMR-free survival, we performed a multivariate Cox regression analysis. Statistical significance was set at $P \leq 0.05$. All data analyses were performed with GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA) and IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY).

RESULTS

Patient Characteristics

Our study involved 400 of the 554 adult recipients of renal allografts at our center from January 2011 to December 2015. Reasons for exclusion are shown in the study flow chart (Figure 1).

The median follow-up was 52 mo (range, 3–105 mo). Table 1 summarizes the demographic and clinical characteristics of the study patients. The average age of recipients was 51 y; 52 (13%) of the patients had undergone a previous renal transplant. Before transplant, lymphocytotoxic panel-reactive antibodies were detected in 34 (9%) recipients, and preformed anti-HLA antibodies were detected by Luminex in 151 recipients (38%). Comparison of relevant baseline characteristics between *CYP3A5* expressers and nonexpressers showed no statistically significant differences between the groups except for a higher number of deceased donors, female donors and a higher frequency of HLA-DR mismatches among nonexpressers (Table 1).

Characteristics of renal allograft outcome and infectious complications after renal transplant among *CYP3A5* expressers and nonexpressers are summarized in Table 2. During the posttransplant period, de novo anti-HLA antibodies developed in 107 (27%) allograft recipients, whereas de novo anti-HLA DSAs appeared in a total of 46 (12%) recipients. Data on typing of de novo anti-HLA antibodies and de novo DSAs are provided in **Table S1** (**SDC,** http://links.lww.com/TP/C259). Allograft failure occurred in 48 (12%) recipients, and 78 (20%) recipients experienced a GFR reduction >50% during the 5 y follow-up. Histologically proven rejection episodes occurred in 129 (32%) patients, whereas cellular rejection (Banff category 4) was detected in 76 (20%) patients and AMR (Banff category 2) was detected in 25 (6%) patients.

The cohort included 69 (17%) *CYP3A5* expressers. Four patients (1.0%) carried the rare homozygous *1/*1 genotype, whereas 65 patients (16%) carried the heterozygous *CYP3A5**1/*3 genotype.

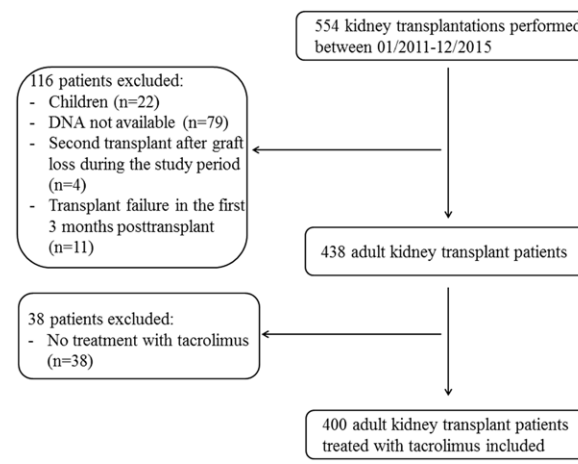

FIGURE 1. Study population flowchart.

TABLE 1.

Baseline characteristics of 400 renal allograft recipients

	All patients (n = 400)	*CYP3A5* expressers (n = 69)	Nonexpressers (n = 331)	χ^2	OR	*P*
Recipient						
Age, median (range)	51 (18–81)	50 (18–80)	51 (18–81)			0.49
No. of women, n (%)	165 (41)	27 (39)	138 (42)	0.15	0.9	0.35
Previous transplants, n (%)	52 (13)	5 (7)	47 (14)	2.44	0.47	0.06
CMV status, positive	244 (61)	42 (61)	202 (61)	0.0006	0.99	0.49
CMV high risk (D+/R–), n (%)	74 (19)	13 (19)	61 (18)	0.006	1.03	0.47
PRA, n (%)	34 (9)	5 (7)	29 (9)	0.17	0.81	0.34
Preformed anti-HLA antibodies, n (%)	151 (38)	25 (36)	126 (38)	0.08	0.92	0.39
Class I, n (%)	125 (31)	21 (30)	104 (31)	0.03	0.96	0.44
Class II, n (%)	70 (18)	9 (13)	61 (18)	1.12	0.66	0.14
Class I and II, n (%)	46 (12)	7 (10)	39 (12)	0.15	0.85	0.35
Preformed anti-HLA DSAs, n (%)	37 (9)	8 (12)	29 (9)	0.37	1.29	0.27
Rest diuresis (ml), median (range)	500 (0–2800)	250 (0–2800)	500 (0–2800)			0.48
Delayed graft function, n (%)	87 (22)	13 (19)	74 (22)	0.41	0.81	0.26
Cold ischemia time (min), median (range)	636 (0–3420)	527 (58–1592)	650 (0–3420)			0.16
Warm ischemia time (min), median (range)	25 (7–75)	26 (12–43)	25 (7–75)			0.84
Donor						
Deceased donors, n (%)	285 (71)	44 (64)	241 (73)	2.28	0.66	0.07
Age, median (range)	52 (0–85)	50 (0–79)	52 (0–85)			0.71
No. of females, n (%)	186 (47)	26 (38)	160 (48)	2.61	0.65	0.05
CMV status, ±	228 (57)	45 (65)	183 (55)	2.3	1.52	0.07
ABO-incompatible transplantat, n (%)	31 (8)	7 (10)	24 (7)	0.67	1.44	0.21
Immunosuppression at transplant						
IL-2 receptor antagonist, n (%)	369 (92)	62 (90)	307 (93)	0.67	0.69	0.21
ATG, n (%)	23 (6)	4 (6)	19 (6)	0.0003	1.01	0.49
Tacrolimus extended-release formulation, n (%)	26 (7)	3 (4)	23 (7)	0.64	0.61	0.21
mTOR inhibitor, n (%)	53 (13)	7 (10)	46 (14)	0.7	0.7	0.2
MMF/MPA, n (%)	345 (86)	61 (88)	284 (86)	0.33	1.26	0.28
Steroids, n (%)	400 (100)	69 (100)	331 (100)			
Rituximab	5 (1)	3 (4)	2 (1)	6.48	7.48	0.005
Other, n (%)	4 (1)	1 (1)	3 (1)	0.17	1.61	0.34
HLA mismatches						
MM (A/B), n (%)	332 (83)	56 (81)	276 (83)	0.2	0.86	0.33
HLA class I MM (A/B): 1-2	196 (49)	33 (48)	163 (49)	0.05	0.95	0.42
HLA class I MM (A/B): 3-4	136 (34)	23 (33)	113 (34)	0.02	0.97	0.45
MM (DR), n (%)	286 (72)	46 (67)	240 (72)	0.96	0.76	0.16
HLA class II MM (DR): 1	182 (46)	24 (35)	158 (48)	3.86	0.58	0.03
HLA class II MM (DR): 2	104 (26)	22 (32)	82 (25)	1.5	1.42	0.11
Causes of renal failure						
1. Diabetic glomerulosclerosis, n (%)	39 (10)	3 (4)	36 (11)	2.77	0.37	0.05
2. Chronic glomerulonephritis, n (%)	114 (29)	19 (28)	95 (29)	0.04	0.94	0.42
3. Nephrosclerosis, n (%)	47 (12)	11 (16)	36 (11)	1.41	1.55	0.12
4. Polycystic kidney disease, n (%)	60 (15)	14 (20)	46 (14)	1.83	1.58	0.09
5. Tubulointerstitial nephritis, n (%)	16 (4)	3 (4)	13 (4)	0.03	1.11	0.44
6. Congenital anomalies, n (%)	31 (8)	5 (7)	26 (8)	0.03	0.92	0.43
7. Autoimmune disease, n (%)	18 (5)	4 (6)	14 (4)	0.33	1.39	0.28
8. Amyloidosis, n (%)	3 (1)	1 (1)	2 (1)	0.55	2.42	0.23
9. Reflux nephropathy/recurrent pyelonephritis, n (%)	27 (7)	2 (3)	25 (8)	1.97	0.37	0.08
10. HUS, n (%)	6 (2)	0 (09	6 (2)	1.27	0	0.13
11. Other, n (%)	39 (10)	7 (10)	32 (10)	0.01	1.06	0.45

ATG, anti-thymocyte globulin; CMV, cytomegalovirus; D, donor; DSA, donor-specific antibody; HUS, hemolytic uremic syndrome; IL-2, interleukin-2; MM, mismatch; MMF, mycophenolate mofetil; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; OR, odds ratio; PRA, panel-reactive antibodies; R, recipient.

TABLE 2.

Characteristics of renal allograft outcome and infectious complications among *CYP3A5* expressers and nonexpressers after renal transplant

	All patients (n = 400)	*CYP3A5* expressers (n = 69)	nonexpressers (n = 331)	χ^2	OR	*P*
Rejection (Banff categories 2, 3 and 4), n (%)	129 (32)	26 (38)	103 (31)	1.13	1.34	0.14
Rejection (Banff categories 2 and 4), n (%)	91 (23)	16 (23)	75 (23)	0.009	1.03	0.46
AMR (Banff category 2), n (%)	25 (6)	8 (12)	17 (5)	4.06	2.42	**0.02**
TCMR (Banff categories 3 and 4), n (%)	123 (31)	24 (35)	99 (30)	0.64	1.25	0.21
TCMR (Banff category 4), n (%)	76 (20)	12 (17)	64 (19)	0.14	0.88	0.35
Mixed AMR/TCMR, n (%)	8 (2)	1 (1)	7 (2)	0.13	0.68	0.36
Transplant failure, n (%)	48 (12)	9 (13)	39 (12)	0.09	1.12	0.39
Decrease in eGFR, n (%)	78 (20)	17 (25)	61 (18)	1.4	1.45	0.12
Death, n (%)	69 (17)	10 (15)	59 (18)	0.44	0.78	0.25
De novo anti-HLA antibodies, n (%)	107 (27)	24 (35)	83 (25)	2.75	1.59	**0.05**
Class I, n (%)	69 (17)	17 (25)	52 (16)	3.19	1.75	**0.04**
Class II, n (%)	68 (17)	17 (25)	51 (15)	3.5	1.8	**0.03**
De novo anti-HLA DSAs, n (%)	46 (12)	13 (19)	33 (10)	4.42	2.1	**0.02**
Class I, n (%)	25 (6)	8 (12)	17 (5)	4.01	2.42	**0.02**
Class II, n (%)	30 (8)	8 (12)	22 (7)	1.67	2.46	0.1
Class I and II, n (%)	9 (2)	3 (4)	6 (2)	0.15	1.11	0.35
Infections						
CMV infection, n (%)	146 (37)	25 (36)	121 (37)	0.003	0.99	0.48
CMV disease, n (%)	31 (8)	4 (6)	27 (8)	0.44	0.69	0.25
BKV viremia, n (%)	97 (24)	15 (22)	82 (25)	0.29	0.84	0.3
BKV nephropathy, n (%)	24 (6)	4 (6)	20 (6)	0.006	0.96	0.47
HEV infection, n (%)	11 (3)	3 (1)	8 (2)	0.78	1.84	0.19
EBV reactivation, n (%)	76 (19)	12 (3)	64 (19)	0.14	0.88	0.35
Influenza A and B infections, n (%)	18 (5)	1 (0.25)	17 (5)	1.81	0.27	0.09
Norovirus infection, n (%)	9 (3)	1 (0.25)	8 (2)	0.24	0.59	0.31
HSV infection, n (%)	15 (4)	3 (1)	12 (4)	0.08	1.21	0.39
Pyelonephritis, n (%)	108 (27)	14 (20)	94 (28)	1.91	0.64	0.08
More than 1 episode, n (%)	58 (15)	8 (2)	50 (15)	0.57	0.74	0.23
Pneumonia, n (%)	55 (14)	13 (19)	42 (13)	1.82	1.6	0.09
More than 1 episode, n (%)	20 (5)	3 (1)	17 (5)	0.08	0.84	0.39
Sepsis, n (%)	78 (20)	12 (17)	66 (20)	0.24	0.85	0.31
More than 1 episode, n (%)	20 (5)	4 (6)	16 (5)	0.11	1.21	0.37

Data were analyzed with 1-tailed χ^2 tests.
AMR, antibody-mediated rejection; BKV, BK virus; CMV, cytomegalovirus; DSA, donor-specific antibody; EBV, Epstein-Barr virus; eGFR, estimated glomerular filtration rate; HEV, hepatitis E virus; HSV, herpes simplex virus; OR, odds ratio; TCMR, T cell–mediated rejection.

Tacrolimus trough levels were significantly lower in *CYP3A5* expressers than in nonexpressers 14 days (7.4 ± 2.4 versus 8.5 ± 2.8; $P = 0.02$) and 1 mo (7.6 ± 2.0 versus 9.0 ± 2.6; $P = 0.0005$) after transplant, although they received significantly higher tacrolimus dosages than did nonexpressers (14 d, 12.8 ± 5.4 versus 8.8 ± 4.4; $P < 0.0001$; 1 mo, 10.8 ± 4.0 versus 7.2 ± 4.0; $P < 0.0001$) (Figure 2A and B). At 14 days and at 1 mo after transplant, a significantly higher portion of patients with tacrolimus trough levels below the target value of 6 ng/mL were observed among *CYP3A5* expressers (14 d, 33%; 1 mo, 22%) than among nonexpressers (14 d, 17%; 1 mo, 9%; $P = 0.001$) (**Table S2, SDC,** http://links.lww.com/TP/C259). More than 1 mo after transplant, *CYP3A5* expressers tended to exhibit lower tacrolimus trough levels than did nonexpressers, although the difference was not statistically significant (Figure 2A). However, *CYP3A5* expressers still required tacrolimus dosages as much as 80% higher than those required by nonexpressers (3 mo, 8.5 ± 3.5 versus 4.9 ± 2.6, $P < 0.0001$; 6 mo, 7.6 ± 3.0 versus 4.2 ± 2.2, $P < 0.0001$) (Figure 2B). Mean C/D ratios were significantly lower among *CYP3A5* expressers than among nonexpressers within the first 6 mo posttransplant (Figure 2C).

CYP3A5 Genotype Is Associated With Development of De Novo Anti-HLA Antibodies and De Novo DSAs

As indicated in Table 2, the incidence of the development of de novo class I and II anti-HLA antibodies after transplant was significantly higher among recipients carrying the expresser *CYP3A5* genotype (24/69 [35%] versus 83/331 [25%]; $P = 0.05$). De novo anti-HLA antibody-free graft survival was significantly worse for *CYP3A5* expressers than for nonexpressers ($P = 0.03$; Figure 3A). In particular, de novo class I anti-HLA antibody-free graft survival was significantly lower among carriers of the *CYP3A5* variant, whereas the difference between groups in de novo class II anti-HLA antibody-free survival did not achieve statistical significance (Figure 3B and C). It is

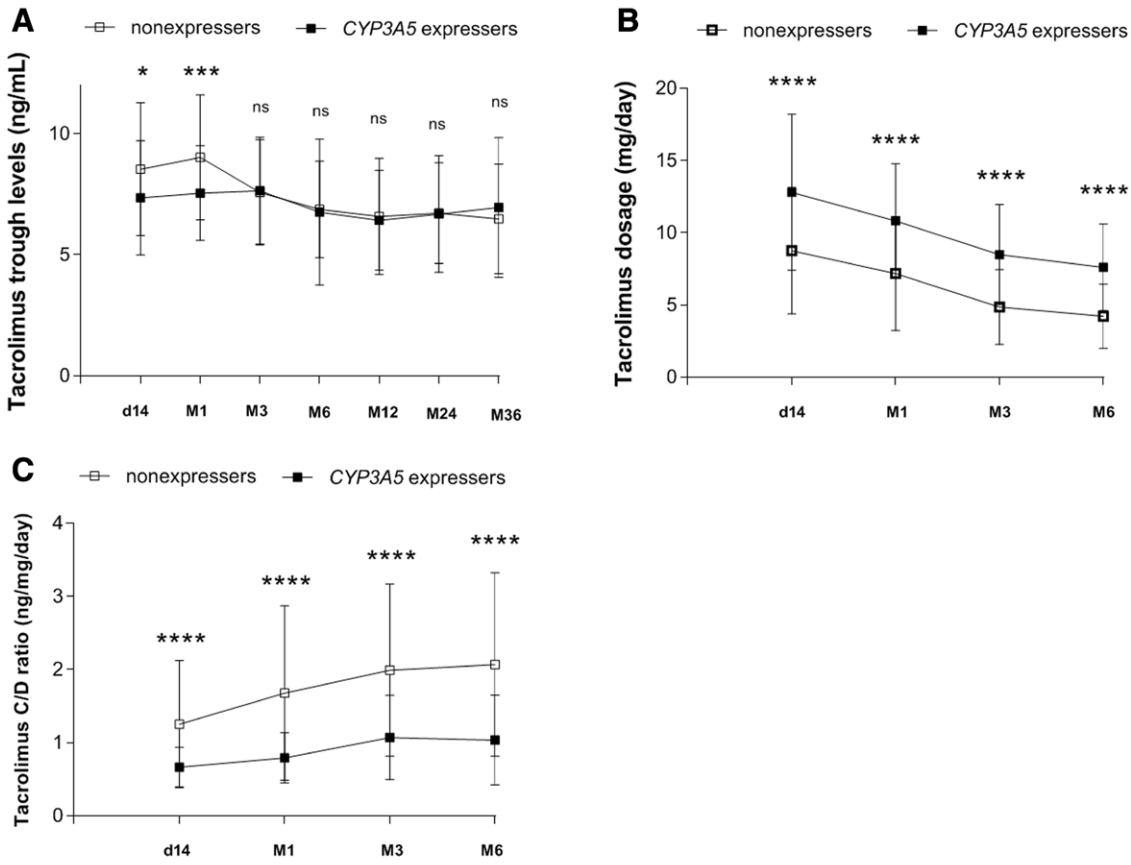

FIGURE 2. Posttransplant tacrolimus trough levels and tacrolimus dosages for *CYP3A5* expressers and nonexpressers. A, Tacrolimus trough levels for *CYP3A5* expressers and nonexpressers during 3 y after transplant. B, Tacrolimus dosages after transplant for *CYP3A5* expressers and nonexpressers during the first 6 mo after transplant. C, Tacrolimus concentration-to-dose ratios during the first 6 mo after transplant for *CYP3A5* expressers and nonexpressers (data are presented as means). *P = 0.05; ****P < 0.001. C/D, ratio of serum concentration of tacrolimus to daily dosage of tacrolimus.

noteworthy that the frequency of de novo anti-HLA DSA development was significantly higher among *CYP3A5* expressers than among nonexpressers 5 y after transplant (13/69 [19%] versus 33/331 [10%]; P = 0.02; Table 2). Regarding median MFI values and peak MFI values of immunodominant de novo anti-HLA DSAs, no significant difference was detected between the 2 groups (**Figure S1, SDC,** http://links.lww.com/TP/C259). Moreover, de novo anti-HLA DSA-free graft survival was significantly worse among *CYP3A5* expressers than among *CYP3A5* nonexpressers (P = 0.019; Figure 3D). Further distinction between de novo class I and II DSAs showed both a significantly higher incidence of de novo class I DSAs and a significantly lower de novo class I DSA-free survival rate for *CYP3A5* expressers than for nonexpressers (8/69 [12%] versus 17/331 [5%]; Table 2; P = 0.02; **Figure S2A, SDC,** http://links.lww.com/TP/C259). The results for de novo class II DSAs showed a trend but failed to reach statistical significance (Table 2 and **Figure S2B, SDC,** http://links.lww.com/TP/C259).

Univariate analysis showed that the presence of either the *CYP3A5**1/*1 or the *CYP3A5**1/*3 genotype was associated with the posttransplant occurrence of de novo anti-HLA DSAs (relative risk, 1.89 [95% confidence interval, 1.05-3.4]; P = 0.04; Table 3). Previous renal transplant, the number of HLA-A and -B mismatches, preformed anti-HLA DSAs, and preformed class II anti-HLA antibodies were also risk factors for the development of de novo anti-HLA DSAs (Table 3). A multivariate Cox regression analysis showed that the effect of *CYP3A5* genotype on the development of de novo anti-HLA DSAs reached statistical significance, a finding indicating that the *CYP3A5* genotype is an independent risk factor for de novo DSA development (relative risk, 2.34 [95% confidence interval, 1.22-4.5]; P = 0.01; Table 3).

CYP3A5 Genotype Is Associated With AMR but Has Limited Effect on Allograft Loss 5 y After Transplant

We found no differences between expressers and nonexpressers in the occurrence of biopsy-proven allograft rejection episodes and in rejection-free survival rates during the 5-y follow-up period (Table 2; Figure 4A). However, the odds ratio of rejection events according to *CYP3A5* genotype was 1.34, a finding reflecting a trend toward an association between biopsy-proven allograft rejection and *CYP3A5* expression. Distinguishing between cellular rejection (Banff category 3 or 4) and AMR revealed similar incidences of cellular rejection and the cellular rejection-free survival rates in both groups (Table 2; Figure 4B). We observed a significantly higher rate of AMR in the group of *CYP3A5* expressers than in the nonexpressers at 5 y after transplant (8/69 [12%] versus 17/331 (5%); P = 0.02; Table 2). Similarly, among patients with AMR we

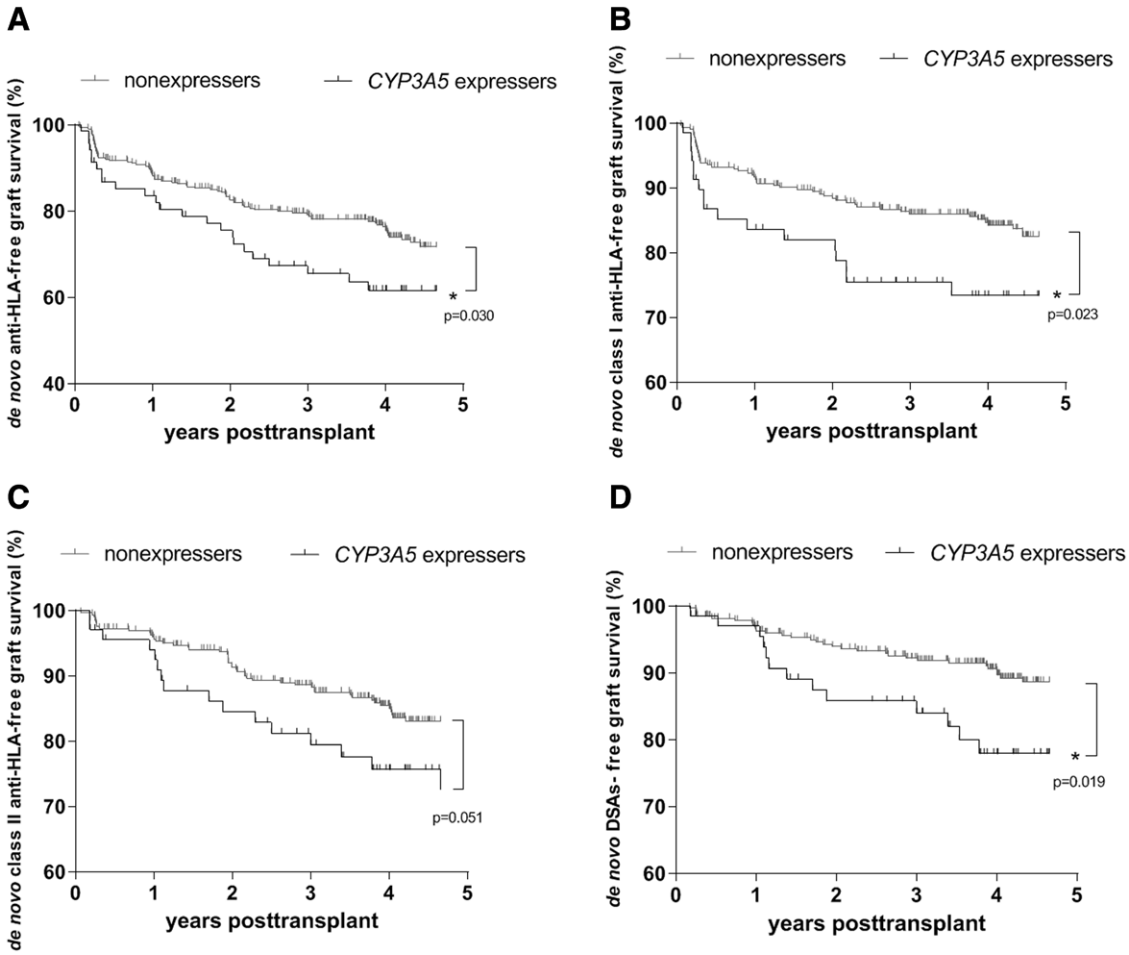

FIGURE 3. Development of de novo anti-HLA antibodies after renal transplant in relation to the *CYP3A5* genotype during 5-y follow-up after transplant. A, Graft survival for occurrence of de novo anti-HLA antibodies according to *CYP3A5* genotype ($P = 0.03$). B, Graft survival for development of de novo anti-HLA class I antibodies ($P = 0.023$). C, Graft survival for development of de novo anti-HLA class II antibodies ($P = 0.051$). D, Graft survival for appearance of de novo anti-HLA DSA antibodies ($P = 0.019$). *$P = 0.05$. DSA, donor-specific antibody.

saw lower survival rates for *CYP3A5* expressers than for nonexpressers ($P = 0.035$; Figure 4C).

Differences in allograft survival between *CYP3A5* expressers and nonexpressers were not statistically significant (Figure 5A; Table 2). Similarly, we found no difference between *CYP3A5* expressers and nonexpressers in rates of GFR reduction after transplant (Table 2; Figure 5B).

Multivariate Cox regression analyses, which were adjusted for potential confounding factors such as previous transplants, HLA mismatches, preformed anti-HLA antibodies, and performed anti-HLA DSAs, showed that *CYP3A5* expresser status is an independent risk factor for the development of de novo anti-HLA antibodies, de novo anti-HLA DSAs, or AMR (Table 4 and **Tables S3 and S4, SDC,** http://links.lww.com/TP/C259).

Cox regression analysis of de novo DSAs free-survival adjusted for underimmunosuppression with tacrolimus trough levels at 1 mo after transplant revealed that early underimmunosuppression abrogated the effect of *CYP3A5* expresser status on de novo DSA-free survival (**Table S5, SDC,** http://links.lww.com/TP/C259).

Analysis of the effect of recipient *CYP3A5* genotype on the onset of viral infections found no significant differences between *CYP3A5* expressers and nonexpressers (Table 2).

DISCUSSION

This large retrospective study was designed to assess the relationship between *CYP3A5* gene variant and clinical outcome parameters during a 5 y period after renal transplant. *CYP3A5* expressers had lower tacrolimus levels during the first month after transplant and tended to have lower tacrolimus levels during the first 2 y after transplant, although they were receiving tacrolimus dosages as much as 80% higher than those received by nonexpressers. The frequencies of de novo anti-HLA antibodies and de novo DSAs were significantly higher among *CYP3A5* expressers than among nonexpressers. AMR-free graft survival rates were lower among *CYP3A5* expressers than among nonexpressers. Multivariate analysis showed that the *CYP3A5* variant is an independent risk factor for the development of de novo anti-HLA DSAs. However, we found no difference between *CYP3A5* expressers and nonexpressers in terms of allograft loss, the occurrence of T cell–mediated rejection, or infections.

The key finding of the present study was that the *CYP3A5* rs776746 variant confers a higher risk of the development of de novo anti-HLA DSAs. We postulate that the functional *CYP3A5* genotype also increases the risk of underimmunosuppression, considering that *CYP3A5* expressers

TABLE 3.

Results of univariate and multivariate analyses identifying risk factors for development of de novo donor-specific antibodies among 400 patients after renal allograft transplant

	De novo anti-HLA DSA-positive (n = 46)	Patients without de novo anti-HLA DSAs (n = 354)	Univariate relative risk (95% CI)	*P*	Multivariate relative risk (95% CI)	*P*
Women, n (%)	16 (35)	149 (42)	0.76 (0.43-1.38)	0.34		
Previous transplants, n (%)	12 (26)	40 (11)	2.36 (1.29-4.13)	**0.005**	2.63 (1.21-5.7)	**0.015**
Preformed anti-HLA antibodies, n (%)	20 (43)	131 (37)	1.27 (0.73-2.19)	0.39		
Class I, n (%)	17 (37)	108 (31)	1.29 (0.74-2.26)	0.37		
Class II, n (%)	13 (28)	57 (16)	1.86 (1.03-3.34)	**0.04**	1.45 (0.64-3.3)	0.38
Preformed anti-HLA DSAs, n (%)	8 (17)	29 (9)	2.07 (1.04-4.09)	**0.04**	1.22 (0.49-3.04)	0.67
MM (A/B), n (%)	43 (93)	289 (82)	2.94 (0.94-9.19)	**0.04**	3.57 (1.08-11.75)	**0.036**
MM (DR), n (%)	35 (76)	251 (71)	1.27 (0.67-2.41)	0.46		
ABO-incompatible transplant, n (%)	4 (9)	27 (8)	0.96 (0.44-2.95)	0.96		
Autoimmune disease as cause of ESRD, n (%)	2 (4)	16 (5)	0.92 (0.25-3.67)	0.9		
CYP3A5 variant, n (%)	13 (28)	56 (16)	1.89 (1.05-3.4)	**0.04**	2.34 (1.22-4.5)	**0.01**
Deceased donors, n (%)	29 (63)	256 (72)	0.69 (0.39-1.20)	0.19		
No. of female donors, n (%)	26 (57)	160 (45)	1.5 (0.86-2.59)	0.15		

CI, confidence interval; DSA, donor-specific antibody; ESRD, end-stage renal disease; MM, mismatch.

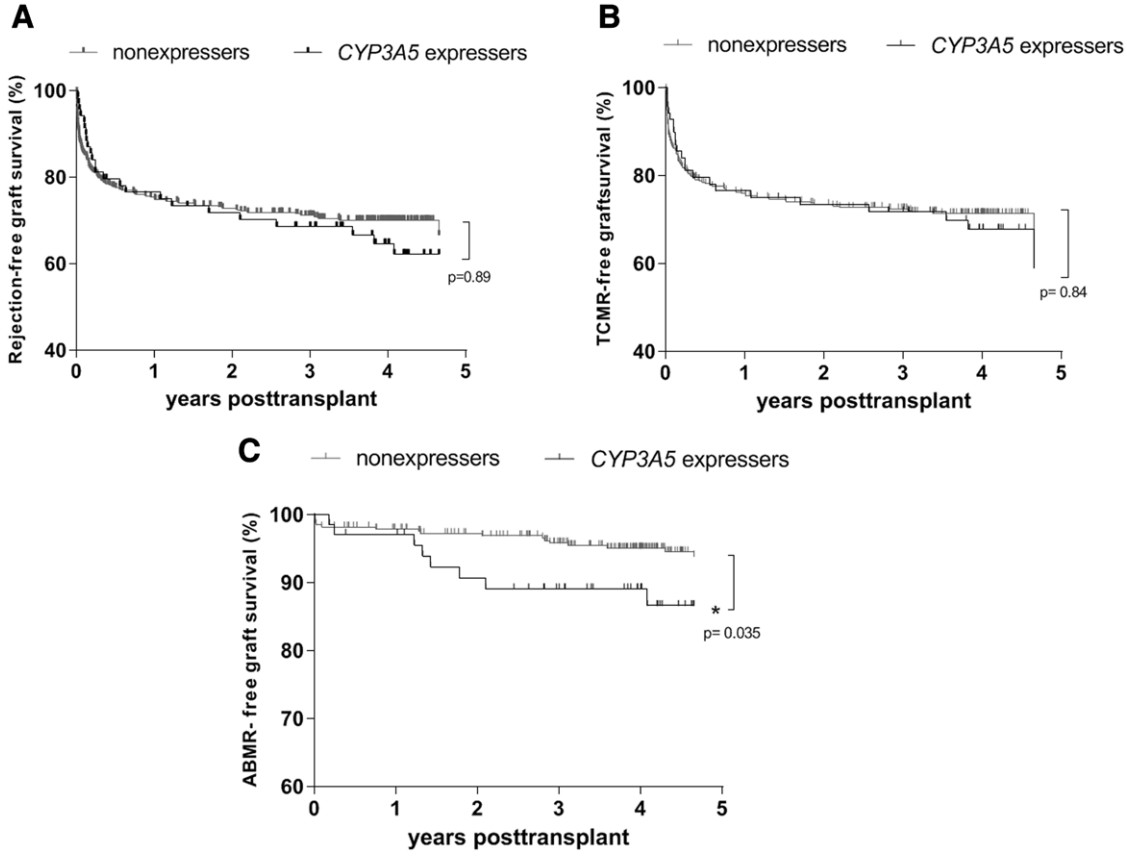

FIGURE 4. Biopsy-proven rejection-free graft survival among renal transplant patients according to *CYP3A5* genotype during 5-y follow-up after transplant. A, Rejection-free graft survival rates for *CYP3A5* expressers and nonexpressers ($P = 0.89$). B, T cell–mediated rejection–free graft survival rates for *CYP3A5* expressers and nonexpressers ($P = 0.84$). C, Antibody-mediated rejection–free graft survival rates for CYP3A5 expressers and nonexpressers ($P = 0.035$). *$P = 0.05$. AMR, antibody-mediated rejection; DSA, donor-specific antibody; TCMR, T cell–mediated rejection.

require nearly double the tacrolimus dose as that required by nonexpressers. Underimmunosuppression may promote the development of de novo anti-HLA DSAs. Low tacrolimus exposure, which is linked to *CYP3A5* expression, has been shown to be associated with a higher risk of the development of de novo anti-HLA DSAs.[31,32]

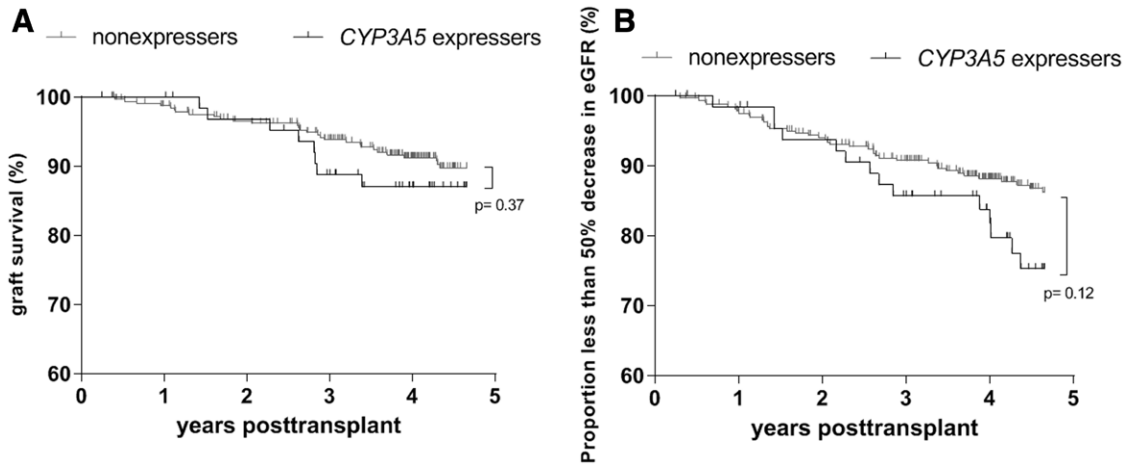

FIGURE 5. Outcome of renal allograft transplant in 2 patient groups categorized by *CYP3A5* genotype during 5-y follow-up. A, Allograft survival rate for *CYP3A5* expressers compared to that for nonexpressers (P = 0.37). B, Proportion of patients with stable allograft function among *CYP3A5* expressers and nonexpressers (P = 0.12). Stable graft function was defined as the loss of <50% in eGFR compared with baseline eGFR after transplant. eGFR, estimated glomerular filtration rate.

TABLE 4.

Results of univariate and multivariate analyses identifying risk factors for development of antibody-mediated rejection among 400 patients after renal allograft transplant

	Patients with AMR (n = 25)	Patients without AMR (n = 375)	Univariate relative risk (95% CI)	*P*	Multivariate relative risk (95% CI)	*P*
Women, n (%)	13 (52)	152 (41)	1.54 (0.72-3.3)	0.26		
Previous transplants, n (%)	7 (28)	45 (12)	2.60 (1.15-5.67)	**0.02**	0.95 (0.36-2.49)	0.91
Preformed anti-HLA antibodies, n (%)	17 (68)	134 (36)	3.50 (1.55-7.92)	**0.001**	1.77 (0.68-4.62)	0.25
Class I, n (%)	15 (60)	111 (30)	3.26 (1.51-7.06)	**0.002**		
Class II, n (%)	12 (48)	59 (16)	4.28 (2.04-8.98)	**0.0001**		
Preformed anti-HLA DSAs, n (%)	14 (56)	23 (6)	12.49 (6.12-25.49)	**0.0001**	12.08 (4.94-29.57)	**0.00001**
MM (A/B), n (%)	24 (96)	308 (82)	4.92 (0.68-35.72)	0.07		
MM (DR), n (%)	21 (84)	265 (71)	2.09 (0.73-5.96)	0.15		
ABO-incompatible transplant, n (%)	3 (12)	28 (8)	1.62 (0.51-5.12)	0.41		
Autoimmune disease as cause of ESRD, n (%)	2 (8)	16 (4)	1.86 (0.47-7.23)	0.38		
CYP3A5 variant, n (%)	8 (32)	61 (16)	2.26 (1.02-5.02)	**0.04**	2.53 (1.08-5.9)	**0.032**
Deceased donors, n (%)	18 (72)	267 (71)	1.04 (0.44-2.42)	0.93		
No. of female donors, n (%)	12 (48)	174 (46)	1.5 (0.5-2.27)	0.88		

AMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific antibody; MM, mismatch.

In fact, we observed significantly lower tacrolimus trough levels among *CYP3A5* expressers than among nonexpressers during the first month after transplant, although *CYP3A5* expressers were already receiving significantly higher tacrolimus dosages than nonexpressers. These findings indicate underimmunosuppression in *CYP3A5* expressers during the early period after transplant despite meticulous adjustment of tacrolimus dosage. In long-term follow-up, the difference between expressers and nonexpressers faded, although *CYP3A5* expressers tended to have slightly lower tacrolimus trough levels than did nonexpressers during the first 2 y after transplant, although their tacrolimus dosage was nearly twice as high. To control for tacrolimus trough levels in a Cox regression analysis, we performed adjustment for tacrolimus trough levels at 1 mo after transplant abrogating the independent influence of *CYP3A5* genotype on the risk of de novo DSA development. We can assume that *CYP3A5* expressers are more prone to underimmunosuppression than are nonexpressers, and this underimmunosuppression may lead to alloimmunization.

The prevalence of the *CYP3A5**1 allele in 17% of our study population was not significantly different from the reported frequencies of this genetic variant (8%–11%) among other European populations of renal allograft recipients.[33,34] This finding excludes bias in the reported results of our study. The *CYP3A5* rs776746 variant is believed to increase the intrapatient variability of tacrolimus metabolization.[9] Seibert et al[35] demonstrated the contribution of *CYP3A5* loss-of-function variants to intrapatient variability in tacrolimus pharmacokinetics. Several studies have associated an accelerated incidence of de novo anti-HLA DSAs with a higher variability in tacrolimus levels among recipients of solid-organ transplants.[36-39] Rodrigo et al[37] reported that a high degree of intrapatient variability in tacrolimus trough levels is an independent risk factor for

de novo anti-HLA DSA development within the first year after kidney transplant. Therefore, it is conceivable that recipients expressing the *CYP3A5*1* allele are predominantly exposed to episodes of subtherapeutic tacrolimus concentrations leading to humoral immune activation, including the formation of DSAs. Unfortunately, our retrospective study did not assess the intrapatient variability of tacrolimus trough levels.

Insufficient immunosuppression also impairs allograft outcome and leads to the occurrence of acute rejection. Low tacrolimus C/D ratios are associated with decreased allograft survival.[40] Moreover, some reports suggest that the high intrapatient variability of tacrolimus trough levels as a surrogate for under immunosuppression, may be responsible for allograft failure and T cell–mediated rejection, as well as for acute AMR.[32] However, studies involving European cohorts of renal allograft recipients found no significant association between *CYP3A5* gene variant and either allograft survival or the occurrence of biopsy-proven rejection.[9,24,41-43] A significantly higher rate of acute rejection events was found in Asian cohorts and may be due to the higher frequency of the expresser allele among Asians.[24,42] Accordingly, the previously published results of a meta-analysis pooling 25 studies of the association between *CYP3A5* genotype and the risk of acute rejection found no significant effect.[42] However, the findings about the impact of *CYP3A5* genotype on rejection episodes differ strongly between various studies. This difference may first be attributed to the fact that most of the studies did not require routine renal allograft biopsies, used inhomogeneous histologic diagnostic criteria for acute rejection, and did not consider recent modifications in these criteria. Second, in most of the studies, the follow-up period was <12 mo after transplant, and this short period resulted in an inadequate estimation of the incidence of rejection. Moreover, these studies may have failed to detect a significant effect of *CYP3A5* genotype on acute rejection because their cohorts included mostly patients with a low risk of rejection.

The present study provided a long follow-up period of at least 5 y and included a large number of patients. Compared to our study, most of the 25 previous studies involved fewer study subjects and chose a much shorter follow-up period, observing patients for 1 y or less after transplant.[24,42] In addition, previous studies considered acute cellular rejection or all rejection episodes after transplant without detailed differentiation between TBMR and AMR. No previous studies determined the development of de novo DSAs.

Only 3 of 25 studies considered the risk of rejection during a long-term period after transplant. As demonstrated by Khan et al,[42] who evaluated the influence of *CYP3A5* genotype on acute rejection by clustering the existing studies into subgroups with similar study periods, there is a significant association between *CYP3A5* genotype and the risk of acute rejection within 36–60 mo after transplant in the 3 selected studies.[44-46] In short-term follow-up period, *CYP3A5* expressers had a comparable risk to develop acute rejection compared to nonexpressers.[42] In 2007, a study by Quteineh et al,[44] which involved 136 renal allograft recipients with a follow-up of 1 y, found that acute rejection, classified as Banff category 1 (cellular) according to the 2005 Banff criteria, was more common among *CYP3A5* expressers than among nonexpressers. A 2009 study by Singh et al[45] also found a significantly higher rate of acute biopsy-proven rejection, as classified by the 1997 Banff criteria, among *CYP3A5* expressers. However, the number of patients treated with tacrolimus-based therapy was low with 73 recipients, and the rate of acute rejection was still very high, ranging from 41% to 63%. The 2010 study by Kuypers et al[46] involved 304 renal allograft recipients and found a slightly higher frequency of acute rejection episodes among expressers than among nonexpressers. In contrast to our study, all 3 large studies with long-term follow-up of transplant recipients focused on acute cellular rejection and did not analyze the occurrence of AMR or determine the appearance of de novo DSAs.[44-46]

We found a higher risk of the development of de novo HLA-antibodies, de novo DSAs, and subsequent AMR among patients expressing the *CYP3A5* allele than among nonexpressers. Additionally, AMR occurred earlier among *CYP3A5* expressers than among nonexpressers, a finding corresponding to lower AMR-free allograft survival rates among carriers of the *CYP3A5* variant. Contradicting the results of previously cited studies, we detected no association between *CYP3A5* expresser status and acute cellular rejection.[44-46]

When the results of the current study are compared with those of previous studies, it should be taken into consideration that many previously published studies were performed before 2010 and, unlike our study, used previous Banff classification criteria to diagnose rejection. In several studies, no clear differentiation between TBMR and AMR was considered, and data about acute rejection episodes were pooled. The Banff diagnostic criteria for AMR have also changed profoundly over the past 10–15 y; therefore, episodes of AMR that were included in our study were unrecognized elsewhere.

To verify our results regarding the association of de novo DSAs and AMR with *CYP3A5* expresser status in terms of possible confounders, we adjusted the analysis for known confounders such as previous transplants, the presence of HLA mismatches, and preformed HLA DSAs, as well as differences in baseline characteristics of the cohort in a multivariate Cox model analysis. *CYP3A5* expresser status remained an independent risk factor, a finding indicating its clinical relevance for the development of de novo DSAs and AMR.

Our results must be considered in light of several other limitations of the study. One of the most important limitations of the study is its retrospective design, which made it impossible to assess intrapatient variability of tacrolimus trough levels. Moreover, we did not perform protocol biopsies. Renal transplant biopsy was generally performed only when rejection was clinically suspected. As a consequence, we may have underestimated the frequency of AMR rejection. Finally, we also cannot completely exclude other potential residuals or unmeasured confounders affecting the reported data.

We found a higher rate of AMR among *CYP3A5* expressers, but this rate was not associated with renal allograft survival or the incidence of cellular rejection; these findings confirm the results of recent studies.[41] One possible explanation for this finding is that allograft failure is a rather late event after the development of de novo DSAs and the appearance of AMR; therefore, a long follow-up period

would be necessary for detecting a statistically significant difference. Thus, the tacrolimus metabolism phenotype seems to exert an essential impact on allograft survival, an effect that is partly attributed to the pharmacogenetics of tacrolimus, including *CYP3A5* genotype. Apart from pharmacogenetic variations, other factors, such as medication nonadherence, drug-drug interactions, nutritional interferences, and concurrent diseases, are known to affect tacrolimus absorption and elimination and to determine the variability in tacrolimus exposure.[9]

Although the *CYP3A5* rs776746 variant is assumed to be the most important genetic variant in tacrolimus metabolism, other genetic variants, such as *CYP3A4**22, *CYP3A4**1B, or *POR**28, may account for additional differences in tacrolimus metabolization and may affect the variability of tacrolimus.[16] In line with this hypothesis, a combination of functional genetic variants of *CYP3A5* and *CYP3A4* in kidney transplant recipients was linked to an extensive clearance of tacrolimus requiring very high tacrolimus doses.[47]

Concerning the association between the *CYP3A5* genotype and infectious complications after transplant, the frequencies of viral and bacterial infection were comparable in *CYP3A5* expressers and nonexpressers. Two previous studies on renal and liver transplant recipients demonstrated elevated number of CMV and bacterial infections among *CYP3A5* expressers that were explained by potential overimmunosuppression.[48,49] Conflicting results of our study might be attributed to the fact that *CYP3A5* expressers in our study may have been underexposed rather than overexposed to tacrolimus.

Taken together, the results of our retrospective study emphasize the importance of *CYP3A5* expression status as an independent risk factor for the development of de novo anti-HLA DSAs and AMR after renal transplant. However, *CYP3A5* expression did not affect allograft survival, probably because of the relatively short follow-up period of 5 y after transplant. Our findings indicate that early detection of high-risk patients carrying the *CYP3A5* rs776746 variant allows tacrolimus dosing changes that can minimize the risk of de novo anti-HLA DSA development after renal transplant and can improve long-term renal allograft survival. Clinicians can appropriately avoid increasing the tacrolimus dose because they are alert to tacrolimus toxicity in cases of fluctuating tacrolimus trough levels. The patient subgroup of *CYP3A5* expressers requires close and regular therapeutic drug monitoring with subsequent correction of subtherapeutic ranges of tacrolimus trough levels during long-term follow-up. Our findings show that the link between genetic variations in tacrolimus metabolizing enzymes and a patient's disposition to alloimmune responses is important, and they indicate that the pathophysiology behind the described association between *CYP3A5* expresser genotype and de novo anti-HLA DSA development should be investigated in further mechanistic studies.

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