The MDR1/ABCB1 Gene, a High-Impact Risk Factor for Cardiac Transplant Rejection

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Background. Variations in the expression and activity levels of the multidrug-resistance MDR1/ABCB1 encoded P-glycoprotein (P-gp) have an impact on the therapeutic efficacy of many drugs. C3435T and G2677 polymorphisms of the MDR1/ABCB1 gene correlate with cellular expression levels of P-gp, a membrane-bound efflux pump which removes a multitude of drugs, including chemotherapy drugs and immunosuppressants, from cells. We aimed to investigate whether the phenomenon of drug resistance, mediated by the MDR1/ABCB1 gene and seen in tumor cells to chemotherapeutic agents, is important in the field of transplantation, predisposing some patients to resistance to immunosuppressants.
Methods. G2677 and C3435T polymorphisms of the ABCB1 gene were determined by PCR in 170 heart transplant recipients. We examined the relationship between MDR1/ABCB1 polymorphisms and endomyocardial biopsy-proven rejection (EBPR) determined by biopsy performed at set intervals according to a standard protocol.
Results. A significant relationship was found between a patient's C3435T genotype and freedom from first grade ≥3A rejection episode. 3435-CC recipients were 1.8 times (1.05–3.09; $P=0.03$) more likely to undergo a ≥3A rejection episode in the first 12 months. Haplotypes derived from the G2677 and C3435T polymorphisms (GG/CC, GT/CT and TT/TT) amplified this phenomenon further (log rank, $P=0.03$; HR 2.18; 1.21–4.26; $P=0.02$).
Conclusions. ABCB1 polymorphisms correlate with freedom from grade ≥3A EBPR and we believe that this may be attributed to MDR1/ABCB1 encoded P-gp mediating the efflux of immunosuppressants out of leukocytes, with depleted immunosuppressant levels in leukocytes manifesting as increased cellular rejection.

Keywords: Gene polymorphism, Heart transplantation, P-glycoprotein, MDR1/ABCB1, Rejection.

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The MDR1/ABCB1 gene encodes the membrane-bound efflux pump P-gp. P-gp is expressed in the apical membranes of excretory tissues, such as liver, kidney, and intestine and contributes to the elimination of xenobiotics and drugs into bile and urine and limits drug absorption from the gastrointestinal tract (*1*, *2*). P-gp removes a multitude of drugs, including chemotherapy drugs and immunosuppressants, from cells. G2677, located at exon 21, and C3435T, located at exon 26, of the MDR1/ABCB1 gene are single nucleotide polymorphisms (SNPs) which correlate with cellular expression levels of P-gp on leukocytes (*3*). As P-gp expression has also been demonstrated in T cell membranes (*4*) and it is a major transporter of immunosuppressant drugs out of cells it may locally alter drug response in lymphocytes. Variation at the exon 21 locus leads to amino acid modification (Ala893Ser/Ala893Thr) which has been linked with in vitro enhanced efflux of digoxin from cells (*5*). The C3435T SNP is a silent mutation and the mechanism by which it exerts its influence is as yet unknown (*6*), although patient handling of P-gp substrates is most varied where groups are studied according to this polymorphism of the MDR1/ABCB1 gene (*7*). Numerous other SNPs have been characterized (*8*), however, little clinical effect has been demonstrated. Strong linkage disequilibrium (allelic association beyond that expected between two distinct loci) has been detected between several MDR1/ABCB1 polymorphisms, and inconsistencies in the literature may be due to the attention on the power of SNP variations instead of on linked nucleotide variations (*9*).

In oncological studies, a survival advantage for patients carrying the 3435-TT genotype of the exon 26 MDR1/ABCB1 polymorphism has been shown in a study of 405 patients with acute myeloid leukemia (*10*) and similar improved survival has been seen in childhood acute lymphoblastic leukemia (*11*), breast cancer (*12*), and renal epithelial tumors (*13*).

Many reports have found only equivocal results regarding immunosuppressant levels (*14*, *15*) and few studies have reported a definitive affect on rejection episodes or other outcomes, although most studies have focused on small populations of patients. The aim of this study was to determine whether SNPs of the MDR1/ABCB gene have any impact on the levels of rejection that are encountered by patients, the rejection potentially signifying a form of drug resistance to immunosuppressive agents.

METHODS

We examined the relationship between MDR1/ABCB1 polymorphisms and endomyocardial biopsy-proven rejection (EBPR) determined at set intervals according to a standard protocol for 170 heart transplant patients.

Study Patients

Cardiac allograft recipients transplanted between April 1987 and March 2005 at the Wythenshawe Cardiothoracic

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Transplant Centre, Manchester, UK, were studied. Informed written consent was obtained from all patients in compliance with South Manchester Local Regional Ethics Committee Regulations. Immunosuppression consisted of a standard triple-drug regimen of cyclosporine A (CyA), azathioprine, and prednisolone. Target CyA blood trough level were 200–300 ng/ml for the first three months, 150–300 ng/ml for the first year, and 100–150 ng/ml from 12 months onwards. Transplant rejection was assessed by endomyocardial biopsy at weeks 1, 2, 3, 4, 6, 8, 10, and 12 and then monthly between 3 and 6 months and then at months 9 and 12. Additionally, biopsy was undertaken if there was clinical evidence of rejection. The risk associated with polymorphisms in the MDR1/ABCB1 gene was compared using Cox regression analysis with other known risk factors in the same cohort of patients, namely cytomegalovirus (CMV) mismatch (*16*), human leukocyte antigen (HLA) mismatch (*17*), sex mismatch (*18*), ischemic time and age for comparison (*19*). Patient immunosuppressant regimen, drug dosage, and plasma immunosuppressant levels were analyzed at 3 months and 12 months from the date of transplantation. This was a retrospective study. Patients were managed without knowledge of their MDR1/ABCB1 genotype.

Sequence Specific Polymorphism: Polymerase Chain Reaction (PCR) Exon 21 and 26 (ABCB1)

Specific oligonucleotide primers were designed based on the published DNA sequence (Genosys, UK); exon 21 A allele, G allele and T allele 5′-GTTTGACTCACCTTCCCAG T-3′; C-3′; and A-3′ respectively with generic 5′-GCAGGC-TATAGGTTCCAGG-3′; exon 26 C and T allele 5′-GTGGT-GTCACAGGAAGAGAT C-3′ and T-3′ respectively with generic, 5′-CCAGATGCTTGTATACAGGTA-3′. Internal control primers to the human growth hormone sequence ensured amplification had occurred; 5′-GGT CTT CCA GCT GGA GAA-3′ and 5′-TAA ATA GAG GGA GCT GGC-3′. The primers were made to 50 mM by the addition of measured quantities of PCR grade water. PCR was performed according to a standard technique previously described in our laboratory (*20*).

Statistical Analysis

Patient clinical data is presented as means±SD for continuous variables and as the number of occurrences for noncontinuous variables. One way analysis of variance (ANOVA) was used to compare differences between groups for continuous data and the Mann Whitney *U* test (SPSS for Windows 11.0; SPSS Inc, Chicago, IL) was used to test for significance between independent samples for noncontinuous data. Univariate analysis in terms of freedom from rejection was explored with the use of Kaplan-Meier and log-rank test analyses. Risk factors for time to first ≥3A endomyocardial rejection were explored using univariate and multivariate Cox regression analyses. A value of *P* less than 0.05 was used to indicate significance. Arlequin version 2.000 (*21*) was used to calculate genotype frequencies and to check Hardy Weinburg distribution.

RESULTS

In all, 170 cardiothoracic transplant patients were studied. Polymorphism distributions are shown in Tables 1 and 2 and are in the Hardy-Weinberg equilibrium. Exon 21 AT and AG polymorphisms are rare and represent typically less then 5% of patients in previously described studies (*8*, *22*). No significant demographic or baseline physiological differences were found between the subgroups according to univariate analysis.

Analysis of the exon 21 gene polymorphism and its influence on freedom from ≥3A grade rejection showed no significant overall differences by the log rank test, however, there was a significant difference in the number of ≥3A rejection episodes between the GG group and the TT group ($P=0.038$). A significant relationship was found between a patient's exon 26 genotype and freedom from first grade ≥3A rejection episode. 3435-CC homozygous recipients were 1.8 times (1.05–3.09, $P=0.03$) more likely to undergo a ≥3A rejection episode in the first 12 months.

Analysis of the exon 21 and exon 26 haplotypes GG/CC (n=17), GT/CT (n=57), and TT/TT (n=20) showed that the overall difference between actuarial curves in terms of free-

TABLE 1. Patient data: G2677T (exon 21) and C3435T (exon 26) of the ABCB1 gene

	Exon 21						Exon 26			
	AG	AT	GG	GT	TT	*P* value	CC	CT	TT	*P* value
Observed recipients (%)	6 (3.7)	5 (3.1)	36 (22.1)	78 (47.1)	37 (22.1)		29 (17.1)	105 (62.0)	36 (21.1)	
Expected recipients (%)			37.3 (24.5)	76.2 (49.4)	38.9 (25.5)		39.1 (23.0)	84.8 (50.0)	45.9 (27.0)	
Age at transplant (years)	42.5 (±5.9)	52.6 (±4.5)	48.1 (±2.0)	48.9 (±1.3)	45.8 (±2.0)	0.47	46.6 (±2.8)	47.2 (±1.2)	50.5 (±1.6)	0.31
Male recipient	66.70%	80%	91.90%	82.30%	86.49%	0.41	69.00%	58.10%	75.00%	0.15
HLA-A mismatch 0/1/2	1/3/2	1/2/1	7/17/13	15/32/31	3/18 /15	0.97	5/16/8	21/45/37	3/16/18	0.21
HLA-B mismatch 0/1/2	0/2/4	0/2/2	0/17/20	5/28/45	0/12/24	0.62	0/11/8	3/38/62	2/14/21	0.93
HLA-DR mismatch 0/1/2	0/2/4	0/2/2	2/18/17	7/27/44	2/15/19	0.77	3/12/14	5/34/64	3/18/16	0.21
Sex mismatch	16.70%	0%	25%	34.2%	32.4%	0.44	34.50%	34.30%	22.20%	0.38
Donor age (years)	36 (±4.4)	42.3 (±9.6)	29.2 (±1.9)	33 (±1.4)	29.3 (±2.0)	0.14	29.2 (±2.6)	32 (±1.2)	31.4 (±1.9)	0.19
Ischemic time (min)	218 (±42)	223.2 (±15)	191.9 (±8)	201.4 (±6)	210.4 (±8)	0.43	194 (±10)	199 (±5)	215 (±10)	0.55
Donor CMV-positive	16.7%	40%	36.10%	51.9%	49.2%	0.28	44.80%	42.90%	41.70%	0.44
Recipient CMV-positive	50%	60%	44.40%	48%	40.50%	0.95	55.20%	52.40%	63.90%	0.48

Values expressed as a percentage or mean (±SE). There were no statistically significant differences between groups.

TABLE 2. Patient data: exon 21 and 26 haplotypes

	Exons 21 and 26			
	GG/CC	GT/CT	TT/TT	*P* value
Number of recipients (%)	17 (18%)	57 (60.6%)	20 (21.2%)	
Age at transplant (years)	50.2 (±3.3)	49 (±1.6)	50.5 (±2.3)	0.87
Male recipient	82.3%	84.2%	80.0%	0.91
HLA-A mismatch 0/1/2	2/10/5	13/22/21	2/9/9	0.46
HLA-B mismatch 0/1/2	0/7/10	3/19/34	0/7/13	0.69
HLA-DR mismatch 0/1/2	1/9/7	3/16/37	1/9/10	0.36
Sex mismatch	23.5%	36.8%	33.3%	0.44
Donor age (years)	27.1 (±2.7)	32.9 (±1.5)	29 (±2.7)	0.14
Ischemic time (min)	188 (±13)	205.8 (±7)	227.7 (±12)	0.07
Donor cytomegalovirus-positive	41.1%	50.9%	60.0%	0.61
Recipient cytomegalovirus-positive	35.2%	50.9%	65.0%	0.41

Values expressed as a percentage or mean (±SE). There were no statistically significant differences between groups.

dom from grade 3A rejection by the log rank test was significant ($P=0.03$; Fig. 1) and that the difference between the two homozygous groups was highly significant ($P=0.009$). The increased risk of having a grade ≥3A rejection episode for the GG/CC haplotype was 2.18 times greater than the GT/CT and the TT/TT haplotypes (95% CI 1.21–4.26; $P=0.022$). The greatest risk factor for grade ≥3A rejection by univariate analysis was the MDR1/ABCB1 gene haplotype exon 21 GG and exon 26 CC (Table 3); however, by multivariate analysis exon 26 was the only significant independent risk factor (HR 2.203; 95% CI 1.13–4.30; $P=0.02$).

Mean weight-adjusted prednisolone dose at 12 months (0.17±0.04 mg/kg) for the exon 26 CC genotype was 19% and 33% higher then for the CT and TT groups respectively ($P=0.015$). No CC patients were weaned from Prednisolone, one patient was weaned from the heterozygous group 0.095% and five were weaned from the TT group 13.9% by 12 months ($P=0.19$). Mean weight adjusted CyA dose (Fig. 2) at 3 months was 4.53±2.11 mg/kg for the exon 26 CC genotype and was 0.9% and 46.8% higher then for the CT and TT groups respectively ($P=0.001$) and at 12 months at 5.29± 1.89 mg/kg was 32.8% and 49.7% higher then the CT and TT groups, respectively ($P=0.003$). There was no significant difference between CyA trough plasma levels between groups or between creatinine clearance at 3 or 12 months for any genotype group. By 12 months from the time of transplant 13.8%, 16.2% and 13.9% of exon 26 CC, CT and TT patients had been converted to tacrolimus from CyA due to repeated rejection episodes or CyA toxicity ($P=0.177$) and 11%, 26.25% and 27.8% of patients had been converted from azathioprine to mycophenolate ($P=0.010$). The total number of ≥3A rejection episodes over the 12-month period was 146. For the exon 26 genotype 17, 50 and 11 patients had one or more ≥3A rejection episodes for the CC, CT, and TT genotypes respectively ($P=0.024$).

DISCUSSION

This is the first study to investigate the influence of polymorphisms of the MDR1/ABCB1 gene on freedom from rejection episodes in heart transplant recipients. Onset and severity of rejection in transplant patients is variable, suggesting that individual genetic differences may be highly relevant. The results demonstrate an increased risk of rejection for heart transplant patients carrying the CC genotype at exon 26, the GG genotype at exon 21, and an increased risk of rejection for the CC/GG haplotype at exon 26 and exon 21. This research shows that the MDR1/ABCB1 polymorphism that a patient carries is an important risk factor for significant EBPR in the first 12 months with an impact greater then HLA mismatch, CMV mismatch, sex mismatch, and age of recipient. Kaplan Meier analysis of ABCB1 genotype with freedom from ISHLT grade 1 or grade 2 rejections was not significant. The threshold for treatment of EBPR with high-dose steroids in most centers would be a grade ≥3A rejection episode and it is this threshold that most clinicians would take as the significant level.

With higher expression of P-gp being described on the membranes of leukocytes of patients carrying the MDR1/ABCB1 3435-CC genotype (*3*) more frequent rejection episodes were expected. P-gp is a transmembrane ion pump involved in the handling of CyA, tacrolimus, and prednisolone as well as many other drugs. The high expression of P-gp on PBMCs may enhance the rate at which these cells pump out the immunosuppressive drugs targeted at them. Calcineurin inhibitors have a principally intracellular role. Rapid clearance from the target cells, in those with a high P-gp producing phenotype, would therefore leave this group with suboptimal intracellular T cell immunosuppression and explain the higher levels of rejection that we have seen in this study.

Patients carrying the exon 26 CC genotype received higher doses of prednisolone and CyA. CyA trough levels did not vary between patients according to genotype. Variation between patients in prescribing patterns may be explained by the higher levels of rejection seen in the exon 26 CC group; however, the higher drug dosages administered did not result in a significantly higher trough plasma CyA level. In liver transplant patients the 3435-TT genotype requires approximately 50% lower weight adjusted doses of CyA then patients who are 3435-CC to maintain the same peak CyA concentration and the CC patients demonstrate a tendency towards more biopsy-proven rejection episodes (*23*). In pediatric heart transplant patients more rapid steroid weaning has

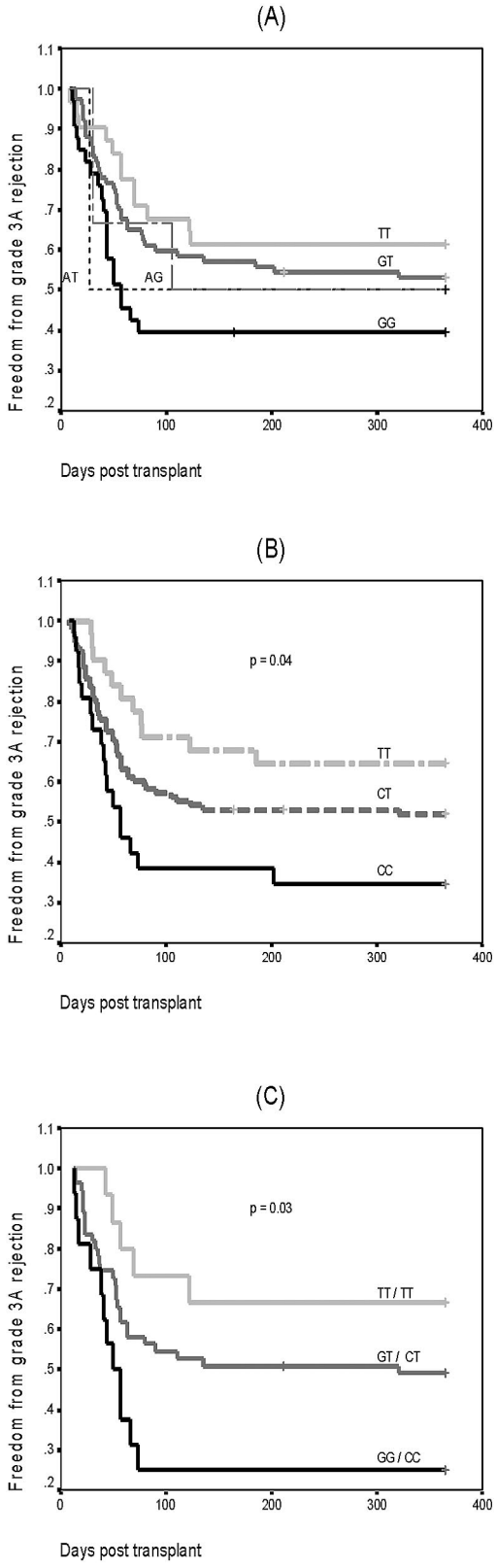

FIGURE 1. Actuarial (Kaplan Meier) freedom from grade ≥3A rejection according to ABCB1 exon 21 genotype (A), exon 26 genotype (B), and the exon 21/26 haplotype (C). The *P* values refer to the overall difference between actuarial freedoms from grade ≥3A rejection by the log-rank test for each genotype.

TABLE 3. Risk factors for grade 3A rejection episodes in the first 12 months after heart transplantation

	Hazard ratio	95% CI	*P* value
Exon 26 and exon 21 haplotype CC/GG vs. T-/T-	2.18	(1.21–4.26)	0.022
Exon 26 CC vs. T-	1.80	(1.05–3.09)	0.034
Exon 21 GG vs. T-	1.68	(1.01–2.92)	0.048
CMV mismatch vs. match	1.64	(1.05–2.57)	0.042
CMV (+D) to CMV (–R) vs. CMV (–D) to CMV (–R)	1.79	(0.89–3.56)	0.980
CMV (–D) to CMV (+R) vs. CMV (–D) to CMV (–R)	1.57	(0.83–2.98)	0.160
Female donor to male recipient	1.46	(0.89–2.39)	0.137
Male donor to female recipient	0.56	(0.18–1.77)	0.323
Heart recipient age >54 vs. <54	0.84	(0.58–1.55)	0.839
Ischemic time >200 vs. <200 min	1.15	(0.73–1.80)	0.552
HLA DR 1 or 2 vs. 0 mismatches	5.78	(0.80–41.6)	0.081
HLA A 1 or 2 vs. 0 mismatches	1.32	(0.76–2.29)	0.344
HLA B 1 or 2 vs. 0 mismatches	2.88	(0.4–20.73)	0.206

D+, donor positive; R+, recipient positive.

been observed to occur in patients who are 3435-TT genotype (*24*), however many reports have found only equivocal results regarding immunosuppressant levels (*14*, *15*, *25*) and few studies have reported a definitive effect on rejection episodes or other outcomes.

In this study, we deliberately focused on patient freedom from the first ≥3A rejection episode in order to avoid the influence of the impact in changes to drug regimen that occur from the first rejection episode onwards. Postrejection, immunosuppressive regimens, whether with steroids in the short term, alter a tendency in the doctor dosing the patient from the point of rejection onwards to increase immunosuppressant levels given a rejection history or a considered decision to change immunosuppressive regimen. Some patient's immunosuppressive regimes were altered from the original during the 12 months of the study. Despite this the number of ≥3A rejection episodes was still higher overall in the 3435-CC group.

Survival

This study was based on a cohort of patients who were recruited at a single time point. Retrospective in nature, the study has favored the recruitment of survivors and this may confound the mix of genotypes that were observed; favoring genotypes less susceptible to drug resistance, rejection or early or mid term mortality. Definitive conclusions regarding the influence of the C3435T and G2677 polymorphisms on

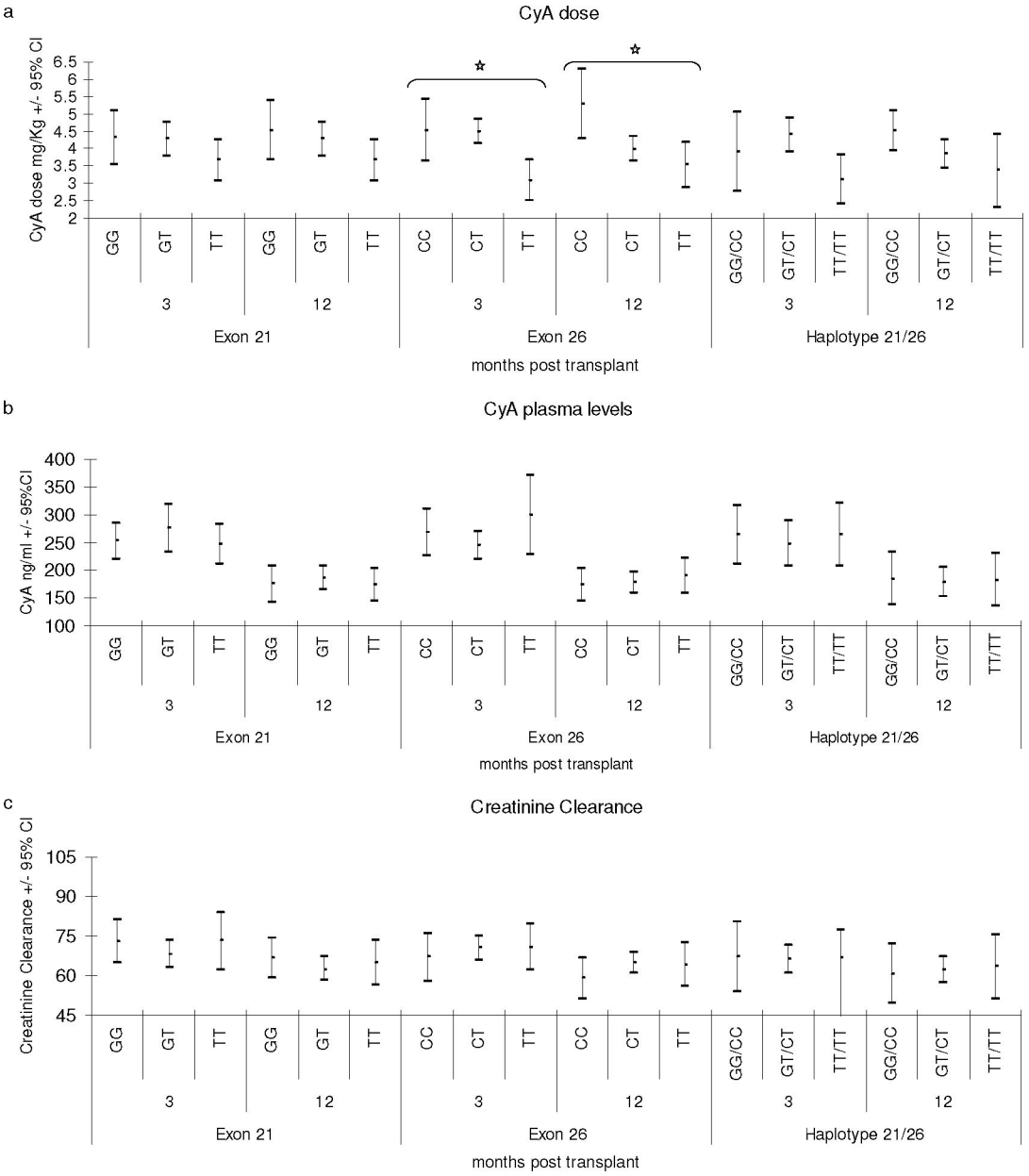

FIGURE 2. Values are means±95% confidence intervals (CI). Data intervals are at 3 and 12 months posttransplant. Results are categorized according to exon 21, exon 26, and exon 21/26 genotypes. One-way analysis of variance (ANOVA) was used to compare differences between groups. *$P<0.05$.

survival are, therefore, not feasible from this study. In oncological studies of multidrug resistance it has been found that there is a link between survival in acute childhood lymphoblastic leukemia and the C3435T polymorphism, the 3435-CC individuals having a poorer outcome in terms of disease relapse and overall survival (*11*).

Implications for Practice

Several studies have found that it is possible to reverse the MDR phenotype (*26–28*). Tsuruo et al. initially discovered that it was feasible to reverse the MDR phenotype with the calcium antagonist verapamil (*27*) and this would provide a mechanism to asses whether drug resistance could be diminished clinically in the setting of a clinical trial.

This research is important because the ability to identify the individual differences between patients, which produce demonstrable differences in terms of their handling of immunosuppressive drugs and their susceptibility to rejection, potentially offers the opportunity to modify immunosuppressive regimens according to a patient's genetic makeup. It is also possible that identification of a drug-resistant cohort will be useful in drug trials where exclusion of this group will allow more meaningful analysis of the remainder of the population.

In conclusion, we believe that the MDR1/ABCB1 gene and P-gp are significant risk factors for transplant rejection. This is likely to be caused by the intracellular extrusion of immunosuppressants from leukocytes by P-gp. This phe-

nomenon is indicative of a multidrug resistance in transplantation akin to tumor resistance to chemotherapy agents in the field of oncology. We believe it may now be feasible to tailor immunosuppression regimes to include immunosuppressants not handled by P-gp.

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