Donor ABCB1 Variant Associates with Increased Risk for Kidney Allograft Failure

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ABSTRACT

The impact of variation within genes responsible for the disposition and metabolism of calcineurin inhibitors (CNIs) on clinical outcomes in kidney transplantation is not well understood. Furthermore, the potential influence of donor, rather than recipient, genotypes on clinical endpoints is unknown. Here, we investigated the associations between donor and recipient gene variants with outcome among 4471 white, CNI-treated kidney transplant recipients. We tested for 52 single-nucleotide polymorphisms (SNPs) across five genes: CYP3A4, CYP3A5, ABCB1 (MDR1; encoding P-glycoprotein), NR1I2 (encoding the pregnane X receptor), and PPIA (encoding cyclophilin). In a discovery cohort of 811 patients from Birmingham, United Kingdom, kidney donor CC genotype at C3435T (rs1045642) within ABCB1, a variant known to alter protein expression, was associated with an increased risk for long-term graft failure compared with non-CC genotype (hazard ratio [HR], 1.69; 95% confidence interval [CI], 1.20–2.40; \( P = 0.003 \)). No other donor or recipient SNPs were associated with graft survival or mortality. We validated this association in 675 donors from Belfast, United Kingdom (HR, 1.68; 95% CI, 1.21–2.32; \( P = 0.002 \)), and in 2985 donors from the Collaborative Transplant Study (HR, 1.84; 95% CI, 1.08–3.13; \( P = 0.006 \)). In conclusion, these data suggest that an ABCB1 variant known to alter protein expression represents an attractive candidate for future study and risk stratification in kidney transplantation.

Calcineurin inhibitors (CNIs) remain principal components of most immunosuppression regimens in kidney transplantation. CNI dose adjustments based on CNI blood levels is the traditional approach to drug administration; however, this is an imperfect approach because CNIs have a narrow therapeutic index and high pharmacokinetic variability.1 The unpredictable response to CNI drug dosing has been attributed to interindividual differences in expression of drug-metabolizing enzymes and transporters. Important components of this CNI disposition “axis” are the nuclear pregnane X receptor (PXR, encoded by the nuclear receptor subfamily 1, group I, member 2 [NR1I2] gene), which is proposed to be a “master regulator” of hepatic drug handling; cytochrome P450 (CYP) enzymes

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3A4 and 3A5; the drug transporter P-glycoprotein (P-gp, encoded by the ATP-binding cassette, sub-family B, member 1 [ABCB1] gene, also known as the multidrug resistance 1 [MDR-1] gene); and cyclophilin A (Cyp, encoded by the peptidylprolyl isomerase A [PPIA] gene), the target of cyclosporine²–⁶.

Although data are inconsistent, there is evidence that single-nucleotide polymorphisms (SNPs) within these genes are associated with variation in cyclosporine and tacrolimus pharmacokinetics.⁷–⁹ Preliminary and unreplicated reports also suggest that recipient genotype for ABCB1 is associated with delayed graft function¹⁰ and acute rejection,¹¹,¹² and recipient CYP3A5 genotype is associated with mortality.¹³ However, with the exception of this report by Kreutz and colleagues,¹³ no studies have addressed the relationship between recipient genotype and the important “hard” clinical endpoints of renal transplantation (i.e., allograft failure or mortality), with limitations stemming from small sample sizes and short follow-up.¹⁴

In contrast to studies of recipient genotype, far less information is available on the relationship between SNPs within genes of the kidney transplant donor and subsequent outcome. Although reduced expression of CYP3A5 and P-gp within the transplanted kidney have been associated with CNI toxicity,¹⁵,¹⁶ only two small published studies to date have addressed the relationship between donor SNPs and clinical outcomes in cyclosporine-treated patients. These provide some evidence for an association between genetic variation in donor ABCB1 and acute cyclosporine nephrotoxicity¹⁷ and allograft failure.¹⁸ Both studies focused on the C3435T polymorphism within the ABCB1 gene, an exonic SNP whereby the TT genotype is associated with reduced P-gp expression.¹⁹

The purpose of this study was to assess the relationship of both donor and recipient genotype with kidney allograft survival and recipient mortality. The study was not restricted to the limited number of SNPs previously studied but rather involved a comprehensive survey of common sequence variation in the target genes involved in CNI disposition. This was accomplished by interrogating the International HapMap resource²⁰ and selecting 52 tagging SNPs to capture the majority of common variation within five candidate genes. Preliminary positive findings from an initial discovery cohort were examined in two further independent populations, incorporating a total of 4471 renal transplant recipients followed for up to 20 years.

### RESULTS

#### Characteristics, Studied SNPs, and Outcome in the Discovery Cohort (Birmingham)

Demographic characteristics of this cohort are shown in Table 1. The entire multiple-gene SNP panel included 52 SNPs (Table 2). ABCB1 rs2032582 was the only triallelic SNP; the remainder were biallelic. All SNPs with the exception of ABCB1 rs6979885 and NRII2 rs119299668 (failed assays) were genotyped successfully in the 811 donors and their respective recipients, with genotyping call rates of >95%. All ABCB1, CYP3A4, CYP3A5, PPIA, and NRII2 SNPs were within Hardy-Weinberg equilibrium (HWE) bounds ($P>0.05$), apart from NRII2 rs2461818, which was excluded from additional analysis. To reduce the impact of population stratification, all donors were white, and analysis of recipient genotype was limited to the 670 (82.6%) white recipients. There were 79 deaths and 184 death-censored graft failures during 163 months’ follow-up.

#### Table 1. Demographic characteristics of the study cohorts

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Birmingham (n=811)*</th>
<th>Belfast (n=675)*</th>
<th>CTS (n=2985)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>42.5±15.5</td>
<td>41.4±16.8</td>
<td>47.7±12.8</td>
</tr>
<tr>
<td>Men</td>
<td>61.8</td>
<td>62.4</td>
<td>61.8</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>82.6</td>
<td>99.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Black</td>
<td>5.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>South Asian</td>
<td>16.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Other</td>
<td>0.5</td>
<td>0.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Prior transplant</td>
<td>14.4</td>
<td>0.0</td>
<td>14.6</td>
</tr>
<tr>
<td>Diabetes</td>
<td>16.1</td>
<td>15.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Duration of dialysis (yr)</td>
<td>4.1±2.2</td>
<td>—</td>
<td>3.9±2.1</td>
</tr>
<tr>
<td>Panel-reactive HLA antibodies</td>
<td>0 (0–5)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td><strong>Donor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>43.0±15.3</td>
<td>36.4±16.8</td>
<td>41.3±17.2</td>
</tr>
<tr>
<td>Men</td>
<td>54.4</td>
<td>59.3</td>
<td>59.8</td>
</tr>
<tr>
<td>Live donor</td>
<td>14.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HLA mismatch</td>
<td>2.1±1.1</td>
<td>2.2±1.1</td>
<td>3.0±1.4</td>
</tr>
<tr>
<td>Cold ischemia time (hr)</td>
<td>—</td>
<td>—</td>
<td>19 (16–24)</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>91.2±25.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>15.9</td>
<td>—</td>
<td>12.1</td>
</tr>
<tr>
<td>Region (North America versus Europe)</td>
<td>—</td>
<td>—</td>
<td>15 versus 85</td>
</tr>
<tr>
<td>Delayed graft function*</td>
<td>30.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Biopsy-proven acute rejection</td>
<td>28.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD25 monoclonal antibody</td>
<td>30.9</td>
<td>—</td>
<td>11.0</td>
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<tr>
<td>Anti-thymocyte globulin</td>
<td>—</td>
<td>—</td>
<td>28.5</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>100</td>
<td>82.5</td>
<td>84.8</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>17.5</td>
<td>15.2</td>
<td>15.2</td>
</tr>
</tbody>
</table>

Unless otherwise noted, values are percentages. Data expressed with a plus/minus sign are the mean ± SD.

*Total number of patients undergoing genotyping in Birmingham (discovery) cohort.

¹Number of patients in Belfast cohort successfully genotyped at ABCB1 C3435T (rs1045642).

²Number of patients in CTS cohort successfully genotyped at ABCB1 C3435T (rs1045642).

³Median (interquartile range).

*Delay of requirement in the first postoperative week.
Death-Censored Allograft Survival

C3435T Polymorphism (rs1045642) within ABCB1 of the Donor (Discovery Cohort)

Kaplan-Meier analysis revealed that the donor ABCB1 C3435T genotype (rs1045642) was associated with death-censored allograft survival in the Birmingham cohort (Figure 1; log-rank \( P = 0.008 \)). For this SNP, 777 of 811 (96%) donors were successfully genotyped, with CC, CT, and TT genotypes found in 20.9%, 50.0%, and 29.1%, respectively. Death-censored graft survival was worse when kidneys were transplanted from donors with the CC genotype. This difference persisted in a Cox proportional hazards model (donor CC versus non-CC genotype: hazard ratio [HR] for graft failure, 1.69; 95% confidence interval [CI], 1.20–2.40; \( P = 0.003 \)), adjusted for the relevant baseline covariates described in the Concise Methods section. Further adjusting the model for the post-transplant events of delayed graft function and biopsy-proven acute rejection (any grade of severity; modeled as a time-dependent covariate) resulted in no material change to the effect of genotype on outcome (donor CC versus non-CC genotype: HR, 1.61; 95% CI, 1.19–2.48; \( P = 0.005 \)).

C3435T Polymorphism (rs1045642) within ABCB1 of the Donor (Replication Cohorts)

This association was next examined in the two replication cohorts. In the Belfast cohort, ABCB1 C3435T genotypes were within HWE bounds in the 675 donors who were successfully genotyped (97% call rate). CC, CT, and TT genotypes were found in 21.2%, 49.5%, and 29.3% of donors, respectively. All donors were white. There were 189 death-censored graft losses during 238 months’ follow-up, with Kaplan-Meier estimates demonstrating an association between this donor genotype and death-censored graft survival (Figure 2; log-rank \( P = 0.01 \)). This association persisted in the Cox regression analysis (donor CC versus non-CC genotype: HR for graft failure, 1.68; 95% CI, 1.21–2.32; \( P = 0.002 \)), adjusted for baseline clinical covariates described in the Concise Methods section.

In the Collaborative Transplant Study (CTS) cohort, genotyping was successful in 2985 of 3000 donors (call rate >99%) at the studied ABCB1 C3435T locus; genotypes were within HWE bounds. CC, CT, and TT genotypes were found

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Table 2. Details of SNPs tested

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP Tested</th>
<th>Additional SNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>rs2242480 (CYP3A4*1G), rs4646437, rs2246709</td>
<td>rs2740574 (CYP3A4*1B)</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>rs4646450, rs776746 (CYP3A5*1/3)</td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>rs17064, rs6979885, rs2235048, rs12720066, rs418735, rs2235013, rs2032588, rs11772987, rs868755, rs6950978, rs12334183, rs10264990, rs13226726, rs1202172, rs418733, rs1202184, rs17327624, rs7802773, rs2888599, rs2214102, rs13233308, rs10276499, rs2157930</td>
<td>rs1045642 (3435C&gt;T), rs2032582 (2677G&gt;T/A), rs1128503 (1236C&gt;T), rs3213619 (129T&gt;C), rs2229109 (1199G&gt;A)</td>
</tr>
<tr>
<td>PPIA</td>
<td>rs6970925, rs6463247, rs9638978</td>
<td>rs8177826 (−11G&gt;C), rs6850 (+36G&gt;A)</td>
</tr>
<tr>
<td>NR1I2</td>
<td>rs2056630, rs10934498, rs1357459, rs2461822, rs2461818, rs7643645, rs2472681, rs11917714, rs6785049, rs11929668, rs3732359, rs3732360, rs1054190</td>
<td>rs6785049 (7635A&gt;G), rs2276707 (8055C&gt;T), rs3814055 (−25385C&gt;T)</td>
</tr>
</tbody>
</table>

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Figure 1. Kaplan-Meier survival curve for Birmingham cohort. This demonstrates the association between donor ABCB1 C3435T (rs1045642) SNP and death-censored graft survival (\( P = 0.008; n = 777 \) successfully genotyped at this locus).

Figure 2. Kaplan-Meier survival curve for Belfast cohort. This demonstrates the association between donor ABCB1 C3435T (rs1045642) SNP and death-censored graft survival (\( P = 0.01; n = 675 \) successfully genotyped at this locus).
in 22.8%, 49.2%, and 28.0% of donors, respectively. No difference in genotype frequency was evident between North American and European donors (chi-square $P=0.12$). All donors were white. Among the 2985 recipients there were 715 death-censored graft losses during the first 10 post-transplant years. The death-censored graft survival of these patients was similar to that of 13,630 patients from the CTS who had similar clinical characteristics but were not selected for donor genotyping (log-rank $P=0.46$). Reassuringly, this finding suggests that the group selected was representative of the wider CTS cohort.

In the overall CTS cohort, no evidence of association between donor genotype at $ABCB1$ C3435T and death-censored graft survival was demonstrated (Figure 3; upper panel; log-rank $P=0.82$). However, an association between donor genotype and death-censored graft survival was seen in the subgroup of 452 tacrolimus-treated patients, with worse outcome after transplantation from a donor displaying the CC genotype at this $ABCB1$ locus (Figure 3; lower right panel; log-rank $P=0.01$). Cox regression confirmed this association (donor CC versus non-CC genotype: HR, 1.84; 95% CI, 1.08–3.13; $P=0.006$) in the adjusted model.

No evidence of an association between donor genotype and graft survival was seen in the subgroup of 2533 cyclosporine-treated patients within the CTS cohort (Figure 3; lower left panel; log-rank $P=0.85$).

**Linkage Disequilibrium between Alleles and Outcome**

It has been suggested previously that alleles at the loci C3435T, G2677T/A, and C1236T within exons 26, 21, and 12, respectively, display linkage disequilibrium and form a haplotype within $ABCB1$. This was therefore examined in the current cohorts. In fact, among donors from the Birmingham cohort, there was evidence of recombination (rather than linkage disequilibrium) between alleles at C3435T and C1236T (D', 0.83; 95% CI, 0.79–0.88) and also at C3435T and G2677T/A (D', 0.83; 95% CI, 0.79–0.88). Assessment of linkage between C1236T and G2677T/A was inconclusive (D', 0.93; 95% CI, 0.90–0.96) (see the Statistical Analyses section for definitions). Linkage disequilibrium for these loci is shown in Figure 4A. Therefore, these SNPs did not form a haplotype block.

No relationship between donor gene variation at C1236T (rs1128503) or G2677T/A (rs2032582) and death-censored graft survival was seen in the Birmingham cohort (log-rank $P>0.5$ for both analyses). Similar results were found in the Belfast and CTS cohorts; no evidence of linkage disequilibrium was apparent (Figure 4, B and C), and no evidence for an association between gene variation at C1236T or G2677T/A and death-censored graft survival was found in either cohort (log-rank $P>0.05$ for both gene loci).

**Other Donor and Recipient SNPs within $ABCB1$**

No other donor or recipient SNPs within $CYP3A4$, $CYP3A5$, $NR1I2$, or $PPIA$ were associated with death-censored graft survival in the discovery (Birmingham) cohort ($P>0.05$ for all) and were not evaluated in the replication cohorts.

**Donor and Recipient SNPs within $CYP3A4$, $CYP3A5$, $NR1I2$, and $PPIA$**

None of the studied donor or recipient SNPs within $CYP3A4$, $CYP3A5$, $NR1I2$, or $PPIA$ were associated with death-
censored allograft survival in the discovery cohort (\(P>0.05\) for all) and were not evaluated in the replication cohorts.

**Mortality**

**Donor and Recipient SNPs within ABCB1**

No donor SNPs within \(ABCB1\) were associated with recipient mortality in the discovery cohort. Two recipient SNPs within \(ABCB1\) (rs4148735 and rs1128503) demonstrated evidence of association with recipient mortality (log-rank \(P=0.02\) for both). It was noted that the heterozygosity at both these loci portended the worst outcome. These associations did not reach statistical significance in the adjusted Cox model comparing the heterozygous genotype with other genotypes (\(P=0.08\) and \(P=0.09\), respectively) and were not evaluated further in the replication cohorts.

**Donor and Recipient SNPs within CYP3A4, CYP3A5, NR1I2, and PPIA**

None of the studied donor or recipient SNPs within \(CYP3A4, CYP3A5, NR1I2,\) or \(PPIA\) were associated with mortality in the discovery cohort (\(P>0.05\) for all) and were not evaluated in the replication cohorts.

**DISCUSSION**

To our knowledge, this is the most comprehensive study to date of kidney transplant donor and recipient pharmacogenetic variation within multiple CNI-treated cohorts. It represents clinical outcomes from nearly 4500 transplant procedures. The results demonstrate a replicated association between death-censored graft survival and the \(C3435T\) polymorphism within the donor \(ABCB1\) gene, a SNP known to have functional significance. An initial positive signal detected in a large discovery cohort (Birmingham, \(n=811\)) was seen in a subsequent cohort of 675 patients from Belfast using a distinct genotyping platform. It was also then seen in a further cohort of tacrolimus-treated patients in the CTS cohort. The effect was independent of established confounding risk factors for transplant outcome, and although the analyzed confounders differed slightly between cohorts, this may in fact widen the generalizability of the findings.

Although the association was not evident when the CTS cohort was analyzed as a whole (or the subgroup treated with cyclosporine), this may reflect heterogeneity of this CTS population. Unlike the single-center cohorts in this study, the CTS cohort is drawn from multiple countries and transplant centers that use different treatment approaches and algorithms that cannot necessarily be accounted for in database analyses such as this. Therefore, although the impact may be context-specific, this gene variant certainly represents a promising candidate for future genetic studies in kidney transplantation.

Limited data correlating kidney transplant donor gene variation with outcome exists, and published studies have focused on this same \(ABCB1\) C3435T polymorphism (rs1045642) within exon 26, for which the homozygous TT genotype is associated with reduced renal \(P-gp\) expression\(^{19}\) and potentially an increased risk for CNI nephrotoxicity. These studies have shown evidence for an association between donor TT genotype and adverse outcome,\(^{17,18,21}\) rather than CC genotype as seen in the current study. In the first study of 99 patients, donor TT genotype correlated with the development of acute cyclosporine nephrotoxicity,\(^{17}\) defined on clinical grounds after a retrospective records review. A second study of 259 prevalent transplant recipients with functioning grafts at 12 months post-transplantation suggested that the presence of the homozygous form of a “variant” donor haplotype (which included the TT genotype at \(ABCB1\) C3435T) was associated with increased graft failure rates during a follow-up of 6 years,\(^{18}\) with failures due to “clearly identifiable causes” excluded from analysis. Finally, a histology-based study of the same \(ABCB1\) SNP showed an association between donor TT genotype and the development of interstitial fibrosis and tubular atrophy on protocol biopsy specimens taken up to 3 years post-transplantation in patients treated with tacrolimus.\(^{21}\) This donor genotype was not associated with other chronic histologic lesions, such as transplant glomerulopathy\(^{22}\) or arterial intimal thickening.\(^{23}\) The discrepancies between these
previous studies and the current investigation may be due to differences in study size, patient characteristics, duration of follow-up, and the end points evaluated. Other histologic studies showing an association between cyclosporine toxicity and reduced tubular P-gp expression predominantly addressed arteriolar hyalinosis and tubular vacuolization as markers of toxicity, \(^{15}\) although these lesions may actually have limited impact on graft outcome. \(^{24}\) In addition, the in vivo association between genotype and P-gp expression in biopsy tissue from transplanted kidneys is imperfect \(^{21}\) and may depend on the tissue studied (i.e., there is some degree of genotype-phenotype disconnect). Even in the nontransplant setting there are conflicting data with regard to the role of \(ABCB1\) genetic variation influencing P-gp expression and function. \(^{25}\)

It is possible that the deleterious effect of the donor CC genotype seen in this study may in fact relate to increased renal P-gp expression. It is recognized that P-gp stimulates cell growth, \(^{26}\) displays an antiapoptotic effect in fibroblasts, \(^{27}\) promotes cholesterol ester–mediated vascular damage, \(^{28}\) and is associated with increased propensity to renal injury in an animal model of ischemic injury. \(^{29,30}\) This effect may relate to characteristics of reparative stem cells, which may be mobilized from (donor) bone marrow and other niches, or which may reside within the kidney itself. \(^{31,32}\) Although speculative, it is plausible that these phenomena may lead to the tissue injury and disrepair that are characteristic of chronic kidney damage. In keeping with this is the development of CKD in liver transplant recipients displaying CC genotype at this locus (i.e., kidney genotype CC at \(ABCB1\) C3435T). \(^{33}\)

No associations between death-censored graft failure or mortality and any of the other studied SNPs within donor or recipient \(ABCB1\), \(CYP3A4\), \(CYP3A5\), \(NR1I2\), or \(PPIA\) were evident in the discovery cohort. Thus, they were not investigated in the replication cohorts. We acknowledge that this methodology does not eliminate the risk for type II errors (failing to find a clinically relevant association due to insufficient sample size). However, because the purpose of the study was to identify clinically relevant genetic variation, rather than to conclusively exclude clinically irrelevant genetic variation, this approach was appropriate.

Although it is almost impossible to completely exclude cryptic population stratification in a study such as this, we believe that inclusion of white patients only and the use of replication cohorts (with the observation that allele frequency did not differ between European and North American individuals within the CTS cohort) goes some way toward this. Most patients included in this study were treated with cyclosporine, and although maximum follow-up was considerable, follow-up in others was more limited. Therefore, further long-term studies in contemporary cohorts, particularly in tacrolimus-treated patients, and in patients and donors of different racial backgrounds (while aiming for racial homogeneity within studies to minimize population stratification) are required to shed further light on the relationship between donor and recipient genotype and the important endpoints of renal transplantation. Although pharmacogenetic variation between recipients has previously been linked to other short-term and surrogate clinical endpoints, such as drug levels, delayed graft function, and acute rejection, these were intentionally not addressed in the current study because doing so would have considerably increased the risk of finding chance associations. Such associations may have remained a concern even with use of a replication cohort.

CNI-based protocols are the standard for kidney transplant immunosuppression. Therefore, a greater recognition of the role of variation in genes controlling transport and metabolism of these agents is relevant. However, the effect may be independent of the drug itself (as postulated above) and therefore of potential relevance in emerging CNI avoidance regimens. The gene variant described here may play a role in risk stratification in renal transplantation and in organ allocation policy, although it is likely that a panel of genetic biomarkers may be required to achieve optimal utility. C3435T probably represents an important functional SNP, and further research into the mechanism behind the genotype/phenotype relationship would be welcome.

In summary, this is the largest pharmacogenetic study, with longest follow-up to date, to assess the association of donor and recipient genotypes and important clinical endpoints in kidney transplantation. This study has identified an association between CC genotype of the functional C3435T polymorphism within \(ABCB1\) of the transplant donor genome and worse long-term death-censored graft survival. Although this relationship may be context-specific, this \(ABCB1\) gene variant represents an intuitive and attractive focus for future research efforts to evaluate the generalizability of these preliminary results.

**CONCISE METHODS**

**Initial Discovery Cohort**

All consecutive kidney transplant procedures performed at the Queen Elizabeth Hospital, Birmingham, United Kingdom, between 1996 and 2006 were considered (\(n=890\)). Genomic DNA was available for 811 white transplant donors and their respective recipients (Table 1). To avoid the confounding effect of population stratification in the interpretation of the results, the analyses with regard to recipient genetic variability were conducted in a subset of 670 white recipients and their respective donors (all of whom were white). Analyses of donor genetic variability were conducted for all 811 white donors and their respective recipients, with the analysis adjusted for recipient ethnicity (as well as other covariates). All patients received cyclosporine and corticosteroids as part of the initial immunosuppressive regimen; the antimetabolite used was azathioprine in 77% of patients and mycophenolic acid in 23%.

The study was performed in accordance with the Declaration of Helsinki. The National Research Ethics Service (United Kingdom) approved the study and waived requirement for individual patient consent.
SNP Selection and Genotyping

Genotyping data for each gene of interest (CYP3A4/CYP3A5/ABCB1/PPIA/NR1I2) were downloaded from the International HapMap Project Phase II CEU population (www.hapmap.org; data release 24). In addition, commonly investigated SNPs identified in the transplant literature, and most specifically those with previously reported associations with CNI pharmacokinetics, kidney transplant outcomes, or functional mutations, were also tested.

For the CYP3A4 gene, HapMap revealed three SNPs with minor allele frequencies of >5% in the CEPH (Utah Residents with Northern and Western European Ancestry) (CEU) population present within this 27204-bp region. The Tagger pairwise function within HapMap assigned the three tag SNPs—rs2242480 (CYP3A4*1G), rs4646437, and rs2246709—to capture >80% of the common variation within this gene and surrounding area. Moreover, CYP3A4*1B (rs2740574) was assessed because it is commonly studied in renal transplantation and is known to increase expression of CYP3A4; however, it was not included by HapMap.

For the CYP3A5 gene, HapMap revealed three SNPs with minor allele frequencies of >5% in the CEU population present within this 31804-bp region. The Tagger pairwise function within HapMap assigned the two tag SNPs—rs4646450 and rs776746 (CYP3A5*1/*3)—to capture >80% of the common variation within this gene and surrounding area. Of note, CYP3A5*1/*3 (rs776746) has been commonly investigated in transplant recipients and leads to a splicing defect associated with loss of tissue expression.7

For the ABCB1 gene, HapMap revealed 121 SNPs with minor allele frequencies of >5% in the CEU population present within this 209616-bp region. The Tagger pairwise function within HapMap assigned 23 tag SNPs (rs17064, rs6979885, rs2235048, rs12720066, rs4148735, rs2235013, rs2032588, rs1772987, rs68755, rs6950978, rs12334183, rs10264990, rs13226726, rs1202172, rs4148733, rs1202184, rs17327624, rs7802773, rs2888599, rs2214102, rs13233308, rs10276499, and rs2157930) to capture >80% of the common variation within this gene and surrounding area. In addition, attention was focused on those SNPs previously investigated in transplant recipients: C3435T (rs1045642),10,11,17 G2677T/A (rs2032582),11 C1236T (rs1128503),11 C129T (rs3213619)34 and G1199A (rs2229109).35

For the PPIA (cyclophilin A, Cyp) gene, HapMap revealed six SNPs with minor allele frequencies of >5% in the CEU population present within this 6475-bp region. The Tagger pairwise function within HapMap assigned the three tag SNPs—rs6970925, rs6463247, and rs9638978—to capture >80% of the common variation within this gene and surrounding area. In addition, the previously investigated SNPs A36G (rs68580)36 and -11G>C (rs8177826)36 were also tested.

For the NR1I2 gene, HapMap revealed 42 SNPs with minor allele frequencies of >5% in the CEU population present within this 38001-bp region. The Tagger pairwise function within HapMap assigned 13 tag SNPs (rs2056530, rs10934498, rs1357459, rs2461822, rs2461818, rs7643645, rs2472681, rs1917714, rs6785049, rs1929668, rs3733259, rs3733260, and rs1051490) to capture >80% of the common variation within this gene and surrounding area. In addition, previously investigated SNPs A7635G (rs6785049)37 and C8055T (rs2276707),37 which were represented by the tag SNP −25385C>T (rs3814055),38 were also assessed.

The full panel of tested SNPs is shown in Table 2. DNA was extracted from whole blood or separated splenocytes using an automated DNA separation process (MagNA Pure Compact, Roche Diagnostics GMBH). Purified DNA was adjusted to 50 ng/μl for testing. All SNP assays were purchased from Applied Biosystems (Warrington, United Kingdom) and genotyped on an ABI7900 HT using Taqman (Applied Biosystems) genotyping technology. Information on their locations and the SNPs that they tag is available on request.

Outcome Measures

The primary outcome measures of interest were death-censored allograft survival (time from transplantation to dialysis requirement or retransplantation) and recipient survival (time from transplantation to death). Other relevant clinical and demographic data were retrieved from the prospectively maintained institutional database and analyzed in the multiple regression models (see below) to identify an independent effect of genotype on outcome. The analysis was adjusted for the following covariates: donor age, sex, source (living versus deceased donor), serum creatinine, and hypertension history; recipient age, sex, race, duration of dialysis, diabetes as the cause for renal failure, transplant number, panel-reactive anti-HLA antibodies, donor-recipient HLA mismatch, and induction antiCD25 monoclonal antibody. Race was self-reported. Delayed graft function (defined as the requirement for dialysis in the first postoperative week) and episodes of biopsy-proven acute rejection (any severity; Banff classification) were considered as intermediate endpoints and so were not evaluated in the initial analysis. However, secondary analysis was performed with additional adjustment for these two variables.

Replication Cohorts and Genotyping

Two independent cohorts were used to replicate any genetic associations found in the discovery cohort (Table 1). The first consisted of 697 white renal transplant donors (and their respective recipients, 99.1% of whom were white) at Belfast City Hospital, Belfast, United Kingdom. Only first deceased donor transplant procedures were considered. Genotyping in this cohort was performed using MassARRAY iPLEX (Sequenom, San Diego, CA). As expected for an independent cohort, available covariates differed from the discovery cohort, and the Belfast analysis to determine an independent effect of genotype was adjusted for the following: donor age and sex, recipient age and race, diabetes as the cause for renal failure, donor-recipient HLA mismatch, and transplant year.

The second replication cohort consisted of 3000 representative white kidney transplant donors and recipients drawn from the CTS database by random selection using a computer-generated program; the transplantations took place between 1988 and 2010. Of these, 85% underwent transplantation in European centers, and 15% in North America. All recipients received a CNI as part of their initial immunosuppressive treatment after transplantation. Because of the larger size of this cohort, subanalyses were performed for tacrolimus- and cyclosporine-treated patients. DNA samples were shipped to Belfast and genotyping conducted was described earlier. The assessment of the effect of genotype on outcome in the CTS cohort was adjusted for the following: donor and recipient age and sex, duration of dialysis, diabetes as the cause for renal failure, transplant number,
peak panel-reactive anti-HLA antibodies, donor-recipient HLA mismatch, cold ischemia time, induction antibody (anti-CD25 monoclonal antibody versus Anti-thymocyte globulin versus none), transplant year, and region (North America versus Europe).

In all populations, laboratory technical staff performing the genotyping were blinded to the transplant outcomes. Donor and recipient SNP assays acted as internal controls for the analysis, and all assays performed well with experimental control samples on both platforms. Correlation of genotype and clinical outcome was performed internally in each of the three coordinating centers. All DNA was collected before and independently of the outcome variables.

Statistical Analyses
Data are shown as mean ± SD unless otherwise indicated. Genotype frequencies were assessed for HWE using a chi-squared goodness-of-fit test, with a type 1 error rate set at 5%. Linkage disequilibrium was evaluated using haplview with a default algorithm described by Gabriel et al., whereby strong linkage disequilibrium exists when the one-sided upper 95% confidence bound on D’ > 0.98 (i.e., consistent with no historical recombination), and strong evidence for historical recombination exists when the upper confidence bound on D’ is < 0.90. Upper bounds between 0.90 and 0.98 are considered inconclusive. G2677T/A (rs2032528) is triallelic and was therefore analyzed by comparing the most common variant (G allele) with the two least common variants (T allele and A allele).

Cumulative events were analyzed with Kaplan-Meier method, with the log-rank test used for intergroup comparisons. Gene variants demonstrating evidence for an effect on Kaplan-Meier analysis (P ≤ 0.05) were then analyzed in a time-to-event analyses using a Cox model with the effect of genotype adjusted for relevant clinical confounders described earlier. Covariates demonstrating a skewed distribution underwent logarithmic transformation before analysis; collinearity was assessed by variance inflation; biopsy-proven acute rejections was modeled as a time-dependent covariate.

A type 1 error rate ≤ 0.05 for an association discovered in the initial cohort in the adjusted analysis was the criteria for proceeding to study in the replication cohorts. This statistic was not adjusted for whether the comparisons undertaken in the initial cohort because the methodology of the study was to seek independent replication of positive findings. As discussed in Results section, a single SNP showed an association with outcome, and for this gene variant results are shown comparing CC genotype with non-CC genotype as a recessive term. Stata software (for the Birmingham cohort; Stata Corp., Cary, NC) and SPSS software (for the Belfast and CTS cohorts; SPSS Inc., Chicago, IL) were used for analysis.

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DISCLOSURES
None.

REFERENCES


