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Validation of Genetic Variants Associated with Early Acute Rejection in Kidney Allograft Transplantation

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Abstract

Numerous reports have identified genetic variants associated with kidney transplant outcome; but only a few have been validated in subsequent studies. We analyzed the association of 21 previously reported genetic variants associated with acute rejection (AR), in an effort to validate these associations in our kidney transplant population. All recipients (n=585) received Ab induction, rapid discontinuation of prednisone, and CNI with either MMF or sirolimus. Both univariate analysis and logistic regression were used for determining the association between the genotypes and AR. Univariate analysis detected one significant SNP ($p = 0.03$), rs1801133, within the methylenetetrahydrofolate reductase (MTHFR) gene associated with AR. Logistic regression analysis identified 2 variants associated with AR, the 32 bp deletion within chemokine (C-C motif) receptor-5 gene (rs333) and the p.222A/V variant (rs1801133) within the MTHFR gene. Though our analysis utilized a much larger cohort than used in previous reports, we were only able to detect an association with 2 of these variants. The lack of validation for the other 19 variants may be due to the small effect size, or that they are not associated with AR. These results stress the need for larger cohorts for both future studies as well as for validation studies.

Keywords

MTHFR; CCR5; Acute rejection; Kidney allograft; Polymorphism

Introduction

Reversible acute rejection (AR) episodes have been associated with an increased risk of chronic rejection (interstitial fibrosis/tubular atrophy; IF/TA) and decreased long-term graft survival (1, 2). Numerous risk factors for AR, including differences in immunosuppressive protocols, have been defined. In addition, a number of genetic variants have been associated with either an increased or a decreased risk for AR, many in the form of single nucleotide polymorphisms (SNPs) (3-7). The protein products from many of the genes containing these variants are involved in the regulation and responsiveness of the immune system.

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Conflict of Interest Statement
There are no conflicts of interest to report.

Validation of associated variants to AR has been problematic, with many subsequent studies reporting a lack of association with these same variants in different cohorts of kidney allograft recipients. One possible reason for this is that most studies have used relatively low numbers of individuals in their study cohort, with many studies having study populations of 150 or less (6). Additionally, population and clinical care differences may affect association outcomes of the same variant in different studies, especially when study subjects come from multiple sites. We report an attempt to validate 21 genetic variants previously associated with AR risk, or other adverse outcomes, using a cohort of 585 kidney allograft recipients.

Materials and Methods

Patients

All research subjects were transplanted at the University of Minnesota Transplant Center, Minneapolis, MN. A total of 585 recipients (Table 1), were consented for this analysis under an IRB-approved protocol at the University of Minnesota. All but one individual was transplanted before 1995. All individuals received Ab induction, rapid discontinuation of prednisone, and calcineurin inhibitors (CNI) with either mycophenolate mofetil (MMF) or sirolimus. Within this population, a total of 98 individuals (16.8%) were shown to have biopsy proven acute rejection within 1 year. A description of acute rejection episodes, both T-cell mediated and antibody mediated, are shown in Table 2.

Genotyping

Blood was obtained from individuals who had, or were undergoing a kidney transplant. DNA was extracted using routine laboratory methods, with DNA purity and concentration determined by ultraviolet spectroscopy (Thermo Scientific, Wilmington, DE). Twenty one genetic variants within 15 genes were analyzed (Table 3) which had previously been associated with AR in kidney allografts or with poor outcomes after transplantation (3-7). Genotypes were determined using the TaqMan genotyping assay (Applied Biosystems, Inc. Foster City, CA) with primers designed by Applied Biosystem's Primer-by-Design service. Genotypes were visualized using a PRISM 7500 and data analyzed using the ABI Sequence Detection Software. The Angiotensin I-converting enzyme (ACE) and chemokine (C-C motif) receptor 5 (CCR5) variants were analyzed by PCR amplification and the products sized using agarose gel electrophoresis (8, 9).

The description of the SNPs tested (Table 3; Nucleotide Change) are given as proposed by Antonarakis and den Dunnen (10, 11). The location of the altered nucleotide is numbered from the initial nucleotide of the ATG initiation codon with promoter nucleotides given as negative numbers (e.g. c.-385T/G). This numbering system occasionally results in differences between the location given in this report, and what has been historically used to describe the variant in previous reports. In all cases the reference SNP (rs) number is provided to help eliminate ambiguity.

Statistical Analysis

The Hardy-Weinberg Equilibrium test was performed using the exact test. All recipients were at least 1 year post-transplant at the time of analysis. Contingency table analyses were conducted to assess possible univariate associations between each genotype and rejection at 1 month, 6 months and 1 year. Both Fisher's exact test and the Chi-squared test were used and they tended to give similar results (because of the large sample size) in this study. To assess multivariate associations, we conducted multiple logistic regression with the binary outcome indicating rejection. No adjustment for multiple comparisons was made. All analyses were done in SAS (Version 8) and R.

Our chosen significance cut-off at ≤ 0.05 is only suggestive since no multiple comparison adjustment was made. The Bonferroni adjustment is easy to apply but known to be conservative, while it is unclear how to make a more accurate adjustment to account for the step-wise model selection. As an alternative, we also applied a global test called sum of squared score (SSU) test (12). The SSU test was developed for high-dimensional data and, in particular, has been shown to have high power for multi-locus association testing, while avoiding the multiple testing problem by testing on multiple markers (here 21 SNPs) simultaneously (i.e. at once). The test yielded a p-value of 0.0615, at borderline significance.

Results

The characteristics of recipients with and without rejection are shown in Table 1. Age of transplant ($p < 0.001$), crossmatch at transplant ($p < 0.001$) and antibody induction using OKT3 ($p = 0.033$) were the only characteristics found to be significantly different between the two groups. The significance with OKT3 use is questionable due to only 2 individuals in this group. The type of rejection along with Banff scores for T-cell mediated rejection is noted in Table 2. In individuals with more than one AR event (n = 17) the time to the initial event was used. Only T-cell mediated rejection events were used in this analysis.

All SNPs analyzed were in Hardy-Weinberg equilibrium except for the p.10P/L variant in TGFB1 ($p = 8.7e^{-8}$). This SNP was removed from further analysis. Our initial analysis, using univariate analysis, tested the association of 18 SNPs and 2 insertion/deletions (in/dels) with AR at 1 month, 6 months and 1 year post-transplant. No variants exhibited a significant association with AR at 1 month post-transplant, one SNP provided a significant association ($p < 0.615$) with AR at 6 months post-transplant and no variants exhibited a significant association at 1 year post-transplant (Table 3).

The most significant associations between those variants tested and AR was found at 6 months post-transplant. This involved an amino acid substitution in the methylenetetrahydrofolate (MTHRF) gene. The MTHRF gene, rs1801133 (p.222A/V, c. 665C/T also known as C677T), produced a p value of 0.0356. All other polymorphisms tested produced p values above 0.0615 for all three time points to AR.

Stepwise logistic regression was done using main effects only (Table 4). For AR within 1 month post transplant, the MTHFR variant rs1801133 gave a p value of 0.044 with the C/C genotype being protective of AR with an odds ratio (OR) of 0.47. For AR within 6 months post-transplant, two variants were identified. The MTHFR variant rs1801133 gave a p value of 0.0119 and an OR of 0.51 for the C/C genotype and a variant within the chemokine (C-C motif) receptor 5 (CCR5, rs333) gene gave a p value of 0.0316 and an OR of 2.33 for the Wt/Wt (non deletion) genotype. There were no variants that were found to be statistically significant at 1 year post-transplant.

Discussion

We hypothesize that some individuals have a genetic predisposition to the likelihood of AR and that recipient genotypes will, in part, determine organ transplant outcome (e.g. patient and graft survival, rejection free graft survival, death censured graft survival and chronic rejection free graft survival). The goal of this study was to validate candidate polymorphisms that had previously been associated with AR. We tested 21 variants that had been previously associated with AR. Of those tested, two genes, methylenetetrahydrofolate reductase (MTHFR) and chemokine (C-C motif) receptor 5 (CCR5) provided the strongest association with AR, using stepwise logistic regression.

MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF), a cosubstrate for methionine synthase, which is responsible for the conversion of homocysteine to methionine. The T allele of the MTHFR variant rs1801133 produces a thermolabile enzyme with decreased enzymatic activity resulting in a reduction in the formation of 5-methyltetrahydrofolate (5-MTHF) and a concomitant increase in plasma levels of homocysteine (13). We found that individuals homozygous for the wildtype C allele had a reduction in the risk for both 1 month to AR and 6 months to AR (OR 0.47 for 1 month to AR and OR 0.57 for 6 months to AR). The T allele therefore must increase the risk of AR by producing higher levels of plasma homocysteine. Alternatively, reduced serum levels of 5-MTHF and not increased homocysteine levels may be responsible for the observed increased risk (14). An association with the T allele of rs1801133 with chronic allograft nephropathy has been previously reported (15). Connolly et al. reported that in renal transplant recipients the serum homocysteine concentration was a significant predictor of mortality (16). Additionally, homocysteine may induce inflammatory cytokines such as macrophage inflammatory protein 2 (MIP-2), which could lead to increased inflammation in the kidney (17).

Chemokines and their receptors play an important role in the regulation of the immune system. One such receptor, the chemokine (C-C motif) receptor 5 gene (CCR5) is a receptor of several proinflammatory chemokines and is expressed by infiltrating T cells and macrophages. The rs333 variant of CCR5 is a deletion of 32 nucleotides within exon 3 of the coding region, resulting in a non-functional receptor (18). We found that individuals homozygous for the wild type allele had a greater than 2-fold risk of AR showing the deletion polymorphism to be protective for AR. It has been previously reported that individuals who were homozygous for the deletion had significantly increased graft survival (8). The CCR5 32 bp deletion polymorphism has also been associated with a reduction of AR in liver transplant recipients (19). Others have associated specific haplotypes of CCR5 with acute heart rejection (20). An additional polymorphism within the CCR5 promoter (rs1799987) resulting in an increased expression of the receptor has been associated with increased AR risk in kidney recipients (21).

The majority of the variants tested in this study did not exhibit an association with AR, though most had been previously reported to be associated with AR in kidney allograft recipients. Additionally, we did not take into account multiple comparisons, which would further reduce the significance of the SNPs analyzed. One possible explanation for this discrepancy with previous reports is that these variants do not predispose to AR and that the initial studies reported false positive findings. Many of these studies analyzed small patient populations leading to the possibility of spurious statistical results. Additional explanations for this discrepancy may be the use of different patient populations, clinical regiments and study parameters in previous cohorts. Any of these factors could alter the statistical association of a variant with AR. In most instances, the genetic impact of individual variants on complex disease states is small with relatively modest odds ratios being found for associated SNPs. This has been the case for many studies associating genetic variants with complex disease phenotypes. If this is the case for genetic predisposition of AR, much larger cohorts will be needed to provide the statistical power to identify variants with a small effect. The large cohorts necessary for this type of analysis will most likely require the combining of several cohorts for large scale genotyping and analysis. In our study, all of the variants tested exhibited a lack of association under univariant analysis. It was only by multivariate analysis of genotypes that statistically significant associations were identified. This type of analysis will require even larger cohorts if combinations of genotypes need to be tested to detect significant changes in transplant outcomes. In the final analysis, it is most likely that multiple variants within specific pathways will need to be analyzed and clustered to determine the full genetic impact on transplant outcome. Large cohorts will allow for full

genome wide association studies (GWAS) to be done, eliminating the need to guess which candidate genes are best to study.

Additionally, a second set of variants that could impact transplant outcomes are those associated with the genome of the transplanted kidney (21, 22). Little analysis has been done on donor variants, compared to recipient variants, on their effect on transplant outcome, but some SNPs are being identified (22). Some of these variants may be associated with tissue repair, delayed graft function or risk factor for hypertension.

Only a few clinical parameters (e.g., HLA, ABDR identical sibling transplants and delayed graft function) are used to alter immunosuppressive protocols. If specific genetic variants can be associated with transplant outcome, we will have the opportunity to further individualize therapy. For example, if specific genetic variants are associated with increased likelihood of drug toxicity from a specific immunosuppressive agent, an alternative immunosuppressive regimen can be used for that patient. If we show that specific variants are associated with significantly increased incidence of AR under certain immunosuppressive regimens, we can design clinical trials to randomize transplant recipients with these alleles to different immunosuppressive regimens designed to determine if alternate regiments lower their rejection incidence. Similarly, if we show that specific variants are associated with a significant increase rate of chronic graft rejection (with or without an antecedent rejection episode), we can design clinical trials to randomize recipients as to their gene polymorphisms to regimens designed to reduce the incidence of chronic dysfunction.

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Table 1

Characteristics of recipients defined by rejection

	No AR N=487 (83.2%)	AR in 12 months N=98 (16.8%)	P value
Age at Transplant	48.6 (9.5-79.2)	41.7 (10.5-74.1)	<0.001
Weeks to rejection	N/A	14.7 ± 15.2 weeks	
Donor type			0.325
Deceased	155 (31.8%)	36 (36.7%)	
Living related	219 (45.0%)	36 (36.7%)	
Living unrelated	113 (23.2%)	26 (26.5%)	
Sex			0.596
Female	210 (43.1%)	39 (40.2%)	
Male	277 (56.9%)	58 (59.8%)	
Race			
American Indian	6 (1.2%)	2 (2.0%)	0.137
Asian	7 (1.4%)	4 (4.1%)	
African American	12 (2.5%)	4 (4.1%)	
Caucasian	462 (94.9%)	88 (89.8%)	
Ethnicity			
Hispanic	16 (3.3%)	5 (5.1%)	0.373
Non-Hispanic	471 (96.7%)	93 (94.9%)	
PRA			
0	404 (83.4%)	75 (76.5%)	
1-10	23 (4.8%)	4 (4.1%)	
11-50	30 (6.2%	9 (9.2%)	
>50	27 (5.6%)	10 (10.2%)	
Crossmatch at transplant			<0.001
T-cell- / B-cell -	369 (75.8%)	61 (62.2%)	
T-cell- / B-cell +	2 (0.4%)	8 (8.2%)	
T-cell+ / B-cell +	0 (-)	1 (1.0%)	
T-cell+ / B-cell -	1 (0.2%)	0 (-)	
Unknown	115 (23.6%)	28 (28.6%)	
Induction Antibody			
None	53 (9.9%)	9 (8.2%)	
Thymoglobulin	360 (67.2%)	76 (69.1%)	0.351
Campath	29 (5.4%)	9 (8.2%)	0.364
ATG	44 (8.2%)	4 (3.6%)	0.077
OKT3	0 (-)	2 (1.8%)	0.033
Simulect	3 (0.6%)	2 (1.8%)	0.226
Zenepax	47 (8.8%)	8 (7.3%)	0.459
Steroid free recipients	279 (57.3%)	63 (64.3)	0.200
Delayed graft function	34 (7.0%)	5 (5.1%)	0.658

Table 2

Histopathologic description of acute rejection episodes

Rejection Type	Treated acute Rejection Episodes
T-cell Medicated Rejection Grade (Banff '05)	
Borderline	39 (33.9%)
1A	32 (27.9%)
1B	6 (5.2%)
2A	13 (11.3%)
2B	3 (2.6%)
Unknown	22 (19.1%)
Antibody Mediated Rejection	
C4d positive	30 (79.0%)
C4d indeterminate	1 (2.6%)
C4d negative	6 (15.8%)
C4d not done	1 (2.6%)

Table 3

Univariante analysis of SNPs

Gene	Name	SNP	Protein Change	Nucleotide Change	P value† <1 mo	P value < 6 mo	P value <1 yr
ACE	Angiotensin I-converting enzyme	rs4340	Intron	In/del	0.0689	0.4978	0.5365
AGT	Angiotensinogen	rs4762	p.207T/M	c.620C/T	0.5664	0.3909	0.3052
AGT	Angiotensinogen	rs699	p.268M/T	c.803T/C	0.1162	0.0627	0.1475
CCR5	chemokine (C-C motif) receptor 5	rs333	Truncating mutation	c.554_585del32	0.8392	0.0791	0.1747
F2	Prothrombin	rs1799963	None	c.20269G/A	0.2255	0.1897	0.4328
F5	Coagulation Factor V	rs6025	p.534R/Q	c.1602G/A	0.5046	0.6043	0.4886
GNB3	G protein B3 Subunit	rs5443	p.275S/S	c.825C/T	0.0670	0.2494	0.1230
ICAM1	Intercellular adhesion molecule-1	rs1799969	p.241G/R	c.721G/A	0.2406	0.4366	0.6852
ICAM1	Intercellular adhesion molecule-1	rs5498	p.469K/E	c.1405A/G	0.5013	0.4271	0.4554
IFNG	Interferon-γ	rs2430561	Intron	c.114+760T/A	0.6270	0.2999	0.6950
IL2	Interleukin-2	rs2069762	Promoter	c.-385T/G	0.6240	0.7336	0.7225
IL6	Interleukin-6	rs1800795	Promoter	c.-237C/G	0.9769	1.000	0.9882
IL10	Interleukin-10	rs1800896	Promoter	c.-1117C/T	0.2996	0.5147	0.4032
IL10	Interleukin-10	rs1800871	Promoter	c.-854A/G	0.5953	0.2026	0.4729
IL10	Interleukin-10	rs1800872	Promoter	c.-627G/T	0.8110	0.2101	0.4185
ITGB3	Platelet Glycoprotein IIIA	rs5918	p.59L/P	c.176T/C	0.4851	0.4678	0.4683
MTHFR	Methylenetetrahydrofolate	rs1801133	p.222A/V	c.665C/T	0.0733	0.0356	0.0908
MTHFR	Methylenetetrahydrofolate	rs1801131	p.429E/A	c.1286/C	0.4388	0.3943	0.2826
TGFB1	Transforming Growth Factor-β1	rs1800470	p.10P/L	c.29C/T	ND	ND	ND
TNF	Tumor Necrosis Factor-α	rs1800629	Promoter	c.-488A/G	0.3896	0.2194	0.5421
TNF	Tumor Necrosis Factor-α	rs361525	Promoter	c.-418A/G	0.1324	0.3035	0.4369

† p value for AR within the specific time post-transplant

Table 4

Stepwise logistic regression: main effects only

SNPs	odds ratio	95% Wald CI lower	upper	p value
AR within 1 month				
MTHFR (rs1801133)				
C/C	0.47	0.23	0.98	0.0442
C/T or T/T	1.00			
AR within 6 months				
CCR5 (rs333)				
Wt/Wt	2.33	1.08	5.02	0.0316
Wt/Del or Del/Del	1.00			
MTHFR (rs1801133)				
C/C	0.51	0.30	0.86	0.0119
C/T or T/T	1.00			