Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Validation of single nucleotide polymorphisms associated with acute rejection in kidney transplant recipients using a large multi-center cohort

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Keywords
acute rejection, genetic variation and validation, kidney transplantation, single nucleotide polymorphisms.

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Conflicts of Interest
The authors have declared no conflicts of interest.

*See Appendix 1.

Received: 15 April 2011
Revision requested: 9 May 2011
Accepted: 5 September 2011
Published online: 29 September 2011

doi:10.1111/j.1432-2277.2011.01359.x

Summary

There have been numerous reports proposing a statistically significant association between a genetic variant, usually in the form of a single nucleotide polymorphism (SNP), and acute rejection (AR). Unfortunately, there are additional publications reporting a lack of association with AR when a different cohort of recipients was analyzed for the same SNP. The objective of this report was to attempt replication of these published finding in our own kidney allograft recipient cohort. We analyzed 23 genetic variants, previously reported to have a significant association with AR, using a cohort of 969 clinically well-defined kidney transplant recipients. Only one SNP, rs6025 (Leiden mutation), within the coagulation factor V gene, showed a significant association with a *P*-value of 0.011 in a race-adjusted analysis and a *P*-value of 0.0003 in multiple variable analysis. An additional SNP, rs11706052 in IMPDH2, gave a modest *P*-value of 0.044 using multiple variable analysis, which is not significant when multiple testing is taken into consideration. Our results suggest that careful validation of previously reported associations with AR is necessary, and different strategies other than candidate gene studies can help to identify causative genetic variants associated with AR.

Introduction

Kidney allograft transplantation is the treatment of choice for end-stage kidney disease. Unfortunately, an irreversible decrease in allograft function because of chronic rejection (IF/TA) occurs in some recipients limiting long-term graft survival. A major risk factor for IF/TA is reversible acute rejection (AR) episodes. AR has not only been associated with an increased incidence of IF/TA but also associated with decreased allograft survival [1,2]. Clinical care of kidney allograft recipients could be greatly improved if individuals at risk for AR could be identified before transplantation. Individualized immunosuppression therapy and other preventive measures could then be employed in an attempt to reduce the incidence of AR in those individuals predisposed to AR.

It has been hypothesized that some individuals have increased risk for AR because of the inheritance of specific genetic variants. Several genetic variants have been reported to be significantly associated with AR [3–6]. Most of these are in the form of single nucleotide polymorphisms (SNPs). The protein products coded for by many

of the genes containing these variants are involved in the regulation and responsiveness of the immune system such as interleukin-10 (IL10), transforming growth factor-beta1 (TGFB), and tumor necrosis factor-alpha (TNF).

A major limitation in using these SNPs in clinical trials and eventual clinical care has been the lack of replication for most of these reported polymorphisms in subsequent studies [4,6]. Reasons for lack of replication may include false positive associations in the initial report, perhaps because of insufficient control for multiple comparisons, or insufficient power to detect modest effect sizes because of the use of relatively small cohorts. We have completed a study using a large cohort of kidney allograft recipients ($n = 969$) in an effort to identify SNPs associated with AR [7]. Our initial report focused on the most significant SNPs from our own analyses without reference to SNPs previously reported to be associated in AR in other studies. In this report, we determined the effect of these previously reported SNPs on AR within this cohort of kidney transplant recipients.

Materials and methods

Study cohort

The study cohort consisted of 969 kidney and simultaneous pancreas–kidney (SPK) allograft recipients. Patients were enrolled at five transplant centers between 2005 and 2008 at the time of transplantation (Table 1) as part of the Genomics of Kidney Transplantation study, an ancillary study to the Deterioration of Kidney Allograft Function (DeKAF) study [7]. Informed consent was obtained from all participants as approved by the Institutional Review Boards at each center. All kidney transplant recipients undergoing a SPK or kidney transplant alone were eligible. Immunosuppression and AR treatment was center-specific. Clinical information was collected at the time of transplant and regularly until allograft failure, and maintained in a central database.

Acute rejection in our study was defined by the treating physician. However, 97.2% of our AR events were biopsy confirmed.

Table 1. Characteristics of patients in study.

Characteristic	$n = 969$
Ethnicity	
Caucasian	$n = 739$ (76.3%)
Black	$n = 171$ (17.6%)
Asian	$n = 30$ (3.1%)
Other	$n = 26$ (2.7%)
Unknown	$n = 3$ (0.3%)
Hispanic	$n = 17$ (1.8%)
Male	$n = 602$ (62.1%)
Mean age at transplant	49 ± 14 years
Weight at transplant	81 ± 21 kg
Cause of end-stage renal disease	
Diabetes	$n = 299$ (30.9%)
Glomerular disease	$n = 197$ (20.3%)
Hypertension	$n = 121$ (12.5%)
Polycystic kidney disease	$n = 115$ (11.9%)
Other	$n = 237$ (24.5%)
Simultaneous pancreas–kidney transplant	$n = 62$ (6.4%)
Prior kidney transplant	$n = 135$ (13.9%)
Number of HLA mismatches	
0	$n = 113$ (11.7%)
1 or 2	$n = 146$ (15.0%)
3 or 4	$n = 397$ (41.0%)
5 or 6	$n = 313$ (32.3%)
Cross-match positive	$n = 50$ (5.4%)
PRA Positive	$n = 344$ (35.5%)
CNI	
Tacrolimus	$n = 597$ (61.6%)
Cyclosporine	$n = 345$ (35.6%)
None	$n = 27$ (2.8%)
Antibody Induction	
IL-2	$n = 207$ (21.4%)
Monoclonal	$n = 178$ (18.4%)
Polyclonal	$n = 538$ (55.5%)
Combination	$n = 33$ (3.4%)
None	$n = 13$ (1.3%)
Steroids use 14-day post-transplant	$n = 470$ (48.5%)
Mean donor age at transplant	40 ± 14 years
Living donor transplant	$n = 575$ (59.3%)

Identification of candidate genetic variants from the literature

Candidate genetic variants in kidney allograft recipients associated with AR were identified by searching the research literature. The PubMed database was searched using key words including polymorphism, acute rejection, and SNP among others. Several different searches were done to identify as many publications as possible. Only studies using cohorts containing kidney allograft recipients were considered. Candidate SNPs were those that had a positive association with AR ($P < 0.05$). Twenty-six studies identified 30 genetic variants (29 SNPs) within 24 genes (Table 2). Included in Table 2 is the name of the gene, reference SNP number (rs#), nucleotide location within the gene, the effect on the protein, study size, number of AR events and the number of genetic variants tested, *P*-value, and odds ratio (OR) with 95% confidence interval (CI) along with a reference to the study. Seven variants, including rs4340 within the angiotensin I-converting enzyme (ACE) gene (an insertion/deletion of a 288 bp partial Alu sequence within intron 15) were not genotyped in our original analysis [7].

Genotyping

Single nucleotide polymorphism genotyping of our study cohort was conducted using three different platforms [7].

Table 2. Candidate SNPs associated with acute rejection in the literature.

Gene	Name	SNP	Protein change	Nucleotide change	Study *n*, #AR, # test	*P*-value, OR (95% CI) [ref]
ABCB1	ATP-binding cassette, subfamily B, member 1	rs2032582	p.893S/A/T	c.2677T/G/A	*n* = 232, 64, 21	*P* = 0.003, 3.16 (1.50–6.67) [8]
ACE	Angiotensin I-converting enzyme	rs4340	Intron 15	288 bp in/del	*n* = 206, 19, 4	*P* < 0.05, 5.34 (1.27–22.42) [9]
CYP3A5	Cytochrome P450 3A	rs776746	Intron 3	c.219-237G/A	*n* = 136, 19, 3	*P* = 0.01, 10.1 (1.21–83.3) [10]
CCL2	Chemokine CC motif, ligand 2	rs1024611	Promoter	c.-2582A/G	*n* = 167, 27, 2	*P* = 0.022, 2.6 (1.12–6.01) [11]
CCL5	Chemokine CC motif, ligand 5	rs2107538	Promoter	c.-471C/T	*n* = 261, 92, 3	*P* = 0.035, None, [12]
CCR2	Chemokine CC motif receptor 2	rs1799864	p.64I/V	c.190G>A	*n* = 163, 39, 5	*P* = 0.014, 0.30 (0.12–0.78) [13]
					n = 85, 33, 2	*P* = 0.003, None [33]
CCR5	Chemokine CC motif receptor 5	rs1799987	Intron 1	c.-301 + 246A>G	*n* = 163, 39, 5	*P* = 0.016, 0.37 (0.16–0.85) [13]
					n = 243, 46†, 2	*P* = 0.029, 2.76 (1.11–6.90) [14]
					n = 85, 33, 2	*P* = 0.003, None [33]
F5	Coagulation factor V	rs6025	p.534R/Q	c.1602G/A	*n* = 394, 192, 1	*P* < 0.02, 3.83 (1.38–10.59) [15]
FCGR2A	Fc Gamma receptor IIA	rs1801274	p.167H/R	c.500A/G	*n* = 99, 53, 1	*P* < 0.05, None [16]
ICAM1	Intercellular adhesion molecule-1	rs5498	p.469K/E	c.1405A/G	*n* = 42, 11, 2	*P* = 0.013, 0.23 (None) [17]
IL1B	Interleukin-1-beta	rs1143634	p.105F/F	c.315C/T	*n* = 100, 18, 12	*P* = 0.045, 3.11 (1.02–9.44) [18]
IL2	Interleukin-2	rs2069762	Promoter	c.-385T/G	*n* = 63, 20, 1	*P* < 0.05, None, [19]
IL4	Interleukin-4	rs2243250	Promoter	c.-589C/T	*n* = 120, 14, 4	*P* = 0.02, None, [20]
IL8	Interleukin-8	rs4073	Promoter	c.-352A/T	*n* = 296, 61, 2	*P* = 0.032, 2.7 (1.09–6.69) [21]
IL10	Interleukin-10	rs1800896	Promoter	c.-1117C/T	*n* = 291, 50, 8	*P* = 0.016*, 1.9 (1.1–3.1) [22]
					n = 95, 21†, 2	*P* = 0.045, 2.8 (1.17–6.69) [23]
IL10	Interleukin-10	rs1800871	Promoter	c.-854A/G	*n* = 291, 50, 8	*P* = 0.016*, 1.9 (1.1–3.1) [22]
IL10	Interleukin-10	rs1800872	Promoter	c.-627G/T	*n* = 232, 64, 21	*P* = 0.007, 4.71 (1.52–14.55) [8]
					n = 291, 50, 8	*P* = 0.016*, 1.9 (1.1–3.1) [22]
IL18	Interleukin-18	rs187238	Promoter	c.-9212C/G	*n* = 226, 37, 1	*P* = 0.015, 3.65 (1.24–10.79) [24]
IMPDH1	Inosine 5-prime-monophosphate dehydrogenase 1	rs2278293	Intron 7	c.579 + 119G/A	*n* = 191, 29, 11	*P* = 0.008, 0.34 (0.15–0.76) [25]
IMPDH1	Inosine 5-prime-monophosphate dehydrogenase 1	rs2278294	Intron 7	c.550-106G/A	*n* = 191, 29, 11	*P* = 0.02, 0.40 (0.18–0.89) [25]
IMPDH2	Inosine 5-prime-monophosphate dehydrogenase 2	rs11706052	Intron 7	c.819 + 10T/C	*n* = 232, 64, 21	*P* = 0.006, 3.39 (1.42–8.09) [8]
INFG	Interferon-γ	rs2430561	Intron 1	c.114 + 760T/A	*n* = 118, 20, 4	*P*=None, 2.6 (1.6–6.0) [26]
ITGB3	Integrin, Beta-3	rs5918	p.59L/P	c.176T/C	*n* = 119, 52, 1	*P* = 0.04, 2.75 (1.01–7.93) [27]
TGFB	Transforming growth factor-β1	rs1800470	p.10P/L	c.29C/T	*n* = 291, 50, 8	*P* = 0.043**, 1.8 (1.0–3.0) [22]
					n = 164, 7	*P* = 0.02, 4.0 (None) [28]
TGFB	Transforming growth factor-β1	rs1800471	p.25R/P	c.73C/G	*n* = 291, 50, 8	*P* = 0.043**, 1.8 (1.0–3.0) [22]
					n = 118, 20, 4	*P*=None, 0.09 (0.02–0.49) [26]
					n = 164, 13†, 7	*P* = 0.02, 4.0 (None) [28]
TLR4	Toll-like receptor-4	rs4986790	p.299D/G	c.896A/G	*n* = 238, 57, 2	*P* = 0.01, 0.41 (0.30–0.83) [29]
TLR4	Toll-like receptor-4	rs10759932	Promoter	g.3685T>C	*n* = 216, 42, 6	*P* = 0.001, 0.25 (0.11–0.57) [30]
TNF	Tumor necrosis factor-α	rs1800629	Promoter	c.-488A/G	*n* = 232, 64, 21	*P* = 0.029, 2.18 (1.08–4.41) [8]
					n = 100, 18, 12	*P* = 0.03, 6.277 (1.18–33.2) [18]
					n = 291, 50, 8	*P* = 0.003, 5.0 (3.0–8.3) [22]
					n = 95, 21, 2	*P* = 0.005, 3.92 (1.61–9.57) [23]
					n = 164, 13†, 7	*P* = 0.009, 6.1 (None) [28]
					n = 129, 57, 2	*P* < 0.05, 2.53 (1.19–5.37) [31]
VEGF	Vascular endothelial growth factor	rs699947	Promoter	c.-2578C/A	*n* = 173, 65, 4	*P* = 0.005, 4.1 (1.5–11.3) [32]
VEGF	Vascular endothelial growth factor	rs1570360	Promoter	c.-1154A/G	*n* = 173, 65, 4	*P* = 0.001, 6.8 (1.8–25.0) [32]

'Study *n*' is the number of individuals used in that study to determine the *P*-value.
'# test' is the number of polymorphisms tested in the reported analysis.
AR is the number of acute rejection events in the study population
*Significant *P*-value based on an IL-10 haplotype containing three promoter variants.
**Significant *P*-value based on haplotype containing the codon 10 and codon 25 TGFB variants.
†0 and 1 acute rejection events were combined in the no rejection category for analysis

The majority of the SNPs were genotyped using a customized Affymetrix GeneChip [34]. Genotypes were determined using the Affymetrix GeneChip Scanner 3000 Targeted Genotyping System (Affymetrix, Santa Clara, CA, USA) and inversion probe technology. We genotyped additional SNPs using the SNPlex (Applied Biosystems Inc., Foster City, CA, USA) and Sequenom (Sequenom, Inc., San Diego, CA, USA) platforms, as per manufacturer's recommendation. In all cases, the same SNP as that published in the original report was used for analysis.

Assessment of genotype quality has been previously reported [7]. In brief, SNPs that deviated from Hardy–Weinberg equilibrium ($P < 0.002$; 0.05/23) in either African–American or Caucasian cohorts were excluded from our analysis.

Statistical analyses

Cox proportional hazards models were used to investigate the association of each SNP with time to AR. Time to event (i.e. acute rejection) was used because it was considered to be the most powerful means of detecting a true association. SNPs were coded for the additive genetic model. Individuals were considered at risk for AR beginning on the day of transplant. Censoring occurred at the time of death, graft failure, last date of follow up, or 1-year post-transplant.

We first performed a single-SNP race-adjusted analysis, adjusting for recipient race (African–American versus nonAfrican–American) and stratifying by transplant center. Next, multiple variable single-SNP analysis was conducted as described below. Potential clinical covariates for inclusion in the multiple variable model were first identified using backward selection with a retention *P*-value of 0.10, ignoring SNPs. The final multiple variable model for testing the association of each SNP with time to AR was stratified by clinical center, and was adjusted for those clinical covariates retained in a backward selection process [7]. Clinical factors included in the backward selection algorithms consisted of the following, where an asterisk (*) denotes those clinical factors that were significant and retained in the final model: recipient gender*, age*, smoking status [never, past or current], weight*, blood type, cause of end-stage renal disease*, SPK transplant*, prior kidney transplant, prior nonkidney transplant, number of HLA mismatches*, cross-match positive*, panel reactive antibody (PRA) present*, dialysis prior to transplant, CMV serostatus, calcineurin inhibitor (CNI) type used initially (cyclosporine versus tacrolimus)*, type of antibody induction*, steroids use at day 14*, and donor factors (age*, gender, and donor status [living or deceased]*). In addition, the backward selection algorithms were directed to retain recipient race* at all stages of model selection without regard to level of significance.

Analyses were conducted using SAS/Genetics v9.2 (The SAS Institute, Cary, NC, USA, http://www.sas.com).

In this analysis of 969 recipients, we have >50% power to detect variants with a hazard ratio ≥ 1.3 and risk allele frequency ≥ 0.20 at significance level of 5%, whereas the estimated effect sizes for the 23 variants were much greater (Table 2) [35]. In the case of those variants being truly causal, we should have >99% power to replicate at least 16 of 18 associations based on the reported estimated effect sizes in previous studies (although the power calculation is likely overestimated because of 'winner's curse' effect).

Results

Demographic information of our research cohort is shown in Table 1. A total of 969 recipients were collected from five transplant centers, with 181 (18.7%) recipients diagnosed with AR in the first year post-transplant. A total of 176 of the recipients with AR (97.2%) had biopsies, all of which exhibited AR. As previously reported, several clinical outcomes were found to be associated with AR, including recipient factors (race, age, gender, weight), PRA presence, number of HLA mismatches, T- or B-cell cross-match positive, antibody induction, type of calcineurin inhibitor used, steroid use at day 14 post-transplant, simultaneous kidney–pancreas transplant (versus kidney transplant alone), cause of ESRD, living donor (versus deceased donor), donor age, and transplant center [7].

PubMed was searched to identify publications reporting a significant association ($P < 0.05$) between recipient genetic variants and AR in kidney allograft recipients. Twenty-six studies identified 30 genetic variants (29 SNPs) associated with AR (Table 2). Several SNPs had two or more reports stating a significant association with AR including rs1799864 (CCR2), rs1799987 (CCR5), rs1800896 and rs1800872 (IL10), rs1800470 and rs1800471 (TGFB), and rs1800629 (TNF). In the majority of these reports, the cohort size was relatively small with an average size of 179 recipients (range 42–394). In addition, most of these reports analyzed multiple SNPs (19/25), but did not account for multiple testing when determining the statistical significance of the association of a SNP with AR.

Twenty-three of the 29 SNPs identified from the literature were genotyped in our cohort and tested for association with AR (Table 3). All genotypes were found to be in Hardy Weinberg equilibrium for both African–American and Caucasian populations.

The most significant SNP (rs6025) was within the factor V gene (F5) giving a *P*-value of 0.011 (hazard ratio of

Table 3. Analysis of candidate SNPs previously associated with AR.

						Race-adjusted analysis		Multiple variable analysis	
Gene	SNP	Allele	Freq	Freq nonAA	Freq AA	*P*-values	HR (95% Confidence Interval)	*P*-value	HR (95% Confidence Interval)
ABCB1	rs2032582	T	0.384	0.45	0.07	0.24	0.88 (0.71–1.09)	0.34	0.89 (0.7–1.13)
CYP3A5	rs776746	A	0.182	0.08	0.65	0.79	1.05 (0.75–1.45)	0.94	0.99 (0.71–1.38)
CCL2	rs1024611	C	0.281	0.3	0.19	0.60	1.06 (0.85–1.32)	0.61	1.06 (0.84–1.35)
CCL5	rs2107538	T	0.220	0.19	0.38	0.75	1.04 (0.81–1.34)	0.86	1.02 (0.78–1.35)
F5	rs6025	A	0.025	0.029	0.0029	0.011	1.82 (1.15–2.89)	0.0003	2.54 (1.53–4.24)
FCGR2A	rs1801274	C	0.491	0.47	0.59	0.48	1.08 (0.88–1.32)	0.56	1.07 (0.86–1.32)
ICAM1	rs5498	G	0.383	0.42	0.22	0.82	1.03 (0.81–1.29)	0.95	0.99 (0.77–1.28)
IL1B	rs1143634	T	0.210	0.23	0.13	0.58	0.93 (0.72–1.2)	0.93	1.01 (0.78–1.32)
IL2	rs2069762	G	0.269	0.3	0.12	0.38	0.9 (0.71–1.14)	0.25	0.86 (0.67–1.11)
IL4	rs2243250	T	0.270	0.19	0.66	0.72	1.05 (0.82–1.33)	0.80	1.03 (0.8–1.33)
IL8	rs4073	T	0.470	0.52	0.22	0.07	1.22 (0.98–1.51)	0.11	0.83 (0.65–1.04)
IL10	rs1800896	G	0.436	0.45	0.36	0.40	1.1 (0.89–1.35)	0.71	1.04 (0.83–1.31)
IL10	rs1800871	T	0.281	0.26	0.38	0.54	1.08 (0.85–1.36)	0.46	1.1 (0.86–1.4)
IL10	rs1800872	A	0.281	0.26	0.38	0.67	1.05 (0.83–1.33)	0.60	1.07 (0.84–1.36)
IL18	rs187238	G	0.263	0.27	0.23	0.78	0.97 (0.77–1.21)	0.66	0.95 (0.75–1.2)
IMPDH1	rs2278293	A	0.465	0.46	0.48	0.89	0.99 (0.8–1.22)	0.66	1.05 (0.83–1.33)
IMPDH1	rs2278294	A	0.364	0.36	0.39	0.99	1.0 (0.8–1.25)	0.89	1.02 (0.8–1.29)
IMPDH2	rs11706052	G	0.088	0.1	0.01	0.065	0.68 (0.45–1.02)	0.044	0.64 (0.41–0.99)
ITGB3	rs5918	C	0.122	0.13	0.09	0.66	1.07 (0.79–1.46)	0.86	0.97 (0.69–1.36)
TGFB	rs1800471	C	0.071	0.07	0.06	0.81	1.05 (0.7–1.59)	0.99	1.0 (0.64–1.58)
TLR4	rs4986790	G	0.049	0.05	0.05	0.21	1.32 (0.85–2.03)	0.065	1.54 (0.97–2.44)
TNF	rs1800629	A	0.169	0.18	0.1	0.57	1.08 (0.82–1.42)	0.58	1.09 (0.81–1.47)
VEGF	rs699947	A	0.437	0.49	0.19	0.32	0.9 (0.73–1.11)	0.85	1.02 (0.82–1.27)

AA, African–American; HR, hazard ratio; MAF, minor allele frequency.

1.82) for race-adjusted analysis and 0.0003 (hazard ratio of 2.54) in multiple variable analysis. The minor allele frequency for this SNP was 0.025 (0.029 in nonAfrican–Americans and 0.0029 in African–Americans).

Additional SNPs that showed a modest significance were rs11706052 (IMPDH2) with a *P*-value of 0.065 (hazard ratio of 0.68) in the race-adjusted analysis, a *P*-value of 0.044 (hazard ratio of 0.64) in the multiple variable analysis, and rs4986790 (TLR4) with a *P*-value of 0.065 (hazard ratio of 1.54) in the multiple variable analysis. Neither of these additional SNPs were significant when multiple testing was taken into account using a Bonferroni threshold of 0.0022 (0.05/23).

Analysis was also done on African–American recipients only ($n = 171$) and Caucasian recipients only ($n = 739$). In the African–American recipients only, none of the SNPs were significant with the lowest *P*-value being 0.17. For the Caucasian recipients only, except for SNP rs6025 (*P*-value for multiple variable analysis = 0.0003, hazard ratio 2.6 with 95% CI 1.6–4.4), *P*-values for the remaining SNPs were above 0.05, and several of the SNPs with the smallest *P*-values had extremely large confidence intervals, most probably because of very low minor allele frequencies in Caucasians.

Conclusions

We report the association of one of 23 genetic variants, previously reported to be associated with AR risk, using our cohort of kidney transplant recipients, which is the largest cohort of kidney transplant recipients used for genetic analysis to date. In this study, only rs6025 within the F5 gene exhibited a significant association with AR with race-adjusted analysis, which was similar to the statistical method used in the previous publications and in multiple variable analysis. The gene product of F5 is an essential component of the blood coagulation cascade. The rs6025 polymorphism produces an arginine to glutamine amino acid substitution at codon 534 (p.Arg534Gln) resulting in activated protein C (APC) resistance, and is the most common inherited risk for venous thromboembolism (VTE) [36,37]. This variant has been previously associated with acute rejection, acute vascular rejection, and early graft loss in kidney transplant recipients [15,38]. The hypercoagulable state caused by APC resistance may promote increased inflammatory response in the kidney because of endothelial damage [38]. A problem with the utility of this association is the very low minor allele frequency of this SNP (MAF =

0.025), which would require very large numbers of patients for clinical trials to obtain enough individuals having the minor allele. In addition, treatment for the hypercoagulable state could put patients at risk for bleeding, especially during biopsy procedures.

There was also a weak association with SNP rs11706052 within the inosine monophosphate dehydrogenase 2 (IMPDH2) gene. The gene product of IMPDH2 is responsible for the rate-limiting step in de novo guanine nucleotide biosynthesis, and is a target of mycophenolic acid (MPA). The polymorphism is a cytosine or thymine within intron 7 (c.819 + 10T/C). The presence of the C allele has been reported to result in a reduced response to MPA compared with homozygous T, when tested in a lymphocyte proliferation assay, and could explain poor response to mycophenolic acid response in some individuals [39]. Most recipients (925 of 969, 98.1%) received mycophenolate mofetil at some point in the first 6-month post-transplant, showing that these individuals may be sensitive to polymorphisms in IMPDH2-MPA interactions, resulting in an increased risk for AR.

The majority of the SNPs analyzed in this report failed to replicate their association with AR in kidney transplant recipients. Replication of genetic variants, reported to be associated with AR has been problematic, with most studies attempting to replicate the association reporting a nonsignificant result. It is possible that the originally reported associations are false positives. Most of the reports genotyped more than one SNP, but none took multiple testing into account when determining the significance of individual SNPs with AR. All of the initial studies reporting a positive association (Table 2) used small study cohorts. In addition, our study showed that population and clinical care differences (center-specific clinical risk factors) are associated with AR and need to be taken into account in the statistical analysis [7]. Not controlling for such factors could have led to false positive results in original studies or failure of the replications.

Studies to date have focused on common SNPs within obvious candidate genes. However, we were only able to confirm one SNP associated with AR from previous reports, which has a relatively low minor allele frequency. Our results suggest that variants in genes other than candidate genes or candidate pathways may also play a role in AR and will require different strategies for their identification. One possible strategy is to expand the number SNPs analyzed by genotyping SNPs to include SNPs in additional candidate genes. This strategy was attempted with an additional 3,300+ SNPs, but no statistically significant genetic variants associated with AR were identified after correcting for multiple testing [7]. Another approach is a genome wide association study (GWAS). This type of analysis is not dependent on knowing which gene(s) to select, but requires larger study cohorts than have been previously used because of the necessary correction for the large number of statistical tests. In many GWAS studies, a subset of the initially identified SNPs is reproducible, providing important information on pathways, both known and novel. One problem with this type of analysis is that only common variants are analyzed. In addition, in most GWAS results, the effect sizes have been found to be relatively modest. An alternative strategy is whole exome sequencing. In this case, all common and rare variants will be identified within the coding sequence of all known genes. It is possible that rarer variants clustering in candidate genes or candidate pathways may provide greater individual risks for AR compared with common variants (minor allele frequency >1%) and be of greater utility in predicting AR risk. Whole genome sequencing, along with the identification of insertions/deletions (in/dels) would capture most of the genetic variation, but the cohort size required for such an analysis would be very large. In addition, the analysis is much more complex in that there are no reliable methods for the identification of variants affecting gene expression levels, as compared with several methods for the identification of potentially functional coding SNPs, which is what would be needed for exome sequencing, making whole genome sequencing premature at this time [40].

The identification of genetic variants that predispose individuals to adverse outcomes associated with kidney allograft transplantation, including AR, would greatly aid transplantation success for an organ transplant recipient. These variants could help in the individualization of clinical care of kidney allograft recipients. However, identification of true causal variants that have small effect sizes will require larger cohorts than have been previously used. The size of the cohort will be dependent on the expected effect size and the frequency of the risk allele. In addition, it is likely that AR risk involves several biologic pathways, each involving multiple genes with (potentially) multiple genetic variants. The presence of significant genetic heterogeneity will reduce the impact of any given single variant on a clinical outcome, requiring significant power within the study cohorts to identify predisposing variants.

Authorship

WSO: designed research/study, wrote the paper, performed research/study. DPS: analyzed data, wrote the paper. REL: analyzed data, wrote the paper. PAJ: performed research/study, wrote the paper. WG: analyzed data, wrote the paper. AJM: performed research/study. AI: performed research/study, wrote the paper.

Funding

This work was supported by the National Institutes of Health NIAID Genomics of Transplantation grant U19-AI070119, an ARRA ancillary to this grant and DeKAF grant U01-AI058013.

Acknowledgements

This work was supported by the National Institutes of Health NIAID Genomics of Transplantation grant U19-AI070119, an ARRA ancillary to this grant and DeKAF grant U01-AI058013.

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Appendix 1

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