A genome-wide association study of recipient genotype and medium-term kidney allograft function

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Abstract: Background: We examined, through genome-wide association studies (GWAS), the correlation between recipient genetic variation and renal function at five yr.
Methods: Our cohort contained 326 Irish, first time, kidney-only, deceased donor, transplant recipients on calcineurin inhibitors (263 had a functioning graft at five yr) between 1993 and 2002. Outcomes were creatinine at five yr and long-term graft function.
Results: Two variants were identified showing borderline genome-wide significance – one on chromosome 18 (p = 4.048e-08, rs6565887) and another on chromosome 14 (p = 7.631e-08, rs3811321). Individually, the two SNPs explained up to 8.8% and 11.29% of five-yr creatinine variance, respectively, while together they explained up to 17.4% of trait variance. Both variants were predictors of long-term allograft function (p = 0.004, 70% vs 30% survival at 10 yr). The chromosome 14 variant is located in the intergenic region of the T-Cell Receptor Alpha locus.
Conclusions: Using a genome-wide approach, we have identified two associations with five-yr creatinine levels in renal transplant recipients treated with calcineurin inhibitors. Independent replication is now warranted to clarify the clinical significance of these results.

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Renal transplantation remains the preferred treatment for end-stage renal disease (ESRD) in terms of long-term survival and quality of life. It is also a much more cost-effective treatment compared with dialysis (1). There is a shortage of suitable donors, and therefore, waiting times are continuing to increase. In the era under study (1993–2002), allo-graft half-life was approximately seven yr in the United States (2), and 10 yr in our center. There has been little improvement in long-term graft outcome over the past two decades despite the marked improvements in one-yr graft survival. Exploring the genetics of renal transplant outcome may assist in assessing prognosis before and after

transplantation, aid in the diagnosis of post-transplant complications, and help further tailor treatment in transplantation. The aim here is to identify genetic variants affecting medium-term renal function as measured by a quantitative trait, serum creatinine at five-yr post-transplantation.

The development of genome-wide association studies (GWAS), and more recently, whole-genome sequencing, has revolutionized the field of genetic mapping and greatly increased the capacity for identifying genetic predictors of complex human traits such as creatinine levels. By assaying several hundred thousand genetic variants across the human genome, GWAS provide the mechanism to determine the contribution of common genetic variation to complex traits.

Several large GWAS have examined serum creatinine and renal function in populations with normal renal function and chronic kidney disease (CKD) (3–5). These studies have identified markers associated with kidney function and CKD with strong genome-wide significance. However, the determinants of kidney function in a post-transplant cohort may well be different to a normal population. Donor/recipient immunological interactions, transplant immunosuppressive medications, and opportunistic infections are some of the unique factors associated with allograft function post-kidney transplantation.

In this study, we hypothesize that genetic variation in kidney transplant recipients contributes, in a clinically relevant manner, to the outcome of kidney transplant recipients as measured by various measures of graft outcome including long-term graft survival and allograft function as measured by serum creatinine at five yr. To test this hypothesis, we have applied GWAS to a cohort of 326 renal transplant recipients of Irish descent.

Methods

Ethics

The Beaumont Hospital Ethics Committee approved this study (Protocol No. 06/81 and Protocol No. 07/16). Written informed consent was obtained from living participants, with a waiver of consent from deceased recipients.

Patients

All patients were recruited from Beaumont Hospital, Dublin. Patients were all more than 18 yr of age, and transplants were, first time, kidney-only, deceased donor, conducted between 1993 and 2002. All patients were on calcineurin inhibitor–based immunosuppression. Only allografts that functioned for at least 72 h were included. DNA was extracted from blood using standard protocols.

Phenotypic data were retrieved from the Beaumont Hospital's renal transplant database that is prospectively updated by a database manager. For the purpose of this study, we have focused on the 15 clinical parameters (shown in Table 1) that have previously been suggested to associate with creatinine levels, graft function/survival, and mortality in the clinical setting. As such, these were used in the construction of a linear regression data model.

Genotyping and data cleaning

Genotyping was conducted using the Illumina Human 610-Quad platform, which provides excellent coverage of common variation. The raw intensity data were processed and quality-controlled using Illumina Genome Studio software. Data were cleaned using PLINK (6) employing standard protocols (see Data S1).

Four hundred recipient DNA samples were genotyped on the Illumina 610-Quad platform. After data cleaning and quality control using standard protocols, there were 326 patients available for the primary analyses. A total of 263 patients had a functioning allograft at five-yr post-transplant and had creatinine data that could be included in the five-yr renal function analysis. After data cleaning, there were 511 662 SNPs left available for analysis.

Data analysis

We divided our data analysis in two stages. The first stage was designed to identify and characterize

Table 1. Clinical parameters used in clinical regression

	Description
1	Recipient sex
2	Cause of ESRD
3	Donor cause of death
4	Donor age
5	Donor sex
6	Cold Ischemia time
7	Cytomegalovirus (CMV) serological status of recipient
8	CMV serological status of donor
9	Use of anti-CMV prophylaxis
10	CMV infection post transplant
11	Delayed graft function – need for dialysis in the first week post transplantation
12	Number of HLA mismatches – at A, B and DR loci
13	Acute rejection-biopsy proven
14	Acute Rejection-biopsy proven plus empirically treated
15	Panel reactive antibody level

clinical predictors of five-yr creatinine levels. The second stage consisted of a GWAS analysis of five-yr creatinine but including clinical predictors (identified through stage 1) as covariates.

The first stage of our analysis consisted of linear regression data modeling to determine clinical predictors of five-yr creatinine levels using the R package (7). The R function "Generalized Linear Model" (glm) was used for the model analysis. The method employed starts with full set of all 15 independent terms (see Table 1) and reduces the number of terms until the Akaike's information criterion (AIC) reaches a minimum at the intercept as compared to the other terms. We assessed which model term should be dropped in each round using the R function "drop1" (which selectively removes terms and reports the AIC value for those remaining).

The second stage of the analysis consisted of another linear regression, but this time correlating genotype and serum creatinine levels. We included as covariates significant clinical predictors (from stage one) together with principal component analysis (PCA) to control for population structure calculated using Eigensoft (8). We applied the Wald test, as implemented in PLINK. Results were visualized using WGAviewer (9). Unless otherwise stated, tests were performed on the natural log-transformed creatinine levels taken five-yr post-transplant.

Survival analysis was carried out matching genotypes of significant SNPs (identified in the GWAS) to (i) time to graft failure and (ii) time to death. Records with invalid or missing values were excluded. The Stata package was used to perform, plot, and annotate the survival analyses (10). All computational formulas are those implemented in Stata 12.1, which are consistent with those in Fleiss et al. (11).

Comparative analysis of linkage disequilibrium (LD) patterns between reference data sets and our experimental data set was used to partially validate SNPs within significant peaks. Specifically, LD patterns (r^2 values) from both 1000 Genomes (12) and HapMap3 (13) were calculated using SNAP (14) in conjunction with PLINK. Areas of interest and inter-SNP relationship r^2 values were also calculated using SNAP.

Correlation coefficient calculations between specific genotypes and the quantitative trait were performed in R. We report "adjusted r^2" values in the text.

p Values for all genotypic regressions were calculated using PLINK, and we quote the unadjusted value (Asymptotic p-value for t-statistic), the Bonferroni single-step adjusted p-value to correct for multiple testing, and permutation, which is the family-wise corrected empirical p-value (EMP2).

The statistical power of the GWAS linear regression tests was calculated to give a perspective on the results. QUANTO (15) was used to make statistical power calculations in this analysis.

We estimated the "population attributable fraction" for our significant variants in the survival analysis. It is calculated as a measure of the clinical significance of each prognostic factor. It measures the proportion of all deaths in the patient population that may be attributed to the presence of the risk factor. It thus combines the risk associated with the factor with the prevalence of the factor to give an overall summary of the factor's clinical significance.

Results

The primary cohort for analysis consisted of 326 kidney transplant recipients of whom 124 had died during follow-up. Median follow-up time was 10.7 yr, and maximum follow-up time was 17.5. The summary statistics for the clinical characteristics of this group are detailed in Table 2.

Stage 1: Identification of clinical predictors of five-yr creatinine

To determine significantly contributing clinical factors to creatinine levels, we constructed a linear regression data model that considered a set of 15 variables as predictors (see Table 1 for list) and creatinine level at five yr (log-transformed) as the dependent variable. The model that yielded the best fit contained the terms donor age (p = 8.39e-06), donor sex (p = 0.0661), cytomegalovirus (CMV) infection post-transplant (p = 0.0003), and biopsy-proven rejection episode (p = 0.1420) (see Table 3). As donor age and CMV infection post-transplant were statistically significant, they were included as covariates in the genetic model. Multiple regressions showed that donor age and CMV infection post-transplant combined explained 13.3% of the variation in five-yr creatinine levels. Donor age alone explained 10.4%, while CMV infection post-transplant explained 5.46%. The examination of creatinine levels at five yr reduced the cohort to 263 individuals (63 individuals were missing creatinine levels at five yr due to graft failure at or before five yr). It was encouraging that the clinical covariates we derived from our linear regression data model (CMV infection post-transplant and donor age) had previously been established as major predictors of long-term graft function and creatinine level by

other studies [for CMV infection post-transplant see (16–19) and for donor age see (20, 21)]. Our results concurred with a previous finding that estimated GFR and creatinine at one yr (22) are also predictors of long-term graft function. We did not, however, include these in our model as they are highly correlated with creatinine levels at five yr.

Stage 2: GWAS analysis of five-yr creatinine

Application of the full GWAS regression model (including PCA and clinical covariates) resulted in the identification of one variant on chromosome 18 that was GWAS significant (p = 4.048e-08, rs6565887) and another variant on chromosome 14 that was borderline GWAS significant (p = 7.631e-08, rs3811321). Table 4 shows results, Fig. 1 shows the QQ plot, and Fig. 2 shows the Manhattan plot and gene context. When the analysis was repeated using permutation as a more stringent test, the results were confirmed as borderline genome-wide significant (see Table 4). Having shown a significant result using the more conservative natural logarithm transformation of the quantitative trait, the linear regression data model was reapplied using the untransformed values for creatinine at five yr. The results of this less conservative test showed an increase in significance for both chromosomes 14 and 18 (rs3811321 and rs17113407; p = 3.381e-10, rs6565887 p = 8.551e-09). These results together with those generated from regressions without covariates for both transformed and untransformed creatinine levels can be seen in Data S1 and S2. Our observation that the same two SNPs appeared significant, regardless of the transformation chosen or covariates included, gave us increased confidence in these results.

To further refine the results, we investigated LD patterns between the top hits. The variants driving the chromosome 14 signal (rs3811321 and rs17113407) were in complete linkage ($r^2 = 1$) in our data set. This value was consistent with expectation from LD patterns derived from 1000 Genomes and HapMap3 data. A similar set of analysis was performed for the three variants driving the chromosome 18 signal with a similar result: rs9961326 in full LD ($r^2 = 1$) with rs17355430 and both of these in high LD ($r^2 = 0.925$) with rs6565887. These r^2 values were identical to those calculated in both the 1000 Genomes and experimental data.

From the data observed, it would therefore seem that the two signals are probably driven by one causal variant at each site. We therefore focused follow-up work on the most significant variant for each signal, namely rs3811321 (chromosome 14) and rs6565887 (chromosome 18).

We next fitted a linear model between five-yr creatinine and the sample genotypes for rs3811321 and rs6565887. Individually, the two SNPs explained 11.29% and 8.8% of five-yr creatinine variance, respectively, while together they explained 17.4% of trait variance.

Adding the two derived clinical covariates (donor age and CMV infection post-transplant) to the model gave a result that accounted for 37% of the five-yr creatinine trait variance. The effect sizes we have estimated here are almost certainly overestimated as they have been calculated in the cohort in whom the discovery was made. Selection from many thousands of tests imposes an upward bias on the estimate, a phenomenon known as the Beavis effect or "winner's curse" (23). Independent replication is required for a more accurate estimation of true effect size.

Survival analysis

To assess the results as possible indicators of graft failure and mortality, a survival (time to event) analysis was undertaken for rs3811321 and rs6565887 from the entire cohort of 326 patients.

Table 2. Clinical characteristics of data set patients

Variable	Statistic	Result
Recipient age	Mean (SD)	42.6 (13.2)
Donor age	Mean (SD)	35.8 (15.6)
Recipient sex	Number men/women (%)	216/110(66/34)
Donor sex	Number men/women (%)	199/127(61/39)
Cold Ischemic time (h)	Mean (SD)	22.2 (6.2)
HLA mismatches	Mean (SD)	2.9 (1.2)
Acute rejection	Number Yes/No (%)	102/224 (31/69)
Panel reactive antibodies	% 0–10/11–49/50–100	85/8/7
Modality prior to transplant	% HD/PD/pre-emptive	66/33/1
Time spent on dialysis (months)	Median [range]	15 [1–192]

Table 3. Final results of linear data model best predicting log Creatinine at 5 years. AR=Acute Rejection (biopsy proven)

Coefficients	Estimate	Standard Error	p Value
Donor Age	0.0029272	0.0006396	8.39e-06
Donor Sex (M)	−0.0367718	0.0198992	0.066123
CMV Infection	0.1094029	0.0300470	0.000347
AR	0.0327169	0.0221989	0.142137

Table 4. A summary of five-yr creatinine GWAS results

CHR	SNP	UNADJ	BONF	Perm	Effect allele	Beta	r^2
18	rs6565887	4.048e-08	0.02071	0.03197	G	0.2924	0.08765
14	rs17113407	7.631e-08	0.03904	0.06094	G	0.481	0.1129
14	rs3811321	7.631e-08	0.03904	0.06094	C	0.481	0.1129
18	rs9961326	7.709e-08	0.03945	0.06094	A	0.2928	0.08889
18	rs17355430	7.709e-08	0.03945	0.06094	A	0.2928	0.08889
13	rs9324268	2.456e-06	1	0.7383	A	0.1738	0.03302
11	rs3017493	4.908e-06	1	0.9081	G	0.2563	0.001629

GWAS, genome-wide association studies; UNADJ, Asymptotic p-value for t-statistic; BONF, Bonferroni single-step adjusted p-value; PERM, EMP2, which is the corrected empirical p-value (max(T)/family wise). 1000 permutations used. All EMP1 values (Empirical p-value point wise) were 0.000999. Allele = tested allele (minor Allele), beta = regression coefficient, r^2 = correlation coefficient between genotype and creatinine at five yr.
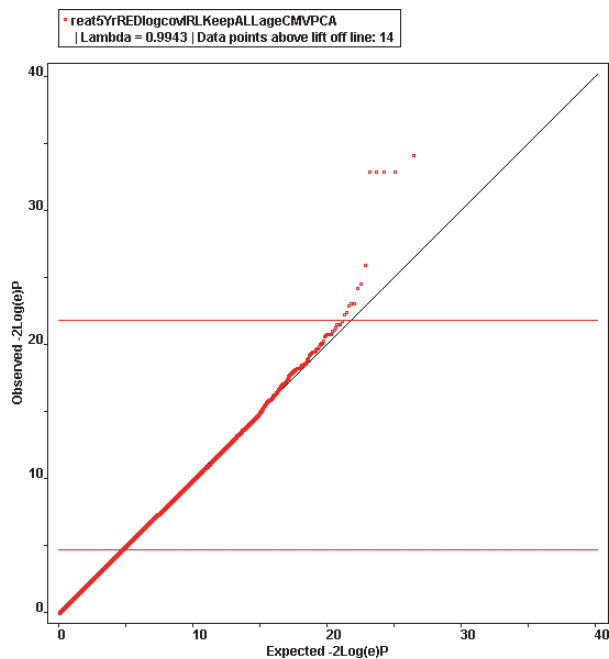

Fig. 1. Q–Q plot from genome-wide association studies of five-yr creatinine includes donor age, cytomegalovirus infection post-transplant, and three principal component analysis vectors as covariates, 511 662 SNPs. Graphic from WGAviewer.

For rs3811321 (chromosome 14 signal), results showed that the effect on allograft failure was to be statistically significant (p = 0.0041, see Fig. 3 and Data S3). The effect of this variant on mortality was not significant (p = 0.082). The analysis showed that the risk of allograft failure in those with the heterozygote genotype is 58%, while the estimated population attributable fraction of failure due to this variant is 7%. The survival curve illustrating time to allograft failure shows 70% vs. 30% failure rate at 10 yr (see Fig. 3).

For rs6565887 (chromosome 18 signal), results showed a statistically significant effect on both failure (p = 0.0252) and mortality (p = 0.0192) (see Data S3). The analysis shows that the risk of fail-

ure in those with the heterozygote genotype is 39.9%, while the estimated population attributable fraction of failure due to this variant is 8.7%. The survival curve illustrating time to failure shows 70% vs. 45% failure rate at 10 yr (see Fig. 4). The survival curve illustrating the risk of death is shown in Fig. 5.

Discussion

This is the first genome-wide association study in renal transplantation and, although modestly powered, it has the advantage that it was carried out on a cohort that is relatively homogeneous both in terms of ancestry and treatment regime. This gives the advantage of having a reduced set of possible covariates compared with larger multi-institute/multinational studies. A possible disadvantage, however, is that any resulting outcomes may be specific to the conditions pertaining to our cohort.

Our hypothesis was that genetic variation in renal transplant recipients contributes to post-transplant renal function, as measured by serum creatinine at five-yr post-transplantation. Serum creatinine levels have previously been examined in normal populations and those with native CKD using GWAS methods (3–5). However, kidney transplant patients represent a biologically unique setting, and determinants of renal function are different to those in healthy populations. Allograft function post-transplant is influenced by immunosuppression toxicity, immune injury (24–26), infectious complications as well as donor factors (19, 20). Therefore, it is highly likely that genetic factors associated with renal function in healthy and renal transplant populations are different. Previous genetic studies in renal transplantation have largely been small candidate gene studies in which a limited number of polymorphisms were studied (27). Moreover, the main outcome variable studied has generally been acute rejection. While acute rejec-
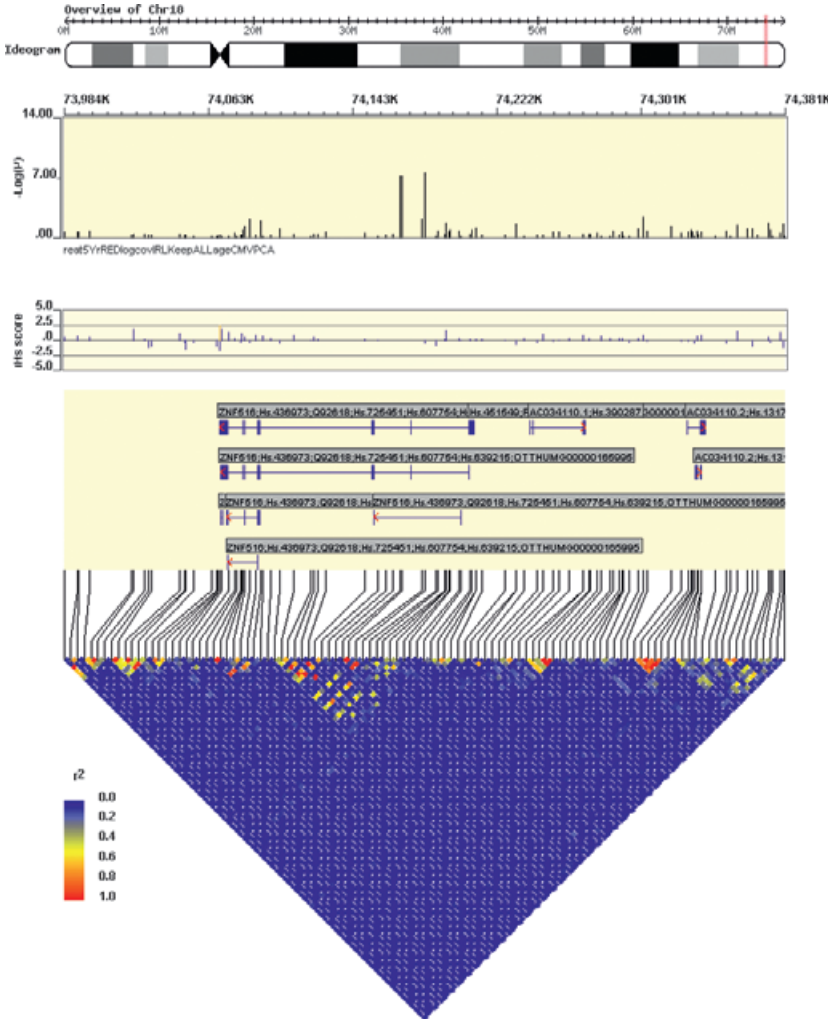
Fig. 2. Annotation of chromosome 18 significant signal – rs6565887 is shown in red above.
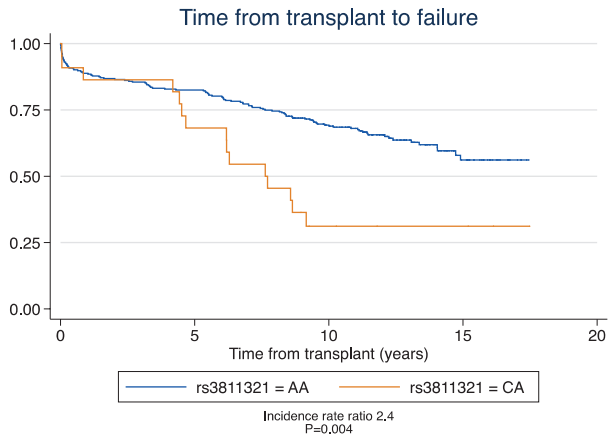

Fig. 3. Survival curve – time to transplant failure.

Fig. 4. rs6565887 – survival curve – time from transplant to failure.

tion does have an influence on long-term allograft survival (24, 25), it is usually very treatable. Long-term allograft function/survival is arguably more relevant clinically.

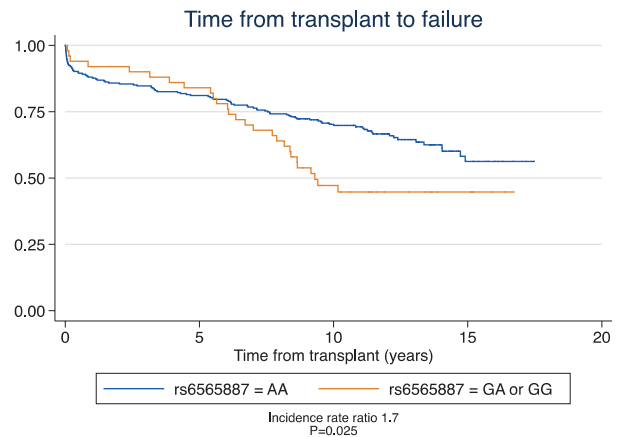A potential criticism of our work is that only transplant recipients were studied. In transplanta-

tion, patient outcome is, in part, determined by the interaction between the recipient and donor genomes. However, there is evidence that recipient genetic factors are independently important (28–33). Another factor to consider is that the patients studied here were transplanted between 1993 and
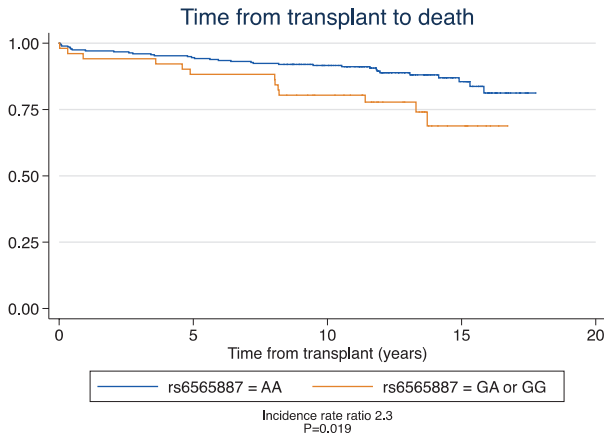
Fig. 5. rs6565887 – survival curve – time from transplant to death.

2002. In the mid-1990s, the main immunosuppressive agent used was cyclosporine. In the late 1990s, tacrolimus was introduced at the recruitment site (Beaumont Hospital) and soon became the cornerstone of renal transplant immunosuppression. Some individuals may question the relevance of our study with a large proportion of patients treated with an older agent. However, cyclosporine is still used in many centers worldwide. Moreover, cyclosporine and tacrolimus are both calcineurin inhibitors and have a similar, if not identical, mechanism of action. They also have a similar side effect profile. Transplant biopsies with cyclosporine- and tacrolimus-induced nephrotoxicity are indistinguishable (34). In our opinion, the longer follow-up time with these patients more than offsets any potential drawbacks.

From a biological and genetic perspective, the first of the two variants (rs3811321/rs17113407) we report is, with respect to renal transplantation, located in a potentially relevant area of the genome. The chromosome 14 signal (rs3811321/rs17113407) covers an intergenic region of the T-Cell Receptor Alpha locus (*TRA@*, HGNC ID:12027) between *TRAV19* and *TRAV20* (see Data S3). The TRA locus encodes proteins for the T-cell receptor alpha chains, which result from the rearrangement/recombination at the DNA level of *TRAV* and *TRAJ* using the deletion of the intermediary DNA to create a rearranged *TRAV-J* gene. The rearranged *TRAV-J* gene is transcribed with *TRAC* and translated to a T-cell receptor alpha chain (35). The TRAV gene family plays an important role in the immune system, and polymorphisms in this region could conceivably alter the immune response to foreign proteins.

Within a T-cell clone, the T-cell receptor (TCR) alpha chain combines with a beta chain, and the resultant heterodimeric T-cell receptor confers antigen specificity on the immune response. The antigen-binding groove of the TCR is primarily encoded by the V gene segments of both alpha and beta chains. Both chains are formed by somatic cell recombination, producing a diverse repertoire of T cells bearing different receptors. Hence, the use of particular V genes is likely to influence the subsequent immune repertoire of the individual. Preferential use of V beta gene families has been demonstrated in a number of immune-mediated diseases, and preferential V-alpha gene usage has been reported in psoriasis (36). Recombination of TCRAV and TCRAJ is thought to involve regulated changes in locus conformation and possibly subnuclear positioning. Hence, if replicated, it is possible that a polymorphism even within a non-encoding portion of the TCRA locus may influence TCRAV gene usage and thus immune responses. It is of interest that GWAS analysis of narcolepsy, a tightly HLA-associated autoimmune disease, identified a strong association with the TCRA locus.

The variant driving the chromosome 18 signal (rs6565887) is a non-coding intronic variant of zinc finger protein 516 (*ZNF516*, see Fig. 2). *ZNF516* is a member of the C2H2-type zinc finger protein family located in the nucleus and cytoplasm and is expressed mainly in kidneys, spleen, and lung. The variant has no obvious functionality.

A potential limitation of the study is the loss of 63 patients who did not have serum creatinine data available (mostly because of allograft failure prior to five yr). A suggestion would be to apply an arbitrary high creatinine value to the early failure patients, but we feel this would not be valid. We feel that allograft function, as universally measured by serum creatinine, at five-yr post-transplantation is a hard endpoint and more clinically relevant than other potential outcome measures such as early acute rejection. Moreover, our significant SNPs from the creatinine analysis were subsequently examined in the allograft survival model, which included all-comers.

Independent replication of these findings is required to confirm or reject their role in renal transplant outcome. Future work may prove the associations we have described here to be useful clinically, either as predictors of outcome or as causal variants driving creatinine levels in transplanted patients. These could provide important insights into the genetic processes at work post-transplantation.

Any attempt to replicate these findings will need to be sufficiently powered. Our calculations indicate that we had 80% power to detect an effect size of 14%. While our largest single genetic effect of

11% suggests that a cohort of 340 would provide 80% power for replication, the true cohort size for an effective replication is more likely to be in the thousands, given the inflation of the discovered effect size calculated here as a result of the "winner's curse" phenomenon (23).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Regression analysis using untransformed five yr creatinine level untransformed with clinical and PCA covariates.

Data S2. Regression analysis results using other combinations of quantitative trait transformation and derived covariates – included to demonstrate consistency of results.

Data S3. Results of STATA survival analysis.