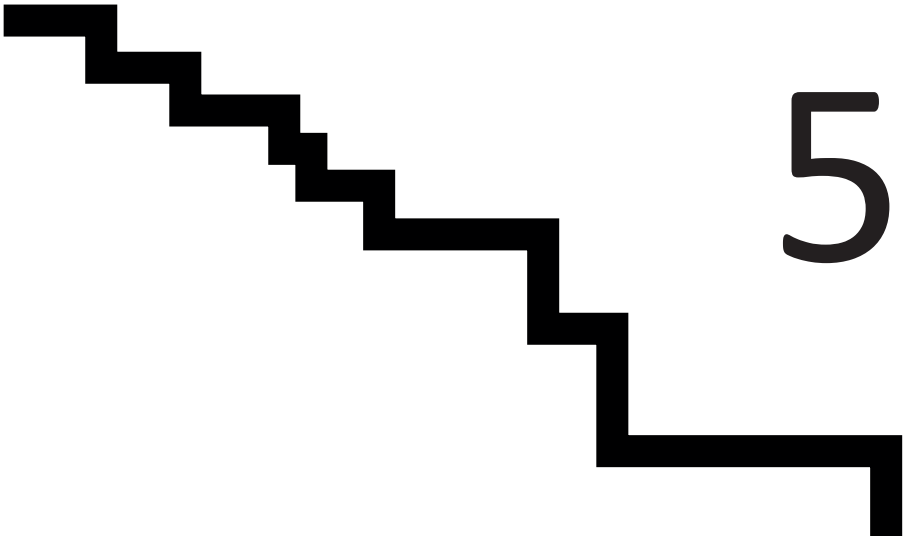
Molecular and genetic markers for the prediction of kidney transplant outcome

Yang, J.

Genome-wide association study of acute rejection in kidney transplantation



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Abstract

Background Biopsy-proven acute rejection (BPAR) is a risk factor for adverse kidney transplant outcome. Previous hypothesis-driven studies have led to identification of genetic variants associated with AR. Here, we conducted a genome-wide association study (GWAS) to search in an unbiased manner for loci and single nucleotide polymorphisms (SNPs) that are associated with BPAR after kidney transplantation.

Method A total of 325 patients and 321 donors, transplanted between 1994 and 2012, were genotyped on the Transplant SNP array v1. The genotyped dataset was imputed based on a combined reference set derived from the 1,000 Genomes Project and Genome of the Netherlands, resulting in seven million analysable SNPs after rigorous quality control. Genetic associations were tested by factored spectrally transformed linear mixed models (FaST-LMM). Identified candidate SNPs in the GWAS discovery cohort were tested in an independent cohort 243 recipients from another transplant center. A set of previously published candidate risk genes of allograft rejection was interrogated in the current GWAS.

Results After correction for clinical risk factors, candidate loci were identified in the patients: top ranking variant rs112775512 (OR=1.44, $P=3.38\times10^{-9}$) in an intron of *COL5A1*, which was associated with BPAR. None of the significant candidate SNPs ($P<5\times10^{-6}$) identified in the discovery cohort could be confirmed in the validation cohort. In previously published genetic association studies, we could confirm the association of rs1801274 in FCGR2 with BPAR.

Conclusion These findings emphasize the importance of validation in genetic association studies. International collaborative studies in the field of kidney transplantation are necessary to increase sample size and identify robust clinically relevant SNPs.

Introduction

Acute allograft rejection remains a risk factor for adverse kidney transplant outcome (1). Human leukocyte antigen (HLA) mismatching between donor and recipient, the presence of anti-HLA antibodies in the patient, delayed graft function (DGF), and younger patient age are considered as risk factors for acute rejection (AR) (2-4). Besides the well-defined clinical and immunological risk factors, genetic variants across the human genome may influence allograft rejection (5, 6). Genetic studies in renal transplant have mainly focused on single nucleotide polymorphisms (SNPs) located within or flanking the genes that encode for cytokines, chemokines, toll-like receptors, ficolins, and complement components, which play a role in immune responses (7-11). These studies have led to inconsistent results, probably due to limited sample size, different population substructures, and the lack of validation in an independent cohort (5, 11).

Identifying genetic variants that underlie allograft rejection is rather complex, since acute rejection is affected by the extent of alloreactivity of the patient's immune system toward the donor organ and the effect of the immunosuppression applied. The choice of candidate genes that have been studied in previous genetic studies has relied heavily on their relationship to the known pathophysiology of rejection, but such studies may not fully explain the genetic basic of allograft rejection (12). Genome-wide association studies (GWAS) represent a hypothesis free approach to identify causal genetic variants by analysing millions of SNPs scattered across genome. GWAS had been successfully applied for identification novel genes in many diseases, such as diabetes and Alzheimer's disease (13, 14). However, in the kidney transplantation field only a few studies have reported novel loci that are associated with acute rejection or long-term kidney function (15, 16). Multicenter GWAS in renal transplants led to identification of two loci, which constitute CCDC67 and PTPRO, associated with biopsy proven acute T cell mediated rejection in both a discovery and validation cohort (15). Another GWAS identified two SNPs (rs3811321 and rs6565887) associated with 5-year creatinine levels and long-term graft survival (16). However, in a larger follow-up study involving 1,638 patients the impact of those two SNPs on long-term graft function could not be confirmed (17). This indicates the importance of validation of GWAS studies and asks for multicenter collaboration in the field of transplantation to increase sample sizes.

In the present study, we applied GWAS to identify novel loci that are associated with the occurrence of biopsy proven acute rejection after kidney transplantation. We also interrogated previously reported SNPs and their possible association with renal transplant outcome in this cohort.

Materials and Methods

Patients and donors

Patients receiving a kidney transplant between 1994 and 2012 in the Leiden University Medical Center (LUMC) were investigated. Thirty-five out of 646 DNA samples were excluded due to poor quality of genotyping (QC call rate < 95%). Finally, there were 611 DNA samples, from 305 patients and 306 donors. Cases were defined as patients developing biopsy proven acute rejection (BPAR) based on Banff classification. Controls were defined as patients with stable graft function, having no indication of clinical rejection. Patients with an episode of BPAR after switching of maintenance medication or with only a clinical indication of rejection without biopsy were excluded (Figure 1).

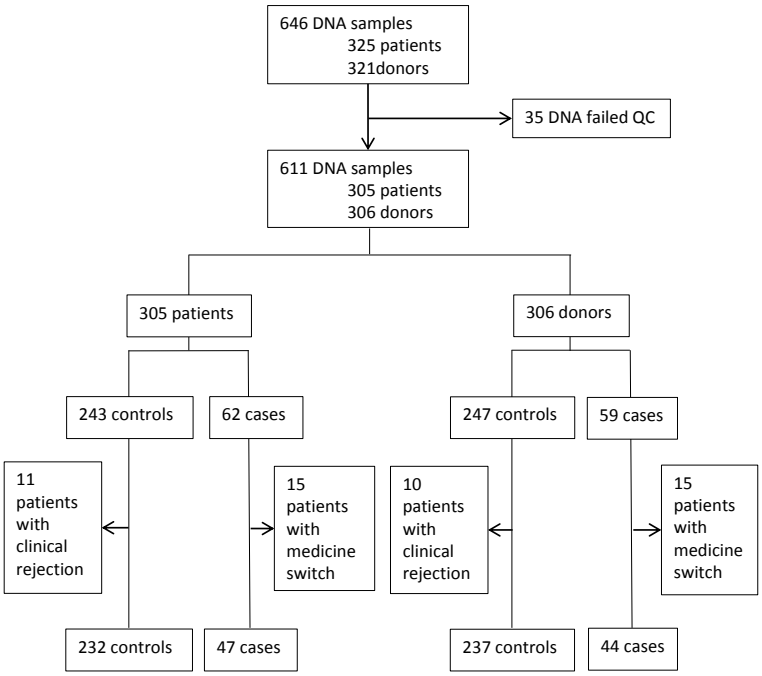

Figure 1. Flowchart of patients and donors included in discover cohort. QC: quality control.

Genotyping

Patient and donor DNA was isolated using chemagic DNA Blood2k Kit by chemagic MSM I equipment (PerkinElmer), and the quantity was measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, Asheville NC). Extracted DNA was diluted to 50 ng/µL with nuclease-free water. All DNA samples were genotyped by transplant SNP

array on the Affymetrix platform, which contain 753,182 SNPs, including fine-mapping SNPs across HLA region and drug-response associated loci (18).

Quality control and Imputation

Normal quality control was applied on raw genotype data to remove low quality samples and SNPs. Five samples with more than 5% missing genotypes were excluded. Individual SNPs with a Hardy-Weinberg equilibrium (HWE) P-value of less than 10^{-3}, monomorphic SNPs, and SNPs with a genotype call rate lower than 95% were excluded. The final dataset contained 611 samples and 592,990 SNPs for imputation.

Imputation was performed using IMPUTE2 based on a combined reference set (1,000 Genomes Project and Genome of The Netherlands, GoNL) (19). A post-imputation quality control was applied to filter out SNPs that did not meet our criteria (info-score < 0.7, HWE p-value < 10^{-3}, SNP call rate < 0.95, MAF < 0.05), which result in a total of 7,067,718 SNPs that were analyzed for association. All the imputed genotyped data were transformed into PLINK format using PLINK software, version 1.9.

Statistical analysis

Associations between clinical phenotypes (biopsy proven acute rejection) and genotypes were tested using factored spectrally transformed linear mixed models (FaST-LMM) to capture population structure, family structure, and cryptic relatedness (20). Clinical risk factors with a P-value less than 0.1 were included in the linear mixed model. The covariant included different therapeutic regimes, patient age and gender, donor type at transplant (living or deceased), and CMV primo-infection. Quantile-quantile (Q-Q) and Manhattan plots were generated using the qqman package (21). Genome-wide distribution of the test statistics showed no systematic inflation by inspecting the Q-Q plot and calculating the genomic inflation factor (λ). Suggestive association threshold ($P<5\times10^{-6}$) and genome-wide significance threshold ($P<5\times10^{-8}$) were used as correction for multiple testing.

Power calculation

The power of the GWAS, demonstrated as the relative risk versus minor allele frequency, was calculated by PGA software (22). We calculated the power using a disease prevalence of 0.15 and statistic power of 0.8, with the threshold of significance $P=5\times10^{-6}$ by default model.

Validation of candidate SNPs associated with kidney transplant outcome

A total of 67 previous published SNPs, located in genes encoding cytokines, chemokines, and innate immune response molecules (reviewed in (23)), were associated with acute rejection after kidney transplantation in the patients. For polymorphisms in the donor, 11 SNPs were reported to be significant association with allograft rejection. These candidate SNPs were validated in the present GWAS in association with BPAR. A P value of less than 0.05 was considered as significant.

Results

Patient characteristics and outcomes

A discovery cohort of 305 patients and 306 donors was genotyped by Affymetrix transplant arrays and passed the quality control. The patient and donor factors (age and gender) were not different between controls and BPAR group. Patients in the studies received different therapeutic regimes, consisting of mycophenolate mofetil (MMF) and calcineurin inhibitor (CNI), after kidney transplantation, which was a risk factor for BPAR. A deceased donor graft and CMV primo infection were risk factors for BPAR, whereas occurrence of DGF, HLA mismatching between donor and recipient, and younger recipient age did not predict occurrence of acute rejection (Table 1).

Table 1. Demographics of study cohort [1]

Variables	Controls (N=232)	Cases (N=47)	P [2]
Recipient age (IQR, year)	54.0 (43.3-60.0)	50.0 (37.0-63.0)	0.501
Recipient gender (female)	33.6%	23.4%	0.171
First transplant	95.7%	93.6%	0.539
Donor age (IQR, year)	49.0 (39.0-59.0)	51.0 (39.0-57.0)	0.893
Donor gender (female)	50.4%	59.6%	0.253
Donor type (deceased)	55.6%	72.3%	0.034*
Cold ischemia time (IQR, h)	17.3 (13.4-20.2) [a]	17.4 (12.8- 22.4) [b]	0.631
DGF (within deceased donor)	61.2% [c]	73.5% [d]	0.185
HLA-A matching	28.5%	36.2%	0.421
HLA-B matching	17. 7%	23.4%	0.322
HLA-DR matching	28.9%	19.2%	0.345
HLA-DQ matching	46.1%	40.4%	0.690
CMV primo-infection	3.9%	10.6%	0.067
Maintenance therapy			0.003*
CNI, MMF	6.5%	12.8%	
Tac, MMF	13.4%	8.5%	
CsA, MMF	43.5%	66.0%	
CNI, MMF, steroid	36.6%	12.8%	

[1] HLA, human leukocyte antigen; DGF, delayed graft function; CMV, Cytomegalovirus; MMF, mycophenolate mofetil; CsA, cyclosporine A; Tac, Tacrolimus; CNI, calcineurin inhibitor.

[2] P-values were calculated using the Mann-Whitney test, Chi-square test or Fisher exact test.

* indicates significance.

[a, b, c, d] data missing for 136, 10, 103, 13 patients

GWAS revealed three loci for BPAR in the recipient

All SNPs passing the QC were tested using the linear mixed model, which was adjusted for clinical risk factors and genetic background. The Q-Q plot showed an effective control of population structure (λ=1.07). The tail of Q-Q curve deviating from the expected distribution may indicate true association (Figure S1). Nine SNPs showed association with BPAR with a $P<5\times10^{-8}$, as shown in a Manhattan Plot (Figure 2). Seven of the nine SNPs identified (top-ranked SNP: rs112775512) are located in the intron of collagen type V alpha 1 (COL5A1). One SNP (rs3057090) is located 20 kb downstream of Dishevelled associated activator of morphogenesis 1 (DAAM1) and one SNP (rs77493583) is located in the intron of DPY19L1.

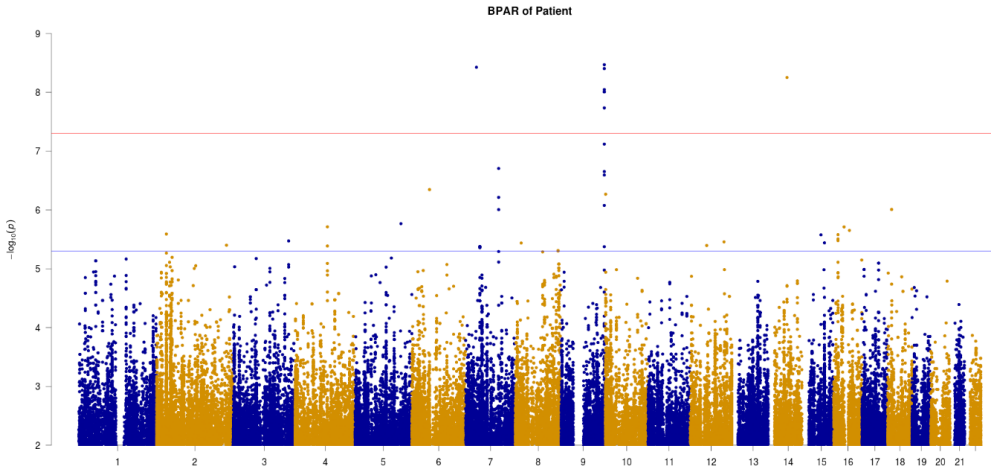

Figure 2. Manhattan plot of SNPs associated with BPAR in patients. Overall, 7 million SNPs were analyzed in relation to BPAR, with correction of clinical risk factors. The figure shows p-values (P<0.01) of each SNP against the chromosomal positions. The red line shows the threshold of genome wide significance ($P<5\times10^{-8}$) and the blue line indicates the genome wide suggestive significance ($P<5\times10^{-6}$).

Genetic variants validation in independent cohort

The validation cohort contained 243 recipients from the transplantation center in Vienna, which had been genotyped using the same Transplant arrays. A total of 44 candidate genetic variants ($P<5\times10^{-6}$) were eligible for association analysis (Table 2). Association of candidate SNPs were tested by the FaST-LMM algorithm, correcting for any significant clinical risk factors. Three SNPs showed significant association with BPAR (P<0.05), but the odds ratio were in opposite direction compared to those in the discovery cohort.

Table 2. Associations of genetic loci with BPAR in two cohorts [1].

SNP			Discovery cohort		Validation cohort	
Chr	SNP ID	Minor allele	P [2]	OR	P [2]	OR
2	rs62178588	G	3.97E-06	0.85	0.012*	1.14
2	rs74765547	TGACTGCTGAAAACAC	2.56E-06	1.24	0.582	0.97
3	rs7627382	T	3.37E-06	1.24	0.106	0.91
4	rs34790532	T	4.10E-06	1.24	0.046*	0.89
4	rs72684896	T	1.93E-06	1.25	0.041*	0.89
5	rs2116800	A	1.71E-06	1.21	0.909	1.01
6	rs76468144	T	4.50E-07	1.48	0.628	0.95
7	rs1826839	A	4.37E-06	1.17	0.301	1.05
7	rs1826840	A	4.37E-06	1.17	0.256	1.06
7	rs2331387	A	4.20E-06	1.17	0.372	1.04
7	rs2331389	T	4.20E-06	1.17	0.372	1.04
7	rs2526975	A	1.97E-07	0.85	0.694	1.02
7	rs2727762	T	6.10E-07	1.18	0.433	1.04
7	rs396600	C	9.86E-07	1.19	0.505	1.03
7	rs4724442	A	4.20E-06	1.17	0.276	1.05
7	rs77493583	C	3.75E-09	1.44	0.67	0.96
7	rs9690070	C	4.20E-06	1.17	0.372	1.04
8	rs1873654	T	3.64E-06	1.28	0.259	1.11
8	rs4909457	T	4.90E-06	1.16	0.923	1
8	rs7386038	T	4.98E-06	1.16	0.927	1
9	rs11103457	T	8.37E-07	1.22	0.886	1.01
9	rs112775512	A	3.39E-09	1.44	0.216	0.9
9	rs118029018	C	9.82E-09	1.42	0.193	0.89
9	rs12001485	A	9.82E-09	1.42	0.284	0.91
9	rs143702384	CA	3.95E-09	1.44	0.18	0.89
9	rs145688704	T	4.22E-06	1.2	0.893	1.01
9	rs372298474	G	2.22E-07	1.36	0.491	0.95
9	rs66698367	T	7.60E-08	1.36	0.223	0.9
9	rs67349136	G	1.84E-08	1.41	0.352	0.93
9	rs72772536	T	9.82E-09	1.42	0.444	0.94
9	rs72772543	C	9.00E-09	1.42	0.573	0.95
9	rs72772548	T	2.55E-07	1.4	0.395	0.92
10	rs35772020	A	5.40E-07	1.43	0.724	0.97
12	rs75575129	A	3.48E-06	1.39	0.831	0.98
12	rs79634630	A	4.01E-06	1.47	0.558	1.09
14	rs3057090	CTTGTTG	5.60E-09	1.44	0.316	1.09

15	rs11632600	T	2.65E-06	1.26	0.473	0.96
15	rs71395028	A	3.62E-06	1.37	0.31	1.13
16	16:32534119	C	1.94E-06	1.57	0.707	0.95
16	rs112139404	AC	2.22E-06	1.43	0.843	0.98
16	rs1230896	T	2.63E-06	1.18	0.666	1.02
16	rs251919	T	3.32E-06	1.18	0.658	1.02
16	rs436054	C	3.11E-06	1.18	0.62	1.02
18	rs67127738	A	9.81E-07	1.2	0.633	0.97

[1] SNP, single nucleotide polymorphism; Chr, chromosome; OR, odds ratio.

[2] P-values were calculated using FaST-LMM algorithm.

Donor GWAS for BPAR

We also performed a GWAS to study the donor genotype in relation to BPAR. The Q-Q plot showed appropriate control of population substructure, but no evidence of association with BPAR (Figure S2). Only one SNP (rs79712820) was significantly ($P<5\times10^{-8}$) associated with BPAR (Figure 3). This SNP was located in the intron of synaptopodin-2, which has actin binding and bundling activity.

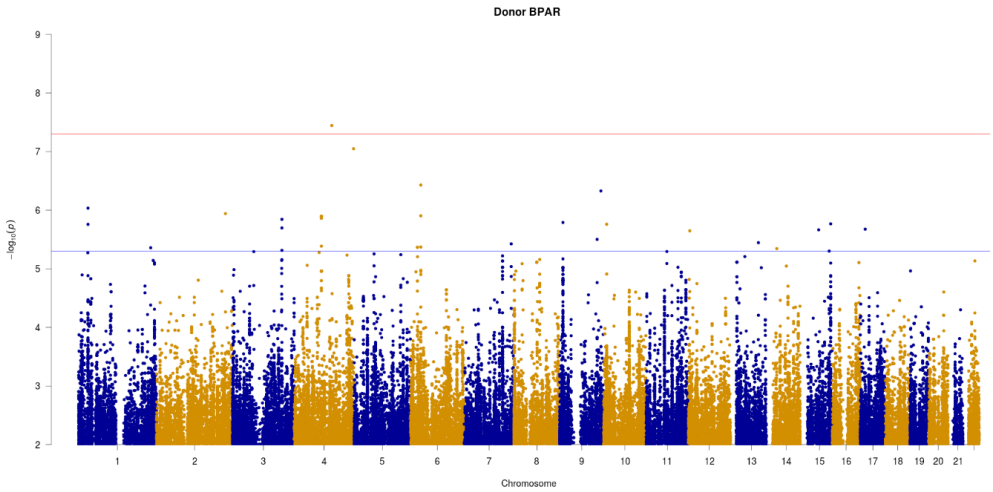

Figure 3. Manhattan plot of SNPs associated with BPAR in donors. The figure shows p-values ($P<0.01$) of each SNP against the chromosomal positions. The red line shows the threshold of genome wide significance ($P<5\times10^{-8}$) and the blue line indicates the genome wide suggestive significance ($P<5\times10^{-6}$).

Validation of previous published candidate SNPs in the current GWAS

We attempted to validate previous publications, which reported 67 SNPs in genes encoding for cytokines, chemokines, cell adhesion molecules, and innate immunity related molecules (23). In total, 59 SNPs were successfully captured in our patient GWAS dataset. Only three of them were significantly associated with BPAR (Table 3). Whereas two SNPs (rs2515641 and rs5742909) showed an opposite effect on AR compared with previous publications. One SNP (rs1801274), located in FCGR2A, was associated with BPAR (P<0.05).

Table 3. Validation of candidate SNPs that were reported in previous genetic association studies of transplant outcome [1]

SNP			Previous publication			Current GWAS		Ref
Chr	SNP ID	Gene	N	P	OR (95% CI)	P [3]	OR	
1	rs2796267	CD46	334	0.012	0.47 (0.26-0.84)	0.75	0.99	(27)
1	rs1801274	FCGR2A	99	<0.045	AR: more C allele [2]	0.002*	0.91	(28)
1	rs1800896	IL10	291	0.016	1.9 (1.1-3.1)	0.06	1.06	(7)
1	rs1800871	IL10	291	0.016	1.9 (1.1-3.1)	0.75	0.99	(7)
1	rs1800872	IL10	291	0.016	1.9 (1.1-3.1)	0.75	0.99	(7)
1	rs1801133	MTHFR	585	0.012	0.51 (0.3-0.86)	0.50	1.02	(29)
1	rs689466	PTGS2	458	0.01	0.59 (0.38-0.91)	0.44	1.03	(30)
2	rs3116496	CD28	270	0.026	1.93 (1.10-3.39)	0.87	0.99	(31)
2	rs733618	CTLA4	167	0.002	0.41 (0.24-0.72)	0.10	1.09	(32)
2	rs5742909	CTLA4	131	0.015	3.45 (1.18-10.1)	0.01*	0.84	(33)
2	rs231775	CTLA4	190	0.037	2.78 (1.07-7.19)	0.06	1.06	(34)
2	rs3087243	CTLA4	72	0.035	4.51	0.47	0.98	(35)
2	rs1143634	IL1B	100	0.045	3.11 (1.02-9.44)	0.10	0.94	(36)
2	rs7574865	STAT4	453	0.003	0.54 (0.36-0.82)	0.71	0.99	(37)
2	rs17868320	UGT1A9	100	0.07	3.62 (0.90-14.5)	0.71	0.97	(38)
2	rs6714486	UGT1A9	100	0.05	4.40 (1.05-18.4)	0.43	0.94	(38)
3	rs5186	AT1R	206	<0.05	8.34 (2.43-28.69)	0.24	0.96	(39)
3	rs1799864	CCR2	163	0.014	0.30 (0.12-0.78)	0.41	1.05	(40)
3	rs1799987	CCR5	243	0.029	2.76 (1.11-6.90)	0.07	0.95	(41)
3	rs1129055	CD86	Meta	0.02	0.35 (0.14-0.85)	0.46	1.03	(42)
3	rs11706052	IMPDH2	232	0.006	3.39 (1.42-8.09)	0.74	0.98	(10)
4	rs4073	CXCL8	296	0.032	2.7 (1.09-6.69)	0.90	1.00	(43)
4	rs2069762	IL2	63	<0.05	NA	0.69	1.01	(44)
4	rs28362491	NFKB1	292	0.001	2.61 (1.50-4.53)	0.10	1.05	(45)
4	rs7439366	UGT2B7	235	<0.046	2.5 (1.00-6.41)	0.05	0.94	(38)
5	rs181781	IL3	330	0.041	0.55 (0.31-0.98)	0.33	0.95	(46)
5	rs40401	IL3	330	0.014	2.18 (1.17-4.05)	0.09	0.94	(46)

5	rs2243250	IL4	120	0.02	NA	0.83	0.99	(47)
5	rs2910164	MIR146A	350	0.04	2.63 (1.04-6.62)	0.94	1.00	(48)
6	rs2269475	AIF	458	0.05	0.61 (0.39-0.97)	0.29	0.95	(30)
6	rs1800629	TNF	623	0.001	4.05 (1.76-9.28)	0.17	0.95	(49)
6	rs699947	VEGFA	173	0.005	4.1 (1.5-11.3)	0.41	1.02	(50)
6	rs1570360	VEGFA	173	0.001	6.8 (1.8-25.0)	0.93	1.00	(50)
7	rs2032582	ABCB1	232	0.003	3.16 (1.50-6.67)	0.26	0.96	(10)
7	rs1800795	IL6	145	0.0002	NA	0.15	0.96	(51)
7	rs2278293	IMPDH1	191	0.008	0.34 (0.15-0.76)	0.32	0.97	(52)
7	rs2278294	IMPDH1	191	0.02	0.40 (0.18-0.89)	0.62	0.98	(52)
8	rs1042032	EPHX2	259	0.015	6.34 (1.35-29.9)	0.62	0.98	(53)
9	rs4986790	TLR4	238	0.01	0.41 (0.30-0.83)	0.82	1.01	(54)
9	rs10759932	TLR4	216	0.001	0.25 (0.11-0.57)	0.77	0.99	(55)
10	rs2515641	CYP2E1	347	0.003	2.55 (1.37-4.75)	0.04*	0.91	(56)
10	rs7096206	MBL2	710	0.01	2.05 (1.16-3.64)	0.86	0.99	(57)
11	rs10765602	CCDC67	778	0.02	1.98 (1.21-3.25)	0.94	1.00	(15)
11	rs187238	IL18	226	0.015	3.65 (1.24-10.79)	0.25	1.04	(58)
12	rs2430561	INFG	118	NA	2.6 (1.6-6.0)	0.48	0.97	(59)
12	rs7976329	PTPRO	778	0.01	1.61 (0.96-2.70)	0.09	0.94	(15)
12	rs11614913	MIR196A2	350	0.027	2.86 (1.12-7.27)	0.68	1.01	(48)
14	rs696	NFKBIA	292	0.007	1.85 (1.18-2.91)	0.79	0.99	(45)
16	rs1801275	IL4R	344	0.019	0.44 (0.21-0.91)	0.11	0.94	(60)
17	rs1024611	CCL2	167	0.022	2.6 (1.12-6.01)	0.57	0.98	(61)
17	rs2107538	CCL5	261	0.035	NA	0.92	1.00	(62)
17	rs5918	ITGB3	119	0.04	2.75 (1.01-7.93)	0.95	1.00	(63)
17	rs1625895	TP53	100	0.009	0.36 (0.16-0.78)	0.25	0.95	(64)
19	rs5498	ICAM1	42	0.013	0.23	0.97	1.00	(65)
19	rs1800470	TGFB	291	0.043	1.8 (1.0–3.0)	0.19	0.96	(7)
19	rs1800471	TGFB	291	0.043	1.8 (1.0-3.0)	0.53	1.04	(7)
20	rs3746444	MIR499A	350	0.027	3.31 (1.14-9.58)	0.37	0.97	(48)
22	rs228942	IL2RB	337	0.0096	2.11 (1.19-3.74)	0.45	1.03	(66)
22	rs228953	IL2RB	337	0.029	1.58 (1.04-2.38)	0.95	1.00	(66)

[1] SNP, single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; NA, not available; CI, Confidence interval; Ref, reference.

[2] OR was not given in study.

[3] P-values in current GWAS were calculated using FaST-LMM algorithm.

From our donor GWAS dataset, a total of 11 previously published SNPs associated with allograft rejection could be successfully captured (Table 4). However, none of these variants was significantly associated with BPAR in the current study.

Table 4. Validation of candidate SNPs that were reported in previous genetic association studies of the transplant donor with outcome [1]

SNP			Previous publications			Current GWAS		Ref
Chr	SNP ID	Gene	N	P	OR (95% CI)	P [2]	OR	
3	rs1799987	CCR5	239	0.029	NA	0.61	1.02	(67)
6	rs1570360	VEGF	173	0.001	2.2 (1.4-3.7)	0.60	1.02	(50)
6	rs1800629	TNFa	120	0.0395	1.4	0.36	1.04	(68)
6	rs699947	VEGF	173	0.005	1.9 (1.2-3.0)	0.66	1.01	(50)
7	rs2069840	IL-6	145	0.0002	8.67	0.27	0.96	(51)
8	rs1042032	EPHX2	259	0.042	5.53 (1.10–27.80)	0.79	0.99	(53)
9	rs4986790	TLR4	122	0.02	NA	0.96	1.00	(69)
9	rs4986791	TLR4	122	0.02	NA	0.51	1.04	(69)
9	rs7851696	Ficolin-2	270	0.048	1.71 (1.02-2.87)	0.66	0.98	(70)
10	rs1800682	FAS	105	0.043	3.27 (1.04-10.32)	0.64	1.01	(71)
10	rs1801157	SDF1	335	0.006	0.39 (0.21–0.76)	0.34	1.04	(72)

[1] SNP, single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; NA, not available; CI, Confidence interval; Ref, reference.

[2] P-values in current GWAS were calculated using FaST-LMM algorithm.

Discussion

We performed a GWAS to investigate genetic variants associated with biopsy proven acute rejection (BPAR) in kidney transplantation. Several SNPs were identified in our discovery GWAS cohort to be associated to BPAR, but unfortunately none of these could be verified in an independent cohort. We could confirm in our cohort the association of rs1801274 in FCGR2 with BPAR, which has been described in a previous genetic association study.

In the transplant recipients, nine SNPs reached genome wide significant level, of which seven are located in the intron of COL5A1 at chromosome 9, and two are lone SNPs located on chromosome 7 and 14. These multiple continuous SNPs were more likely assumed to be true risk variants for BPAR than the lone SNP because of linkage disequilibrium. Although the two lone SNPs were significantly associated with BPAR with high imputation certainty (info>0.9), this finding requires further replication. In an independent validation cohort, the nine candidate SNPs showed no significant association with BPAR. Also, the SNPs

showing association to BPAR with a suggestive P value ($P<5\times10^{6}$) could not be verified in the replication cohort.

Although one SNP reached the significant level in donor GWAS, it did not show large deviation from expected distribution in the Q-Q plot. In addition, the relative small risk effect suggests that this SNP was found by chance. These results suggest that donor polymorphisms confer no big effect on the risk of acute rejection.

The main reason, that findings in the discovery cohort could not be verified in the validation cohort, may be the relatively small sample size and limited power to detect any small effects on outcome of single SNPs. Power calculation showed that SNPs (MAF=0.2) with a relative risk of more than 2.8 could be sufficiently detected (power=0.8) at the genome wide suggestive significance level (Figure 4). However, our results showed that the relative risk calculated by linear mixed model is less than two, indicating that the candidate SNPs may have been identified by chance. In other words, our study is only able to sufficiently detect SNPs (MAF>0.2) that have a relatively big effect on BPAR (RR>2.8). The validation cohort with comparable sample size had modest power to detect variants at the conventional level (P<0.05).

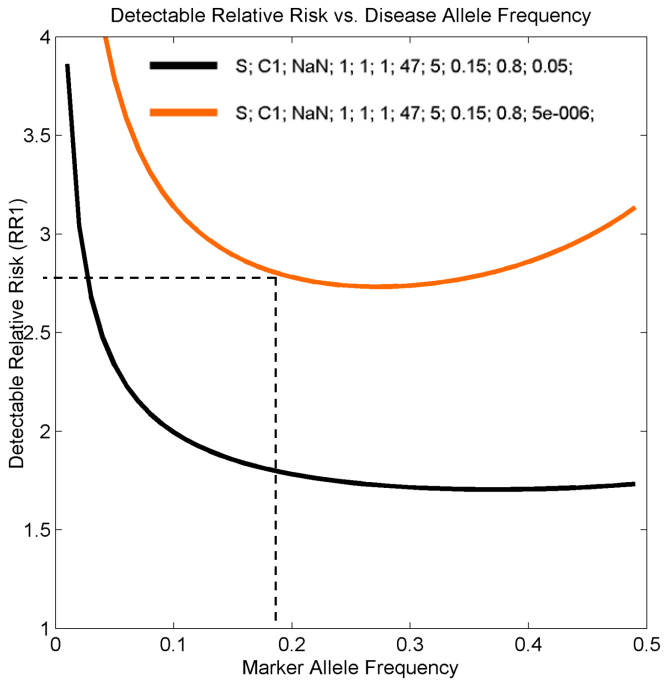

Figure 4. Power of the study. The plot shows relative risk versus minor allele frequency. The power was calculated using a disease prevalence of 0.15 and a statistic power of 0.8 by default model. The orange line and black line show the threshold of suggestive significance ($P=5\times10^{-6}$) and the unadjusted significance (P=0.05), respectively.

Apart from any novel loci, risk loci that have previously been discovered in genetic risk studies were also evaluated in the current GWAS. Variant rs7976329, located in the intron of *PTPRO* and rs10765602 located in the upstream of *CCDC67*, confer a modest effect on BPAR (RR<2) (15). Our study had sufficient power to detect these variants at the conventional significance level (P<0.05). However, these two risk variants showed no association with acute rejection in our study cohort, which suggests that variation between transplant centers may hamper validation in genomic studies. Similarly, two variants (rs3811321 and rs6565887) associated with long-term kidney function in 326 renal transplant recipients could not be confirmed in an independent study on 1,638 patients (16, 17).

Failed validation is quite common in clinical studies especially in the field of transplantation. A well-powered GWAS in blood or marrow transplantation (BMT) demonstrated that a substantial amount of candidate SNPs identified by previous publications showed falsely positive association with survival outcomes (24). Similarly, our current GWAS failed to validate 69 out of 70 genetic variants previously reported to be associated with acute rejection. Only rs1801274 (FCGR2A) could be confirmed at the conventional significance level, whereby the recipients with acute rejection in both studies have higher C allele frequency. Our results highlight the importance of validating SNPs identified by candidate approaches or unbiased genome wide associations, and they demand large collaborative studies of the genetic effect on kidney transplant outcome.

As Stegall and colleagues have mentioned, complex outcomes such as graft loss or acute rejection may have numerous causes, and are unlikely to be fully related to variants in one gene (25). BPAR is associated with the donor organ quality, HLA matching status, and immunosuppressive therapy. Therefore, any effect of genetic variants on acute rejection may be counterbalanced by other potential factors, and thus they may be hard to capture in genetic association studies. At the other hand, the less complex the outcome, the more likely it is to discover robust gene associations. For example, the DeKAF consortium identified by GWAS in African American kidney transplant recipients two additional CYP3A5 alleles that are associated with tacrolimus trough blood concentrations (26). Indeed, the association between pharmacogenetic polymorphisms and tacrolimus concentrations has been widely validated in many studies (23).

In conclusion, we identified several SNPs in the patient and the donor by GWAS, which were associated with occurrence of acute rejection. These could unfortunately not be confirmed in an independent cohort. International collaborative studies are highly recommended to obtain adequate power and overcome any falsely positive findings. Of the SNPs that have previously been described to predict transplant outcome, we could confirm association of rs1801274 in FCGR2 with occurrence of acute rejection. Further studies are needed to establish the function of this molecule in transplantation.

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Supplementary Data

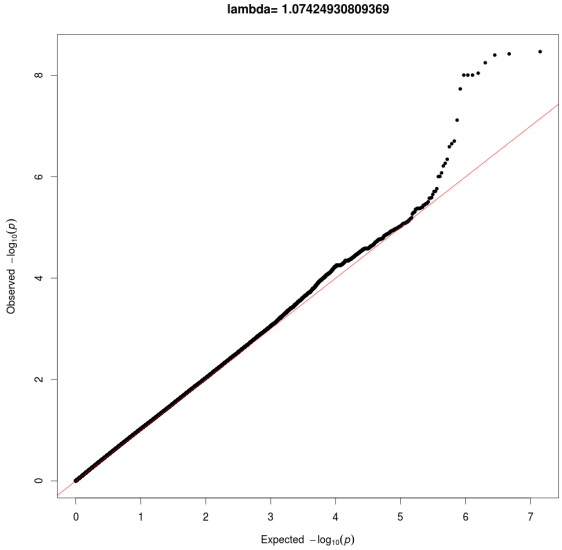

Figure S1. QQ plot for the patient's genetic variants in relation to BPAR. Figure showed an effective control of population structure (λ=1.07). The extreme observed P values may suggest association.

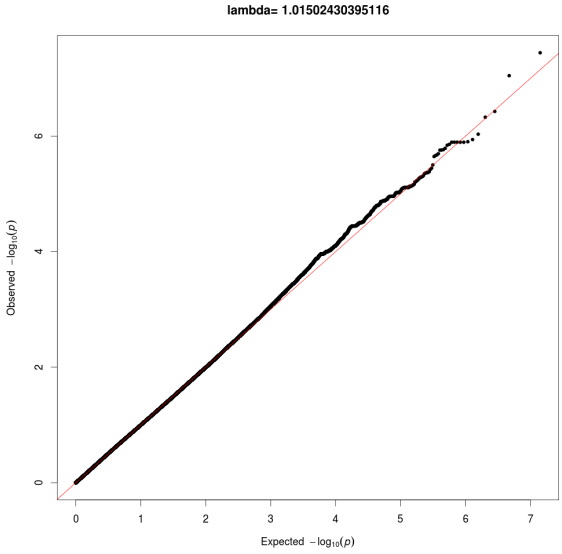

Figure S2. QQ plot for the donor's genetic variants in relation to BPAR. The figure shows the appropriate control of population structure (λ=1.02). The observed P values fall along a straight red line indicates that there is no true association with BPAR.