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Molecular and genetic markers for the prediction of kidney transplant outcome

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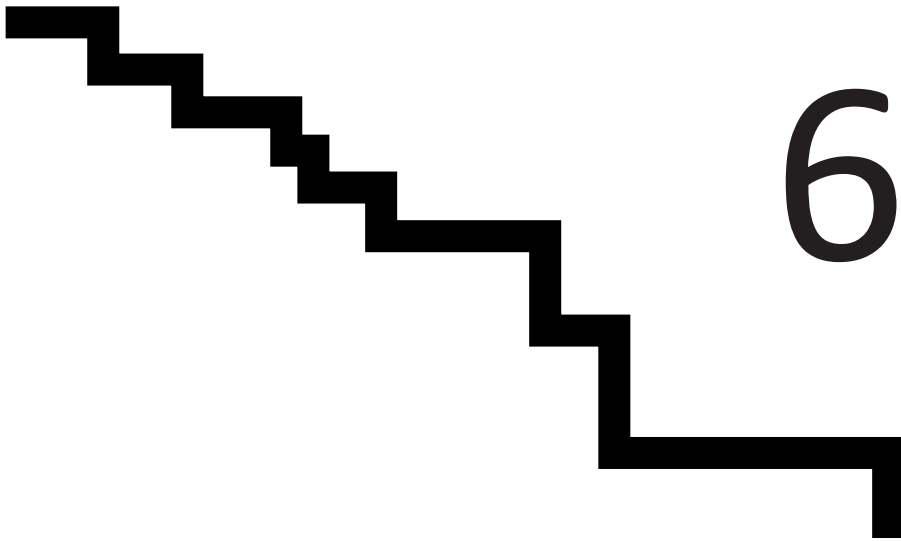
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**The degree of genomic missense SNP mismatching
does not affect outcome after
kidney transplantation**



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Abstract

Background: Human leukocyte antigens (HLA) mismatching has an adverse effect on kidney transplant outcome. But even in fully HLA-compatible donor-recipient combinations graft loss occurs. We suspect that single nucleotide polymorphisms (SNPs) other than those in the HLA genes also play a role. Therefore, we investigated the effect of mismatching of genomic missense SNPs on occurrence of acute kidney allograft rejection and on two-year graft function.

Method: We performed a genome-wide association study to identify the association of mismatching of single missense polymorphisms between donor and recipient with transplant outcome. A total of 300 donor-recipient combinations were genotyped on the transplant SNP array for wide coverage of coding SNPs, insertion-deletion regions, loss of function variants. We pre-selected 10,800 missense SNPs (that cause an amino acid sequence alteration) and examined whether the mismatch of these SNPs is related to biopsy proven acute rejection (BPAR, either yes or no) and two-year estimated glomerular filtration rate (eGFR; either >40 mL/min or ≤40 mL/min). Furthermore, a composite mismatch load score of all the missense SNPs was calculated and related to outcome.

Result: Mismatching of individual missense SNPs and the overall missense SNP mismatch load was not related to BPAR incidence and to graft function at two years.

Conclusion: The results suggested that missense SNPs mismatching does not have a strong effect on acute rejection and graft function in kidney transplantation.

Introduction

Acute allograft rejection remains a risk factor for adverse kidney transplant outcome (1, 2). Human leukocyte antigen (HLA) mismatching is considered as an immunological risk factor for acute rejection. Activated T cells against the incompatible donor HLA antigen secrete cytokines that drive an inflammatory cell infiltrate in the graft and that initiate cellular rejection (3, 4). Humoral alloimmune responses, characterized by the presence of donor specific HLA alloantibodies and complement activation, can lead to destruction of the donor organ (5, 6). Therefore, full HLA matching has a beneficial effect on long term graft survival, both after living and deceased donor transplantation (7, 8). However, allograft rejection and progressive graft loss do occur even in HLA matched transplants (9, 10).

The antigens responsible for rejection and graft loss in HLA identical transplantations are considered as minor histocompatibility antigens (miHA) (11, 12). The miHA are polymorphic peptides that typically arise from single-nucleotide polymorphisms (SNPs) and cause alterations in the amino acid sequence. Alloreactive T cells can recognize these miHA presented by the HLA molecules and subsequently initiate an immune response. Human H-Y antigen as miHA, a well-studied risk factor of graft-versus-host disease (GvHD) in hematopoietic stem-cell transplantation (HSCT), was associated with elevated risk of graft loss after kidney transplantation (9). Disparities between donor and recipient for HLA restricted miHA, which could elicit the GVHD in HLA identical HSCT, have no effect on death censored graft survival in kidney transplantation (13). As identified by a genome-wide association study (GWAS), a mismatch in SNP rs17473423 between donor and recipient was associated with acute GVHD development (12). However, the GWAS-identified locus did not predict amino acid alterations, and the precise pathophysiologic mechanism remains to be determined.

In HLA mismatched transplantations, antigen-presenting cells from the recipient can process and present exogenous antigens, including donor-derived HLA and miHA, to CD4⁺ T helper cells, and initiate graft rejection (14). Similarly, CD8⁺ T cells of the recipient can recognize donor derived peptides, including a SNP not present in the recipient, as a miHA presented by a matched HLA class I antigen on the donor organ. The effect of miHA at the genomic level has not been assessed in the field of kidney transplantation.

In the current study, we applied GWAS analysis on 300 kidney transplantations, attempting to identify mismatching of genomic missense SNPs between the donor and the recipient, and test the relevance in relation to acute rejection and allograft function.

Materials and Methods

Patients and donors

Patients receiving a renal allograft between 1994 and 2012 at the Leiden University Medical Center (LUMC) were investigated (N=325). A total of 644 DNA samples were investigated (325 patients and 319 donors) comprehending 325 transplant cases, whereby six donors donated two kidneys. Twenty-five transplant cases were excluded because of poor quality of DNA (quality control call rate in the GWAS < 95%).

The case group was defined as having at least one biopsy proven acute rejection (BPAR) episode. Controls were defined as patients having stable graft function without any indication of clinical rejection. Twenty-six transplant cases were excluded since they had an episode of BPAR after switching of maintenance medication or they had clinical indication of rejection with no evidence of BPAR (Figure 1). A total of 232 controls and 42 cases with BPAR were analyzed in the database.

The Modification of Diet in Renal Disease (MDRD) was used to estimate glomerular filtration rate (GFR). In this study, eGFR at 2 years below 40 mL/min/1.73m² was used as the cutoff for inferior graft function, since it significantly predicts inferior long term graft survival (Figure S1). A total of 70 patients with inferior graft function and 214 patients with normal graft function were analyzed in the current study.

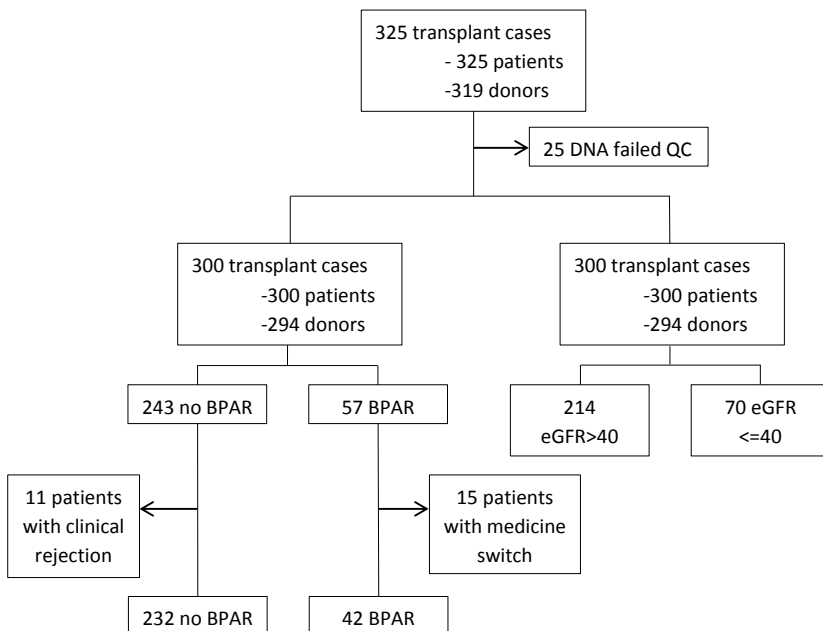


Figure 1: Flow chart of transplant cases included. QC, quality control; BPAR, biopsy proven acute rejection; eGFR, estimated glomerular filtration rate.

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). DNA samples were diluted to 50 ng/ μ l with nuclease-free water. DNA samples were genotyped by transplant SNP array on the Affymetrix platform, which contains 753182 SNPs specially including transplant specific content and function variants module.

Quality control

Genotyping data quality control was performed by Axiom analysis suite based on the 621 individuals (quality control call rate \geq 95%). A total of 152,414 SNPs associated with missense SNP or splice site of gene were extracted. SNPs were filtered out using following parameters: SNPs with less than 95% of individuals successfully genotyped; SNPs with a minor allele frequency (MAF) lower than 5%; SNPs significantly ($P < 0.001$) deviating from Hardy-Weinberg equilibrium; marker alleles poorly clustered (Fisher Linear Discriminant < 3.6); SNPs without reference SNP identifier (ID); SNPs with missing value in more than 5% of transplant cases. This eventually resulted in 10,800 SNPs for analysis.

Mismatching definition of genomic missense SNP

In the current study, mismatching was considered only when recipient had a homozygous genotype and the donor was either heterozygous or homozygous for the other allele (Table S1). A composite score was calculated, reflecting the total amount of mismatching for the missense SNPs between donor and recipient, termed as mismatch load. The transplant cases were divided into three groups based on the mismatch load, based on tertiles (high, intermediate, low).

Statistical analysis

Association between genomic missense SNP mismatching and BPAR or inferior graft function (eGFR \leq 40) were tested using logistic regression corrected for the potential clinical risk factors, including donor age, gender, donor type (DCD, DBD, LRD, LURD), patient age, gender, transplantation number (first transplant), immunosuppressive regimens, HLA -A, -B, -DR matching and primary cytomegalovirus infection using R (3.4.0) package. The Bonferroni method was used to correct for multiple comparisons. The association between mismatch load of missense SNP and BPAR or inferior graft function were analyzed by Pearson chi-squared test. BPAR-free rate curves were created using the Kaplan-Meier method.

Table 1. Demographics of study cohort¹

Variables	no BPAR (N=232)	BPAR (N=42)	P	eGFR<=40 (N=70)	eGFR>40 (N=214)	P
Recipient age (year) ¹	54 (43.25-60)	50 (36.75-62.25)	0.474	56.5 (44-65)	53 (42-59)	0.019*
Recipient gender (% female)	34.1%	23.8%	0.192	35.7%	30.8%	0.448
First transplant (%)	95.70%	92.9%	0.428	92.86%	95.3%	0.537
Donor age (year) ¹	49 (39-59)	51 (38.75-57.75)	0.829	57.5 (49-66.25)	47.5 (37-57)	<0.001*
Donor gender (% female)	50.4%	64.3%	0.098	61.7%	51.0%	0.146
Donor type (%)			0.049*			0.005*
DCD	32.3%	54.8%		50.0%	29.4%	
DBD	23.3%	14.3%		18.6%	24.3%	
LURD	22.4%	16.7%		22.9%	23.4%	
LRD	22.0%	14.3%		8.6%	22.9%	
Cold ischemic time (h) ¹	17 (13.32-20.18) ^a	16.25 (12.3-21.33) ^b	0.844	17 (13.15-21.2) ^e	17 (13.3-20.24) ^f	0.938
DGF (% within deceased donor)	60.5% ^c	72.4% ^d	0.229	79.2% ^g	52.12% ^h	0.001*
HLA-A mismatching (0/1/2)	67/115/50	15/21/6	0.478	20/35/15	65/108/41	0.902
HLA-B mismatching (0/1/2)	42/143/47	9/20/13	0.198	13/37/21	43/130/41	0.247
HLA-DR mismatching (0/1/2)	68/139/25	8/29/5	0.391	16/41/13	63/135/16	0.025*
Primary cytomegalovirus infection	3.90%	9.5%	0.120	10.00%	3.7%	0.042*
Immunotherapy (%)			<0.001*			<0.01*
MMF, CsA	47.8%	85.7%		70.0%	49.1%	
MMF, Tac	16.8%	11.9%		8.6%	16.4%	
MMF, CNI, steroid	35.3%	2.4%		21.4%	34.6%	

HLA, human leukocyte antigen; DGF, delayed graft function; DCD, donor after cardiac death; DBD, donor after brain death; LURD, Living-unrelated donor; LRD, Living-related donor; MMF, mycophenolate mofetil; CsA, cyclosporine A; Tac, Tacrolimus; CNI, calcineurin inhibitor.

^{a, b, c, d, e, f, g, h} data missing for 95^a, 10^b, 103^c, 13^d, 19^e, 91^f, 22^g, 99^h transplant cases.

¹ numbers reflect interquartile ranges.

*P values were calculated using the Mann-Whitney test, Chi-square test or Fisher's exact test.

Results

Patient characteristics and outcomes

A total of 274 Kidney transplant cases passed the quality control for BPAR analysis. Patient- and donor-related variables (age and gender) were not different between controls and BPAR group (Table 1). The donor type and immunosuppressive regime after kidney transplantation did differ ($P<0.05$). The DCD donor were more frequently in BPAR group and inferior graft function group. The patients received MMF and CsA had a higher frequency in BPAR group and inferior graft function group. DGF rate (only in deceased donors), HLA mismatch, and younger age of the recipient did not predict the episode of acute rejection.

A total of 284 transplant cases were included for inferior graft function analysis. The donor and recipient age were higher in inferior graft function group than superior graft function group. The donor type and immunosuppressive regime significantly differed between the two groups. DGF rate and HLA-DR mismatching predict inferior graft function at two years (Table 1).

Missense SNP mismatching association test for BPAR or eGFR progression

After quality control, 10,800 genomic missense variants were tested for association with BPAR using logistic regression corrected for clinical factors. Of these, 584 showed an association with BPAR, with a $P<0.05$ (Table S2). After correction for multiple comparisons, none of these variants by themselves were significantly associated with BPAR. Similarly, a total of 596 missense SNPs showed association with low eGFR at $P<0.05$ (Table S3), whereas none of these SNPs were significantly associated after correction for multiple comparisons. Lack of significant association suggests that the effect of mismatching of single genomic mutations is not strong enough to be detected in this cohort.

Quantitative analysis of genomic missense SNP mismatching

A composite score, reflecting the total amount of mismatching for the missense variants between donor and recipient, was calculated. This score is termed as the mismatch load. As expected, the living related donor (LRD)-recipient combinations had a significantly lower number of genomic mismatching compared to unrelated donor-recipient combinations (Figure 2). The frequency of acute rejection episodes in the LRD group was the same as in the living unrelated donor (LURD) and donor after brain death (DBD) groups (Table 1).

The transplant cases were assigned to one of three groups (tertiles) based on the degree of mismatching with their respective donors. One-third of transplant cases showing the highest degree of mismatching were designated as the 'more mismatching' group. Along the same line, the other transplant cases fell in the 'intermediate mismatching' or 'less mismatching' group (Figure 2). The mismatch load of genomic missense SNPs was not associated with the occurrence of biopsy proven acute rejection and inferior graft function ($eGFR\leq 40$) in either all groups together or with the LRD group excluded (Table 2).

Missense mutation Mismatching

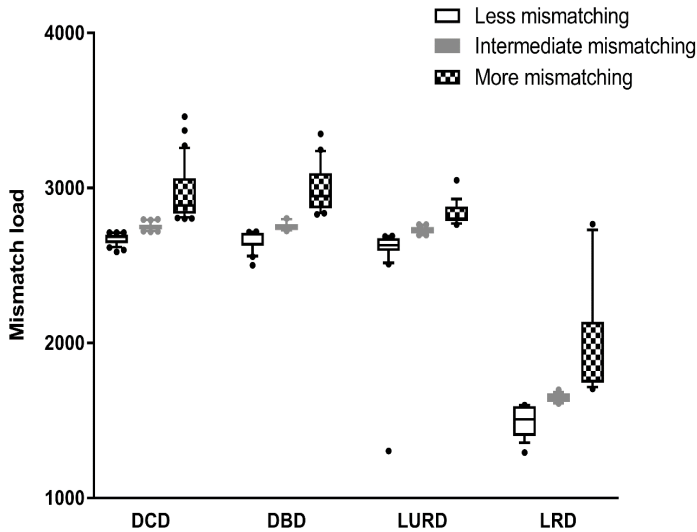


Figure 2: Mismatch load in different donor recipient combinations. Transplant cases were assigned to one of three groups (tertiles) based on the mismatch load: 'more mismatching' group with highest amount of mismatching; 'intermediate mismatching' group with medium amount of mismatching, and 'less mismatching' group with lowest amount of mismatching. Mismatch load of LRD was smaller than in the other donor-

recipient combination groups. Box and whisker plots show medians with 10-90 percentile. LRD, living related donors; LURD, living unrelated donors; DBD, donors after brain death; DCD, donors after cardiac death.

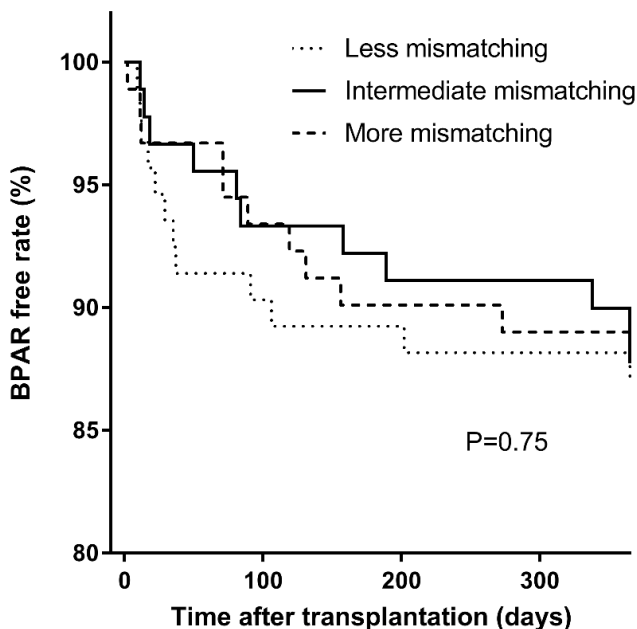
Table 2. Association of genomic mismatch load with acute rejection

Groups	no BPAR (N)	BPAR (N)	P	eGFR≤40(N)	eGFR>40(N)	P
LRD			0.86			0.68
Less mismatching	16	3		3	15	
Intermediate mismatching	17	2		2	17	
More mismatching	18	1		1	17	
DCD,DBD,LURD			0.93			0.67
Less mismatching	61	13		22	54	
Intermediate mismatching	59	12		23	52	
More mismatching	61	11		19	59	
Total cases			0.75			0.57
Less mismatching	77	16		25	69	
Intermediate mismatching	76	14		25	69	
More mismatching	79	12		20	76	

DCD, donor after cardiac death; DBD, donor after brain death; LURD, Living-unrelated donor; LRD, Living-related donor

P values were calculated using Chi-square test or Fisher's Exact Test.

Association of the mismatch load with BPAR-free rate (time to BPAR) at 1 year after transplantation was tested. There was no association between mismatch load and 1 year BPAR-free rate (Figure 3).



Less mismatching	93	85	84	82
Intermediate mismatching	90	85	83	83
More mismatching	91	86	83	82

Figure 3: Association between the mismatching load of missense SNPs and BPAR-free rate. The mismatching load of missense SNPs showed no association to BPAR-free rate.

Association of the analysis of mismatch load under HLA restriction

In fully HLA compatible transplantations, acute rejection may be initiated by miHA differences. In the current study, a series of associations were performed under particular HLA matching conditions. No association was seen between mismatch load and BPAR or inferior graft function in any of the HLA matching subset groups. In full HLA-A-B-DR matching conditions, only two patients had acute rejection, and none of them fell in the ‘more mismatching’ group. Although the number of transplant cases under HLA matching is limited, the mismatch load did not show a clear association trend with acute allograft rejection or allograft function (Table 3).

Table 3. Association of genomic mismatch load with acute rejection under HLA matching restriction

Groups	no BPAR (N)	BPAR (N)	P	eGFR≤40(N)	eGFR>40(N)	P
HLA-A matching			0.52			0.48
Less mismatching	24	8		8	21	
Intermediate mismatching	20	3		7	18	
More mismatching	23	4		5	26	
HLA-B matching			0.62			0.56
Less mismatching	16	5		7	16	
Intermediate mismatching	19	3		4	17	
More mismatching	7	1		2	10	
HLA-DR matching			0.18			0.29
Less mismatching	23	2		5	20	
Intermediate mismatching	28	6		9	24	
More mismatching	17	0		2	19	
HLA-DQ			0.5			0.18
Less mismatching	37	6		10	36	
Intermediate mismatching	36	7		14	29	
More mismatching	36	3		6	33	
HLA-A+B matching			0.71			1
Less mismatching	11	4		4	12	
Intermediate mismatching	9	2		2	9	
More mismatching	5	0		1	6	
HLA-A+B+DR matching			1			1
Less mismatching	9	1		2	10	
Intermediate mismatching	7	1		1	6	
More mismatching	2	0		1	3	

P values were calculated using Chi-square test or Fisher's Exact Test.

Discussion

Acute allograft rejection remains a complication that negatively affects kidney transplant outcome. Although HLA represents a major contributor in the immune response, non-HLA immunity may also contribute to allograft rejection (10, 15). We investigated the possible clinical relevance of the miHA mismatches, characterized as genomic missense SNPs, in kidney transplantation.

Originally, the miHA were identified using cytotoxic T lymphocytes isolated from the recipient with GVHD after HLA identical bone marrow transplantation (11, 13, 16-18). For allorecognition by CD8⁺T cell, the miHA must be presented by those donor HLA antigen, which are shared with the recipient, a phenomenon, which is called HLA restriction (11, 19). The miHA may also be processed and exogenously presented by recipient derived antigen presenting cells to recipient CD4⁺T cells, irrespective of the degree of HLA matching between donor and recipient. Therefore, we tested the possible association between mismatched SNPs between donor and recipient with biopsy proven acute rejection under the hypothesis of no HLA restriction.

Genome-wide association studies are a powerful means to identify causal genetic variants associated with phenotype, by analysing millions of SNPs scattered across the genome (14). Several GWAS have been applied to kidney transplantation, leading to identification of novel SNPs in the recipient associated with transplant outcome (20, 21). However, none of the studies investigated the effect of genetic mismatching between the donor and recipient. In HSCT, a large GWAS identifying allele mismatches with acute GVHD were performed in 1,589 unrelated bone marrow transplants matched for HLA-A, -B, -C, -DRB1, and -DQ1 loci (12). Three discrete positive loci, associated with varying grades of GVHD, were identified under HLA restriction, but none of the SNPs predicted amino acid sequence alterations. The successfully detected loci proved that GWAS can capture the risk allele mismatches relevant with clinical outcome. In order to avoid capturing silent SNPs, we pre-selected the SNPs involving amino acid substitution of encoded proteins, which are considered to generate polymorphic epitopes. Therefore, we performed the genotyping of DNA using transplant specific arrays containing 753K SNPs, of which 152K SNPs related with missense mutation. After strictly filtering, a total of 10,800 SNPs were tested for association with outcome, under the assumption of no HLA restriction. Unfortunately, none of the mismatched missense SNPs could predict BPAR or inferior graft function after multiple corrections, which suggest that the effect of genomic mismatching is not strong enough to detect in the relatively low number of transplants studied.

A higher degree of HLA mismatching leads to an inferior graft survival than HLA matched transplants (7). Therefore, we hypothesized that more mismatching of missense SNPs between donor and recipient leads to a higher chance of rejection. The LRD group indeed showed fewer mismatches, but the frequency of acute rejection was not difference than that of other donor types. Even in subgroup analyses, which divided transplant cases into three group based on mismatch load, the degree of mismatching of genomic variants did not predict BPAR incidence, BPAR-free rate or a poorer graft function. Also when HLA restriction was taken into consideration, the incidence of acute rejection or inferior graft function was not higher in the more mismatched group compared to that in the less mismatched group.

The miHA identified in HSCT studies associated with GVHD were mainly targets for CD8⁺ T cells and recognized in the context of matched HLA class I antigens. Such mismatches did not show any correlation with kidney transplantation outcome (13). In the current study, we found no association between missense SNPs in the genome with BPAR or inferior graft function in kidney transplants. A reasons for our negative finding may be the fact that the additional effect of mismatching of genomic missense SNPs under the condition of HLA mismatching is very low, taking into consideration the efficient immunosuppressive medication given to the patients. The lack of a significant association between allele mismatching and transplant outcome in our study may also be due to the limited number of transplant cases studied. Therefore, our finding needs to be confirmed in an independent, larger-sized cohort.

In conclusion, we found no effect of genomic missense SNP mismatching on biopsy proven acute rejection and inferior graft function in kidney transplantation.

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Supplementary Data

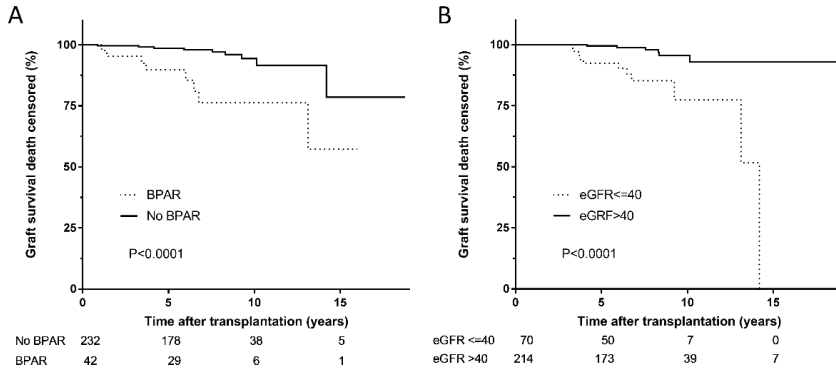


Figure S1: Association of kidney graft survival with the BPAR or eGFR at two year. The BPAR group and progression group with eGFR below 40 mL/min/1.73m² significantly predict the long term graft outcome.

Table S1. Mismatching definition of genomic missense SNP

Recipient	Donor	Risk
AA	AA	0
AA	AB	1
AA	BB	1
AB	AA	0
AB	AB	0
AB	BB	0
BB	AA	1
BB	AB	1
BB	BB	0

Table S2: Top 20 SNPs significantly associated with biopsy acute rejection without multiple correction.

SNP ID	beta	se	z	P	Odds ratio
rs246105	1.4454	0.4112	3.5154	0.000439	4.243549
rs2234970	-1.6544	0.4734	-3.4945	0.000475	0.191207
rs3877899	1.4996	0.4336	3.4588	0.000543	4.479897
rs2229357	-2.0822	0.6125	-3.3994	0.000675	0.124656
rs2007887	1.5177	0.4476	3.3909	0.000697	4.561721
rs17822931	1.5796	0.4682	3.3738	0.000741	4.853014
rs2305780	1.32	0.4167	3.168	0.001535	3.743421
rs11567842	1.3134	0.4196	3.1303	0.001746	3.718796
rs80131293	1.6839	0.5418	3.1081	0.001883	5.386522
rs4503285	-1.7812	0.5741	-3.1025	0.001919	0.168436
rs2259633	-1.9761	0.6404	-3.0856	0.002031	0.138609
rs2394516	-1.5223	0.4962	-3.0677	0.002157	0.218209
rs7148147	1.4098	0.4648	3.0331	0.002421	4.095136
rs9350797	-1.5592	0.5188	-3.0055	0.002651	0.210304
rs3750266	-1.3735	0.4613	-2.9772	0.002909	0.253219
rs10768450	-1.512	0.5084	-2.974	0.002939	0.220469
rs1191778	-1.5299	0.5177	-2.9551	0.003125	0.216557
rs74643365	1.8219	0.6173	2.9514	0.003163	6.183596
rs628524	-1.7013	0.5777	-2.9449	0.00323	0.182446

Table S3: Top 20 SNPs significantly associated with inferior graft function without multiple correction.

SNP ID	beta	se	z	P	Odds ratio
rs34666677	1.7563	0.4443	3.9527	7.73E-05	5.790971
rs16896629	2.2355	0.6027	3.7092	0.000208	9.351156
rs1233387	-1.472	0.4223	-3.4859	0.000491	0.229466
rs3736228	1.2772	0.3869	3.3009	0.000964	3.586583
rs2071950	-1.317	0.4022	-3.2742	0.00106	0.267938
rs11984293	-1.6732	0.512	-3.2679	0.001083	0.187646
rs11155242	1.2122	0.3776	3.2106	0.001325	3.36087
rs10798035	1.1133	0.3481	3.1985	0.001381	3.044388
rs1131600	-1.5637	0.4906	-3.1876	0.001435	0.20936
rs7426114	-1.3851	0.4363	-3.1745	0.001501	0.250299
rs7614116	-1.2953	0.4084	-3.1714	0.001517	0.273816
rs62399429	-1.6219	0.5134	-3.1593	0.001581	0.197523
rs4745571	1.175	0.3739	3.1429	0.001673	3.238143
rs4680	-1.2072	0.3845	-3.1395	0.001692	0.299033
rs41287373	-1.339	0.4271	-3.135	0.001718	0.262108
rs2229362	1.1277	0.3629	3.1079	0.001884	3.088545
rs2257295	-1.2376	0.4033	-3.0682	0.002153	0.29008
rs45535039	1.0662	0.3491	3.0546	0.002253	2.904322
rs17535963	-1.2623	0.4149	-3.0422	0.002349	0.283002