

1-1-2017

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Recommended Citation

Goyal, Rakesh K.; Lee, S J.; Wang, T; Trucco, M; Haagenson, M; Spellman, S R.; Verneris, M; and Ferrell, R E., "Novel HLA-DP region susceptibility loci associated with severe acute GvHD." (2017). *Manuscripts, Articles, Book Chapters and Other Papers*. 503. <https://scholarlyexchange.childrensmercy.org/papers/503>

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Published in final edited form as:

Bone Marrow Transplant. 2017 January ; 52(1): 95–100. doi:10.1038/bmt.2016.210.

Novel HLA-DP Region Susceptibility Loci Associated with Severe Acute Graft-Versus-Host-Disease

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Abstract

Despite HLA allele matching, significant acute graft-versus-host-disease (GVHD) remains a major barrier to successful unrelated donor BMT. We conducted a genome-wide association study (GWAS) to identify recipient and donor genes associated with risk of acute GVHD. A case-control design (grade III-IV versus no acute GVHD) and pooled GWA approach was used to study European-American recipients with hematological malignancies who received myeloablative conditioning non-T-cell depleted first transplantation from HLA-A, -B, -C, -DRB1, -DQ allele level (10/10) matched unrelated donors. DNA samples were divided into three pools and tested in triplicate using the Affymetrix Genome-wide SNP Array 6.0. We identified three novel susceptibility loci in the HLA-DP region of recipient genomes that were associated with III-IV acute GVHD (rs9277378, $p=1.58E-09$; rs9277542, $p=1.54E-06$ and rs9277341, $p=7.71E-05$). Of these three SNPs, rs9277378 and rs9277542 are located in non-coding regions of the HLA-DPB1 gene and the two are in strong linkage disequilibrium with two other published SNPs associated with acute GVHD, rs2281389 and rs9277535. Eighteen other recipient SNPs and 3 donor SNPs

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CONFLICT OF INTEREST

The authors declare no conflict of interest

with a high level of significance (E-07 or lower) were found. Our report contributes to emerging data showing clinical significance of the HLA-DP region genetic markers beyond structural matching of DPB1 alleles.

Keywords

Hematopoietic Cell Transplantation; Graft-Versus-Host-Disease; Genomics; Human Leukocyte Antigen

INTRODUCTION

Allogeneic blood and marrow transplantation (allo-BMT) from volunteer unrelated donors can cure patients with malignant and nonmalignant hematologic diseases who lack a suitable family member donor. Despite optimal supportive care and allele level HLA matching, acute graft-versus-host-disease (GVHD) remains a major barrier to the success of allo-BMT, giving rise to immune suppression, leading to chronic disease and transplant-related morbidity¹. Significant GVHD is associated with increased mortality and once established, often responds poorly to therapy.

The initiation and propagation of GVHD is a multifactorial, multistep process that includes, but is not restricted to, classic HLA mismatch. GVHD is critically dependent on additional pathways including non-classic HLA allorecognition (such as by natural killer cells), immune responses driven by minor histocompatibility antigens (mHAg), and an inflammatory milieu²⁻⁴. Nonsynonymous coding single nucleotide polymorphisms (SNPs) and deletion polymorphisms hold importance as ways of generating potentially immunogenic transplantation antigens. While a wide range of targeted immune response genes have been studied, including polymorphisms in TNF- α , *IL10*, *IFNG*, *IL6*, *IL1*, and *TGFBI*, this approach is inherently biased and results have not been consistently replicated in independent cohorts⁵⁻⁷.

The International HapMap Project (<http://hapmap.ncbi.nlm.nih.gov/>) has cataloged a tremendous level of variation in the human genome⁸. In parallel with this has been the capacity to genotype these SNPs on a massive scale using microarrays, in a cost effective manner. This has led to the unbiased evaluation between genes and phenotypes called Genome Wide Association (GWA). Using this approach there has been a series of genomic discoveries involving complex, non-Mendelian diseases that contribute to risk of common conditions such as Crohn's disease, and play a role in natural history of hepatitis B virus (HBV) and human immunodeficiency virus-1 infections⁹⁻¹².

Here, we report findings of a genome-wide association study (GWAS) that evaluated recipient and donor genomes to identify genes associated with risk of GVHD in the unrelated donor transplant setting.

METHODS

All transplant recipient and donor research subjects had been consented on a research sample repository and a research database protocols of the National Marrow Donor Program (NMDP). This research was also approved by the Institutional Review Board of the University of Pittsburgh.

I. Selection of Study Participants

Unrelated donor transplant recipients who developed grade III-IV acute GVHD (cases) and those with no acute GVHD by day 100 posttransplant (controls) were identified by the Center for International Blood and Marrow Transplant Research (CIBMTR, www.cibmtr.org). Both cohorts were drawn from the same patient population. For this discovery phase of the GWA study, to maximize the probability of identifying highly significantly associated variants, we focused on a homogeneous group of patients.

1. Recipient-donor pairs are allele matched for HLA-A, -B, -C, -DRB1, and -DQB1 loci
2. Recipient-donor pairs are of self-identified European-American ancestry
3. Indication for transplant: leukemia or myelodysplastic syndrome
4. First transplant
5. Standard ablative pretransplant conditioning. Excludes reduced-intensity (mini-) transplants
6. No T-cell manipulation of transplant graft

Acute GVHD was diagnosed using standard clinical criteria, and severity grade (I-IV) assigned by the participating transplant center according to the modified Glucksberg scale¹³. We chose to focus on comparing severe (grade III-IV) acute GVHD cases with no acute GVHD controls to accentuate differences between the two groups and increase the likelihood of identifying genes with significant effects.

II. Sample Size

In the design of a GWA study, two fundamental decisions have to be made: which loci to genotype, and in how many individuals¹⁴. These are affected by a number of factors including number of variants and resolution of the commercially available chips, and budget. Sample sizes are often limited by clinical heterogeneity of study subjects and quality of biological samples.

We hypothesized that a small number of variants in recipient and donor genomes exert detectable influence on acute GVHD status of transplant recipient. Our goal was to have power to detect an odds ratio of 2.0 between cases and controls. Based on selection criteria and number of clinical specimens, our sample size was a compromise between power to detect an effect and cost and we set out to perform significant discovery work and follow-up for some goals, but realized that it will be sufficient only for preliminary data collection for other goals.

III. DNA Extraction, Pooling and Genotyping

All sample processing, DNA isolation and quantitation, pooling of DNA specimens and hybridization to the Affymetrix SNP Array 6.0, was performed in the Human Genomics and Proteomics Core Laboratory of the University of Pittsburgh (www.genetics.pitt.edu). Briefly, high molecular weight DNA was isolated from each individual sample using the Puregene method. Each sample was run on an agarose gel to ensure that the DNA was intact and will not introduce bias into the pools. DNA was quantified fluorometrically in triplicate by the pico-green protocol¹⁵. The DNA was diluted to 5 ng/μl and an equal volume of each added to three independent pools using the Tecan Freedom robotic work station, using methods given in Pearson et al (see below for pooling strategy used)¹⁶.

Each pooled DNA sample was concentrated to 50 ng/μl for hybridization to a separate GWAS chip. Following denaturation with 0.1N NaOH, genomic DNA samples were amplified by incubation at 37° C for 20 hours in a proprietary amplification reaction mix. Amplified DNA was fragmented using vendor supplied reagents by incubation for 1 hour at 37C. Fragmented DNA sample was precipitated and resuspended in hybridization buffer. Arrays were cleaned and activated by washing with ethanol, formamide and vendor supplied pre- hybridization buffers. DNA samples were denatured, applied to the arrays and hybridized 16–24 hours with rocking at 48° C. Unhybridized and non-specifically hybridized DNA is washed away, the chip surface was treated to facilitate primer extension reaction and single base extension performed on bound primers with labeled nucleotides. Bound DNA sample was washed away. Staining steps were performed to attach florescent dyes to the labeled nucleotides and the array surface was sealed to protect the dyes from atmospheric degradation.

IV. Pooled GWAS

A pooled GWAS design was used to reduce cost without sacrificing the power to detect an effect^{16–18}. Subjects were divided into four groups as following: R1, recipients with grade III-IV acute GVHD; R2, recipients with no acute GVHD; D1, donors of patients with grade III-IV acute GVHD, and D2, donors of patients without acute GVHD. The pooling study design is outlined in steps 1–5 below. The same experimental steps were performed for the two donor groups.

1. Three independent pools were created, each containing equimolar concentrations of all samples
2. Each pool was hybridized to three Affymetrix SNP Array 6.0 chips
3. Relative allele frequencies were derived
4. Forty samples were randomly selected from the R1 cohort and
 - Each sample was hybridized individually to an Affymetrix SNP Array
 - Three independent pools of these 40 samples were created and hybridized to three Affymetrix SNP Arrays

- Results from this experiment were used to discover cofactors to refine estimated, pooled allele frequencies
5. A genome wide scan on R2 samples was performed using the steps 1–4

V. Individual genotyping

After results of the pooled GWAS were available, individual genotyping of 18 recipient and 3 donor SNPs was performed. Unfortunately, HLA-DP SNPs were not included in this panel. HLA-DPB1 allele genotyping was performed using 454 pyrosequencing assays and a software program adapted from previously published methods¹⁹.

VI. Statistical Analysis

The study cohort was composed only of patients with grade III-IV acute GVHD or no GVHD, i.e., we excluded patients with grade I-II acute GVHD by design. Since the study cohort was not “population-based”, it could not be used to estimate cumulative incidence of GVHD. As described above, DNA samples were divided into three pools each for recipients and donors and tested in triplicate using the Affymetrix Genome-wide SNP Array 6.0. A smaller subset of pairs from each pool was individually genotyped and comparisons were made between individual and pooled genotyping results. SNPs with B allele frequencies of less than 5% or that differed significantly between pooled and individuals were excluded. Outlier SNPs were excluded or considered missing. An empiric Bayes model was built to analyze the pooled data set. We estimated and contrasted allele frequencies between recipients with and without acute GVHD (R1 versus R2), and between donors of patients with and without acute GVHD (D1 versus D2). The SNPs were ranked according to the P values from the contrast analysis. Please refer to supplementary file named “Biostatistical Analysis” for further details.

The association of specific HLA-DPB1 alleles and acute GVHD was analyzed by logistic regression, adjusting for significant clinical characteristics. To adjust for the higher likelihood of HLA-DPB1 matching in patients with common alleles (particularly HLA-DPB1*04:01), dummy variables that combined HLA-DPB1 allele identification with HLA-DPB1 matching were used in the analysis.

RESULTS

The Statistical Center of CIBMTR identified 864 donor-recipient pairs who met study eligibility criteria. Patient and transplant characteristics are summarized in Table 1. The median recipient age at transplant was 37 years (<1–67) and 58% were male. Acute leukemia was the most common diagnosis. Stem cell sources were similar (59% marrow and 41% peripheral blood) in the GVHD and no GVHD groups. GVHD prophylaxis was cyclosporine-based in 51% of patients and tacrolimus-based in 47%. Blood samples were provided from the CIBMTR Repository on these subjects as frozen aliquots of whole blood, granulocytes, peripheral blood mononuclear cells, or B-lymphoblastic cell lines.

The extracted DNA samples met stringent quality requirements for pooled GWAS experiments from 291 recipients with grade III-IV GVHD, 288 recipients without GVHD,

315 donors for patients with grade III-IV GVHD, and 341 donors for patients without GVHD. An empiric Bayes model was built to analyze the pooled data set. SNPs with p value E-07 or lower are listed in Table 2.

We identified three novel susceptibility loci in the HLA-DP region of recipient genomes that were associated with risk of GVHD (rs9277378, $p=1.58E-09$; rs9277542, $p=1.54E-06$ and rs9277341, $p=7.71E-05$). SNP rs9277378 was the second most significant association in the unbiased analysis, whereas rs9277542 and rs9277341 were of lower significance but of interest because of their location in the HLA-DP region.

To explore whether specific HLA-DPB1 alleles explained the GWAS findings, HLA-DPB1 four digit allele typing was performed on 1726 samples. As expected, only 14.7% of pairs were matched at both DPB1 alleles, 55.4% matched at one, and 29.8% matched at neither. Consistent with prior reports, recipient-donor DPB1 mismatch at one or both allele was associated with greater risk of GVHD (OR 1.52, 95% CI 1.32–1.79, $p=1.5E-07$). When individual HLA-DPB1 alleles were considered, HLA-DPB1*04:01 allele in donors was associated with increased risk of GVHD (44.36% frequency, OR 1.37, 95% CI 1.10–1.65, $p=0.0021$, adjusting for number of mismatched HLA-DPB1 alleles). However, when the analysis was repeated adjusting for the confounding caused by a higher likelihood of HLA-DPB1 matching in donors with the HLA-DP*04:01 allele because of the high frequency of the allele, the association between HLA-DPB1*04:01 and grade III-IV GVHD was no longer statistically significant.

DISCUSSION

Our study globally assessed recipient and donor genomes to identify genes associated with risk of severe acute GVHD using a case-control design and a pooled GWAS approach. We identified three novel susceptibility loci in the HLA-DP region of recipient genomes that were associated with grade III-IV acute GVHD (rs9277378, rs9277542, and rs9277341). Eighteen other recipient SNPs and 3 donor SNPs with a high level of significance should be considered as discovery-level observations and will require further validation work.

We focused on the HLA-DP region results given recent reports on its relevance in the BMT setting and other immune mediated diseases such as HBV. Of the three SNPs identified in our analysis as associated with acute GVHD, rs9277378 is located in the second intron and rs9277542 in the 3'- untranslated region (UTR) of the HLA-DPB1 gene and the two are in strong linkage disequilibrium with each other and with rs9277534 and rs9277535 (Table 3). These SNPs have shown significant genome-wide association with chronic hepatitis B virus (HBV) clearance and persistence^{10, 12, 20–22}. Functional predictions according to SNPinfo suggest a role in HLA-DPB1 expression for rs9277542²³. The 3'-UTR SNPs, rs9277534 and rs9277535 are associated with HLA-DPB1 mRNA expression^{12, 24}. The third variant in our study, rs9277341 is intronic and in LD with rs3077 which lies in the 3'-UTR of HLADPA1. The rs3077 has also shown association with chronic HBV infection and with HLA-DPA1 mRNA expression²⁴. These HLA-DP region SNPs were the only ones identified in the MHC region in our study.

Our results corroborate and extend findings reported by other groups about the importance of the HLA-DP region. Petersdorf and colleagues recently reported an association between the rs9277534 allele and risk of GVHD in unrelated donor transplants with single HLA-DPB1 mismatches in the GVH vector²⁵. Their group had previously identified an association of rs2281389 with acute GVHD; the rs2281389 variant is strongly associated with rs9277534 which is linked to HLA-DPB1 mRNA expression level²⁶. Interestingly, the low expression rs9277534A variant was linked to DPB1 *04:01, *04:02 and *0201 alleles. Based on T-cell epitope cross-reactivity patterns in HLA-DPB1 mismatched transplants, Fleischhauer and colleagues had previously classified these alleles as poorly-immunogenic and associated with a low risk of GVHD²⁷.

While one of our SNPs is in significant linkage disequilibrium with SNPs associated with acute GVHD such as rs9277534 and rs2281389, our study did not detect the same SNPs that were previously reported. This could be related to differences in the chips, study design, or patient and transplant characteristics and would require further testing. Our observations of an association between rs9277378 and rs9277542 with grade III-IV acute GVHD might simply be a reflection of linkage disequilibrium between these two SNPs and rs9277534, or they may reflect independent or synergistic effects on HLA-DPB1 expression or other HLA-DP functions.

Our study has a number of limitations. First, because of its design as a case-control study and pooled GWAS we are limited in the additional analyses we can perform. Since the groups were defined by acute GVHD status and genetic material was pooled for analysis, we were not able to address important questions such as the relationship of these SNPs to specific HLA-DPB1 alleles or other outcomes such as relapse or survival. Since we do not have genetic profiles on individual patients, we cannot look at interactions between the individual HLA-DP SNPs nor adjust for specific clinical characteristics. Second, since our experiments excluded patients with grade I-II acute GVHD, we are unable to provide population-based estimates for the actual risks of severe acute GVHD associated with these SNPs. Finally, this is a discovery study; we believe there are theoretical reasons why these SNPs may reflect important biologic functions but we do not have any empiric data yet in support of this hypothesis.

Our report contributes to emerging data showing clinical significance of the HLA-DP region genetic markers beyond structural matching of DPB1 alleles. Additional studies are needed to fully characterize the functional properties of the SNPs in this region. Improved understanding of multiple genomic variants and their interactions should help identify recipients at the highest risk of severe acute GVHD, develop individualized immunosuppressive strategies, and ultimately improve outcome for allo-BMT recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dan Scheller for research repository management, Deborah Hollingshead for coordinating the GWAS experiments, and Dr. A Kim Ritchey for his support and guidance for this project.

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Table 1

Patient and transplant characteristics

	Grades III-IV acute GVHD by Day 100		No acute GVHD by Day 100	
	N evaluable	N (%)	N evaluable	N (%)
Number of patients	411		453	
Recipient age at transplant, median (range), years	411	39 (<1-67)	453	36 (<1-66)
Male sex	411	246 (60)	453	258 (57)
Performance score prior to transplant > 90	394	299 (76)	401	310 (77)
Indication for transplant	411		453	
AML		103 (25)		145 (32)
ALL		69 (17)		92 (20)
Other leukemia		18 (4)		13 (3)
CML		137 (33)		89 (20)
MDS		66 (16)		82 (18)
NHL		18 (4)		32 (7)
Stem cell source	411		453	
Bone marrow		241 (59)		266 (59)
Peripheral blood stem cells		170 (41)		187 (41)
GVHD prophylaxis	411		453	
Tacrolimus based		197 (48)		244 (54)
Cyclosporine based		212 (52)		196 (43)
Other		2 (<1)		25 (3)
HLA matching at HLA-DQB1	453		411	
Allele-matched		425 (94)		377 (92)
One allele mismatch		28 (6)		34 (8)

Table 2

SNPs with E-07 or Lower Level of Significance were evaluated for their proximity to known genes and possible clues to their biological significance

Recipient (R) or Donor (D) Genome	dbSNP ID	Chromosome	Position	Gene Region	Gene ID	Observed MAF ^a	Minor Allele Frequency ^b	p-value
R	rs1389663	3	22331934	ZNF385D: zinc finger protein 385D	79750	0.391	0.429	7.42E-10
R	rs9277378	6	33158257	HLA-DPB1	3115	NA	0.273	1.58E-09
D	rs13221852	7	3455665	SDK1: sidekick cell adhesion molecule 1	221935	0.4856	0.495	1.56E-08
R	rs16866061	2	225148088	CUL3: cullin3	8452	0.326	0.333	2.47E-08
R	rs12030196	1	59255908	-	-	NA	0.232	3.42E-08
R	rs11023435	11	15084922	-	-	0.164	0.460	4.08E-08
D	rs529967	3	125649193	KALRN: kairin, Rho guanine nucleotide exchange factor kinase	8997	0.406	0.298	4.12E-08
R	rs465530	12	63617033	FLJ41278: uncharacterized LOC400046	400046	0.437	0.490	4.20E-08
R	rs7234719	18	26521532	-	-	0.336	0.278	6.51E-08
R	rs1147805	9	25764342	-	-	0.487	0.439	8.71E-08
R	rs4981053	14	19592561	OR4U1P: olfactory receptor, family 4, subfamily U, member 1 pseudogene	390434	0.472	0.470	1.22E-07
R	rs12641631	4	154884729	RNF175: ring finger protein 175	285533	0.193	0.207	1.39E-07
R	rs6651135	7	53258364	HAUS6P1: HAUS augmin like complex subunit 6 pseudogene 1	653175	0.173	0.136	1.55E-07
R	rs7192392	16	77489879	WVVOX: WV domain containing oxidoreductase	51741	NA	0.394	1.75E-07
R	rs11096855	4	36671033	-	-	0.178	0.172	1.87E-07
R	rs10869777	9	78238977	GCNT1: glucosaminyl (N-acetyl) transferase 1, core 2	2650	0.430	0.490	2.10E-07
R	rs11764561	7	155714863	-	-	0.218	0.232	2.30E-07
R	rs12502114	4	182878208	-	-	NA	0.394	2.89E-07
R	rs10090774	8	141895921	PTK2: protein tyrosine kinase 2	5747	0.449	0.429	3.66E-07
R	rs1360141	9	73481836	-	-	0.403	0.359	7.48E-07

Recipient (R) or Donor (D) Genome	dbSNP ID	Chromosome	Position	Gene Region	Gene ID	Observed MAF ^a	Minor Allele Frequency ^b	p-value
D	rs1387416	6	75023776	LOC101928516; uncharacterized LOC101928516	101928516	0.449	0.465	8.38E-07
R	rs6547818	2	21743084	-	-	0.447	0.444	9.69E-07

^aObserved minor allele frequencies (MAF) from individual SNP genotyping. NA indicates not available

^bFrom The 1000 Genomes Project - Utah residents with ancestry from northern and western Europe (CEPH) <http://browser.1000genomes.org>

Table 3

Linkage Disequilibrium between Current Study SNPs, and between Current Study and Selected Published HLA-DP Region SNPs^a

Gene Region	Current Study	Proxy ^b	Distance, bp	D'	r ²	Minor Allele Frequency ^c
HLA-DPB1	rs9277378					0.273
		rs9277534 (9,12,13)	4528	1	0.96	0.283
		rs9277535 (9,12)	4582	0.96	0.78	0.242
		rs9277542 (11)	4968	1	0.96	0.278
		rs2281389 (14)	9517	1	0.532	0.136
HLA-DPB1	rs9277542					
		rs9277534 (9,12,13)	440	1	1.00	0.283
		rs9277535 (9,12)	386	1	0.82	0.242
		rs9277378 (current study)	4968	1	0.96	0.273
		rs2281389 (14)	4549	1	0.512	0.136
HLA-DPA1	rs9277341					0.298
		rs3077 (12)	6603	1	0.48	0.182

^a Johnson, A. D. et al. SNAP: A web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics*, 2008 24(24):2938

^b Referenced publications are provided in parentheses

^c From The 1000 Genomes Project - Utah residents with ancestry from northern and western Europe (CEPH)