TRANSPLANTATION

Mapping MHC haplotype effects in unrelated donor hematopoietic cell transplantation

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Key Points

- HLA haplotypes encode single nucleotide polymorphisms (SNPs) that are associated with risks after HLA-mismatched unrelated donor HCT.
- SNPs associated with graft-versus-host disease (GVHD) are independent of those associated with relapse.

Life-threatening risks associated with HLA-mismatched unrelated donor hematopoietic cell transplantation limit its general application for the treatment of blood diseases. The increased risks might be explained by undetected genetic variation within the highly polymorphic major histocompatibility complex (MHC) region. We retrospectively assessed each of 1108 MHC region single nucleotide polymorphisms (SNPs) in 2628 patients and their HLA-mismatched unrelated donors to determine whether SNPs are associated with the risk of mortality, disease-free survival, transplant-related mortality, relapse, and acute and chronic graft-versus-host disease (GVHD). Multivariate analysis adjusted for HLA mismatching and nongenetic variables associated with each clinical end point. Twelve SNPs were identified as transplantation determinants. SNP-associated risks were conferred by either patient or donor SNP genotype or by patient-donor SNP mismatching. Risks after transplantation increased with increasing numbers of unfavorable SNPs. SNPs that influenced acute GVHD were independent of those that affected risk of chronic GVHD and relapse. HLA haplotypes differed with respect to haplotype content of (un) favorable SNPs. Outcome after HLA-mismatched unrelated donor transplantation is influenced by MHC region variation that is undetected with conventional HLA typing. Knowledge of the SNP content of HLA haplotypes provides a means to estimate risks prior to transplantation and to lower complications through judicious selection of donors with favorable MHC genetics. (*Blood*. 2013;121(10):1896-1905)

Introduction

The availability of >20 million registered unrelated donors worldwide is an invaluable resource for patients seeking a hematopoietic cell transplant to cure life-threatening blood disorders.[1] In the United States, white patients have a 75% chance of identifying an HLA-A,-C,-B,-DRB1–matched donor, which improves to 94% if criteria are relaxed to donors with a single HLA mismatch; 31% of African-American patients have HLA-matched and 69% have HLA-mismatched donors.[2] Consequently, use of donors with a limited degree of mismatch could greatly increase access of patients to transplantation as therapy. However, HLA-mismatched transplantation is associated with significantly higher risks of graft-versus-host disease (GVHD) and mortality compared with HLA-matched transplantation.[3,4] These risks have limited the general use of HLA-mismatched unrelated donors.

A critical unmet need is to increase the safety of HLA-mismatched unrelated donor transplantation by understanding the immunogenetic basis of transplant-associated complications. The major histocompatibility complex (MHC) is an attractive candidate region for the discovery of clinically important human genetic variation because of the high density of genes with immune-related function.[5] Single nucleotide polymorphisms (SNPs) travel with HLA genes on haplotypes and have been used to map genes within the MHC that cause disease.[6,7] We tested the hypothesis that clinical outcome after HLA-mismatched unrelated donor hematopoietic cell transplantation depends on the cumulative effects of HLA mismatching and of haplotype-associated SNPs.

Methods

Study population

Patients transplanted from unrelated donors mismatched at one HLA locus (HLA-A, -C, -B, -DRB1, or -DQB1) for malignant and nonmalignant blood disorders were retrospectively studied (Table 1).[8,9] HLA haplotype content for SNPs was defined in 163 HLA-A,-C,-B,-DRB1,-DQB1 homozygous cell lines[10] and HLA-matched[11] and HLA-mismatched transplants. Consent from patients and donors was obtained in accordance with the Declaration of Helsinki through the Center for International Blood and Marrow Transplant Research, and the work was approved by the Institutional Review Board, Fred Hutchinson Cancer Research Center.

Genotyping

A total of 5135 samples, including 338 duplicates for intra- and interexperiment quality control, were genotyped for 1228 MHC region SNPs and HLA-

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Table 1. Demographics of the study population

	Transplants (N = 2628)
Age, y	
Patient	
0-19	642 (24%)
20-39	861 (33%)
40 and older	1124 (43%)
Unknown	1 (<1%)
Donor	
18-19	14 (<1%)
20-39	1535 (58%)
40 and older	893 (34%)
Unknown	186 (7%)
Year of transplantation	
1987-1994	349 (13%)
1995-1999	661 (25%)
2000-2003	681 (26%)
2004-2009	937 (36%)
Patient-donor gender	
Male-male	910 (35%)
Male-female	608 (23%)
Female-male	562 (21%)
Female-female	547 (21%)
Unknown	1 (<1%)
Disease/early, intermediate, late, or advanced, other or unknown, no.*	
Acute myeloid leukemia	766 (29%)/216, 246, 296, 8
Acute lymphoblastic leukemia	479 (18%)/142, 221, 113, 3
Chronic myeloid leukemia	574 (22%)/385, 158, 31
Myelodysplastic syndrome	310 (12%)/67, 0, 131, 112
Non-Hodgkin lymphoma	150 (6%)/NA
Other malignancies	51 (2%)/NA
Nonmalignancies	185 (7%)/NA
Patient-donor serologic status for cytomegalovirus	
Negative-negative	816 (31%)
Negative-positive	799 (30%)
Positive-negative	412 (16%)
Positive-positive	556 (21%)
Unknown	45 (2%)
Transplant type†	
Myeloablative	2105 (80%)
Reduced-intensity/nonmyeloablative	505 (19%)
Unknown	18 (1%)
Source of cells	
Bone marrow	1726 (66%)
Peripheral blood stem cells	902 (34%)
GVHD prophylaxis	
Cyclosporine with or without other agents	1244 (47%)
Tacrolimus with or without other agents	896 (34%)
T-cell depletion	329 (13%)
Other combinations	151 (6%)
Missing	8 (< 1%)
Karnofsky performance score (%)	
0-80	643 (24%)
90-100	1614 (61%)
Missing	371 (14%)
Patient-donor HLA-DPB1	
Matched	187 (7%)
GVH mismatch	187 (7%)
HVG mismatch	154 (6%)
Bidirectional mismatch	1025 (39%)
Missing	1075 (41%)
Patient ethnicity	
Hispanic	206 (8%)
Non-Hispanic‡	
White	2141 (81%)
African American	139 (5%)
Asian/Pacific Islander	56 (2%)
Native American	10 (<1%)
Other	5 (<1%)
Unknown	71 (3%)
Donor ethnicity	
Hispanic	159 (6%)
Non-Hispanic‡	
White	1834 (70%)
African American	128 (5%)
Asian/Pacific Islander	54 (2%)
Native American	27 (1%)
Other	57 (2%)
Unknown	369 (14%)
HLA mismatching§	
HLA-A	658 (25%)
Allele	226
Antigen	430
Unknown	2
HLA-B	351 (13%)
Allele	241
Antigen	107
Unknown	3
HLA-C	1006 (38%)
Allele	203
Antigen	802
Unknown	1
HLA-DRB1	159 (6%)
Allele	139
Antigen	19
Unknown	1
HLA-DQB1	454 (17%)
Allele	111
Antigen	343

Data are n (%). Patients received their transplant at the Fred Hutchinson Cancer Research Center (n = 548) or at 1 of 149 other centers in the Center for International Blood and Marrow Transplant Research network (n = 2080).

GVH, graft-versus-host; HVG, host-versus-graft; NA, not applicable.

*Disease status before transplant is categorized as early (first complete remission [CR] of acute myeloid leukemia [AML] or acute lymphoblastic leukemia [ALL], first chronic phase [CP] of CML, refractory anemia [RA] or refractory anemia with ring sideroblasts of myelodysplastic syndrome [MDS]); intermediate (second or higher CR of AML or ALL, second or higher CP or accelerated phase of CML); late or advanced (primary induction failure or first or higher relapse of AML or ALL, blast phase [or blast crisis] of CML, MDS RA with excess blasts or excess blasts in transformation); other (mainly unnamed MDS) or unknown. Other malignancies included Hodgkin lymphoma, plasma cell disorder, multiple myeloma, breast cancer, other malignancies. Non-malignancies included severe aplastic anemia, Shwachman-Diamond anemia, Diamond-Blackfan anemia, Fanconi anemia, sickle cell disease, thalassemia, inherited abnormalities of erythrocyte differentiation or function, other immune system disorders, inherited abnormality of platelets, inherited disorder of metabolism, histiocytic disorders and other nonmalignancies.

†Myeloablative conditioning regimens: cyclophosphamide (Cy) with total body irradiation (TBI) given as a single dose >500 cGY or as fractionated TBI >800 cGY total; CY/etoposide (VP16)/TBI; busulfan (BU)/CY; TBI ≥ 500 cGY single dose; TBI ≥800 cGY fractionated; melphalan >150 mg/m^2; BU >9 mg/kg; BU/melphalan. Reduced intensity regimens: TBI <500 cGY single dose; TBI <800 cGY fractionated; melphalan ≤ 150 mg/m^2; BU ≤9 mg/kg; carmustine (BCNU)/VP16/cytarabine/melphalan (BEAM); CY/BCNU/VP16 (CBV); VP16/CY. Nonmyeloblative regimens: TBI 200 cGY; fludarabine (FLU)/TBI 200 cGY; FLU/CY; FLU/cytosine arabinoside (ARA-C).

‡Definitions follow the US Office of Management and Budget classification.[8]

§HLA allele and antigens were defined according to the official World Health Organization HLA Nomenclature.[9] Following this nomenclature, 5 novel sequences identified in patients (A*02, A*03, B*39, B*40, and C*01) and 2 patient-donor mismatch combinations (HLA-DRB1*03:01/03:05 and HLA-B*40:08/40:11) could not be defined.

A,-C,-B,-DRB1,-DQB1,-DPB1 alleles as previously described.[11] The 338 duplicate samples had a concordance rate of 0.991. Of the 4797 unique samples, 187 (3.9%) failed genotyping or gender control, yielding 2628 transplants (1982 patient-donor pairs and 646 patients or donors) for outcomes analysis. Of the 1228 SNPs, 6 did not meet Hardy-Weinberg equilibrium at $P < .001$, and 114 had interexperimental failures, yielding 1108 SNPs (0.989 genotyping rate) for analysis. To define haplotype content in HLA homozygous samples, SNPs were genotyped using TaqMan chemistry.[12]

Statistical analysis

The clinical end points were grades II-IV and III-IV acute GVHD, chronic GVHD, transplant-related mortality, relapse (malignancies), disease-free survival (malignancies), and survival. Before testing SNPs, we built multivariate models for each end point with appropriately selected clinical prognostic factors (Table 1). The strong linkage disequilibrium across the MHC influenced patient-donor SNP mismatch rates (Figure 1). We therefore included separate variables for HLA-A,-C,-B,-DRB1,-DQB1 antigen or allele mismatches, HLA-DPB1 mismatches, patient natural killer cell immunoglobulin–like receptor (KIR) ligands, and patient-donor KIR ligand mismatching.[13-15] Assessment of these potential risk factors for outcomes was evaluated using Cox proportional hazards models. First, the proportionality assumption was tested for each factor by adding a time-dependent covariate. Factors that violated proportional hazards were adjusted through stratification. A stepwise forward/backward model selection approach was then performed to identify significant clinical prognostic risk factors at a 5% significance level. To control for HLA variation of particular alleles, we further tested association of each end point with 0, 1, or 2 copies of HLA alleles having $\geq$20 counts. On the basis of the multivariate clinical variable models, a second stepwise forward selection procedure was performed on the HLA allele variables with a threshold of $P = .01$ for entering into the preliminary models.

In the single SNP analysis, each of the 1108 SNPs was tested separately for associations with the 7 clinical end points using the preliminary models. We considered 0, 1, or 2 copies of the rare SNP allele carried by each patient or donor. Each SNP was tested 3 ways: patient genotype, donor genotype, and patient-donor SNP mismatching. Samples with missing SNP genotypes were excluded. To control for the familywise error rate, the Bonferroni criterion was applied to adjust for the multiple testing of the 1108 SNPs.

We also explored predictive effects from multiple SNPs to each end point. To circumvent the problem of missing genotypes in the model selection on all the SNPs, we adopted a 2-step approach.[11] For each outcome, we first selected a subset of SNPs with $P < .01$ from the single SNP analysis. On the basis of the preliminary models, we then performed a stepwise forward selection procedure on the selected SNPs to build the final predictive models using a threshold $P = .001$ for the candidate SNPs.

SNP and HLA allele frequency comparisons, and genotypic associations between HLA alleles and SNPs were tested using classical χ^2 tests (or Fisher's exact tests for small cell counts). All analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). Linkage disequilibrium (D' and r^2) between SNPs was estimated using Haploview,[16] PLINK version 1.07,[17] and R package "Genetics" version 1.3.4.[18]

Results

Patient and donor SNP genotypes and mismatching

The minor allele frequencies of the 1108 SNPs were similar to previous reports.[19] Compared with HLA-matched pairs,[11] HLA-mismatched pairs were more frequently mismatched for more SNPs over longer distances across the MHC (Figure 1A-F). These results confirm the highly disparate nature of the MHC in HLA-mismatched transplant patients and donors. Of the 1078 SNPs common to both the HLA-mismatched and the HLA-matched population, there was a higher rate of patient-donor mismatching among HLA-mismatched pairs for 96% of the 1078 SNPs compared with the HLA-matched pairs. Among the HLA-mismatched pairs, the location of the mismatched SNPs depended on the HLA locus that was mismatched, indicative of the positive linkage disequilibrium characteristic of the MHC.[7,20] The difference between the percent of HLA-mismatched pairs and the percent of HLA-matched pairs[11] who were mismatched at each of the 1078 SNP positions shows that a higher percent of HLA-mismatched transplants were mismatched at the SNP compared with HLA-matched pairs (Figure 1G-K).

SNPs associated with clinical outcome

In the single SNP analysis, patient genotype at rs429916 was associated with survival (adjusted $P = .046$), disease-free survival (adjusted $P = .046$), and transplant-related mortality (adjusted $P = .009$) after Bonferroni correction. In the multi-SNP analysis, 12 SNPs were associated with transplant outcome ($P < .001$; Table 2; Figure 2).[21] Some end points were influenced by only one SNP (survival, disease-free survival, grades II-IV acute GVHD), whereas others were affected by more than one SNP (relapse, transplant-related mortality, grades III-IV acute GVHD, chronic GVHD). Each SNP influenced clinical outcome through patient genotype, donor genotype, or patient-donor mismatching specifically as noted in Table 2. These data suggest that both patient and donor SNPs contribute to risks after HLA-mismatched transplantation.

Importance of the number of unfavorable SNPs

Each SNP has either a favorable or unfavorable genotype or a favorable or unfavorable (mis)match status (Table 2). The risks of relapse, transplant-related mortality, grades III-IV acute GVHD, and chronic GVHD each increased with increasing numbers of unfavorable SNPs (Figure 3). Compared with zero unfavorable SNPs for grades III-IV acute GVHD, the effect of 1, 2, and 3 unfavorable SNPs rs209130, rs2075800, and rs394657 was as follows: hazard ratio (HR), 1.58 (95% confidence interval [CI], 1.20-2.09; $P = 1.2 \times 10^{-3}$); HR, 2.17 (95% CI, 1.63-2.88; $P = 1.0 \times 10^{-7}$); and HR, 4.38 (95% CI, 2.44-7.86; $P = 7.8 \times 10^{-7}$), respectively (Figure 3A). The HRs of 1, 2, or $\geq$3 unfavorable rs2523957, rs3830076, rs2071479, and rs107822 SNPs for chronic GVHD relative to no unfavorable SNP were 1.23 (95% CI, 0.87-1.74; $P = .25$), 1.72 (95% CI, 1.20-2.44; $P = 2.3 \times 10^{-3}$), and 2.71 (95% CI, 1.80-4.08; $P = 1.6 \times 10^{-6}$), respectively (Figure 3B). Compared with 0 unfavorable SNP, the impact of 1 or 2 unfavorable rs2244546 and rs986522 SNPs on relapse was as follows: HR, 1.62 (95% CI, 1.34-1.97; $P = 8.1 \times 10^{-7}$) and HR, 2.91 (95% CI, 1.40-6.03; $P = 4.1 \times 10^{-3}$), respectively (Figure 3C). For transplant-related mortality, one unfavorable rs915654 or rs429916 SNP was associated with an HR of 1.40 (95% CI, 1.20-1.64; $P = 2.4 \times 10^{-5}$) and 2 SNPs with an HR of 6.18 (95% CI, 2.42-15.78; $P = 1.4 \times 10^{-4}$; Figure 3D).

To test the hypothesis that some combinations of 2 or more SNPs may be more detrimental than others, we performed a model selection on the 12 SNPs as defined in Table 2. Two SNPs were associated with survival: rs429916 ($P < .0001$) and rs915654 ($P = .0048$). Among the remaining 10 SNPs, combinations of $\geq$2 unfavorable SNPs conferred similar mortality compared with 0 to 1 unfavorable SNPs (HR, 1.01; 95% CI, 0.88-1.16; $P = .86$). In contrast, among 885 patients with $\geq$1 unfavorable rs429916 or rs915654 SNP, the HR of mortality was 1.22 (95% CI, 1.07-1.39; $P = .0038$) compared with patients with 0 to 1 unfavorable SNPs. These results suggest that mortality is influenced mainly by rs429916 and rs915654.

The selection of donors with favorable SNPs is likely to improve outcomes for patients. For example, transplantation of donors with favorable SNPs for relapse (rs2244546CC and rs986522CG) and for

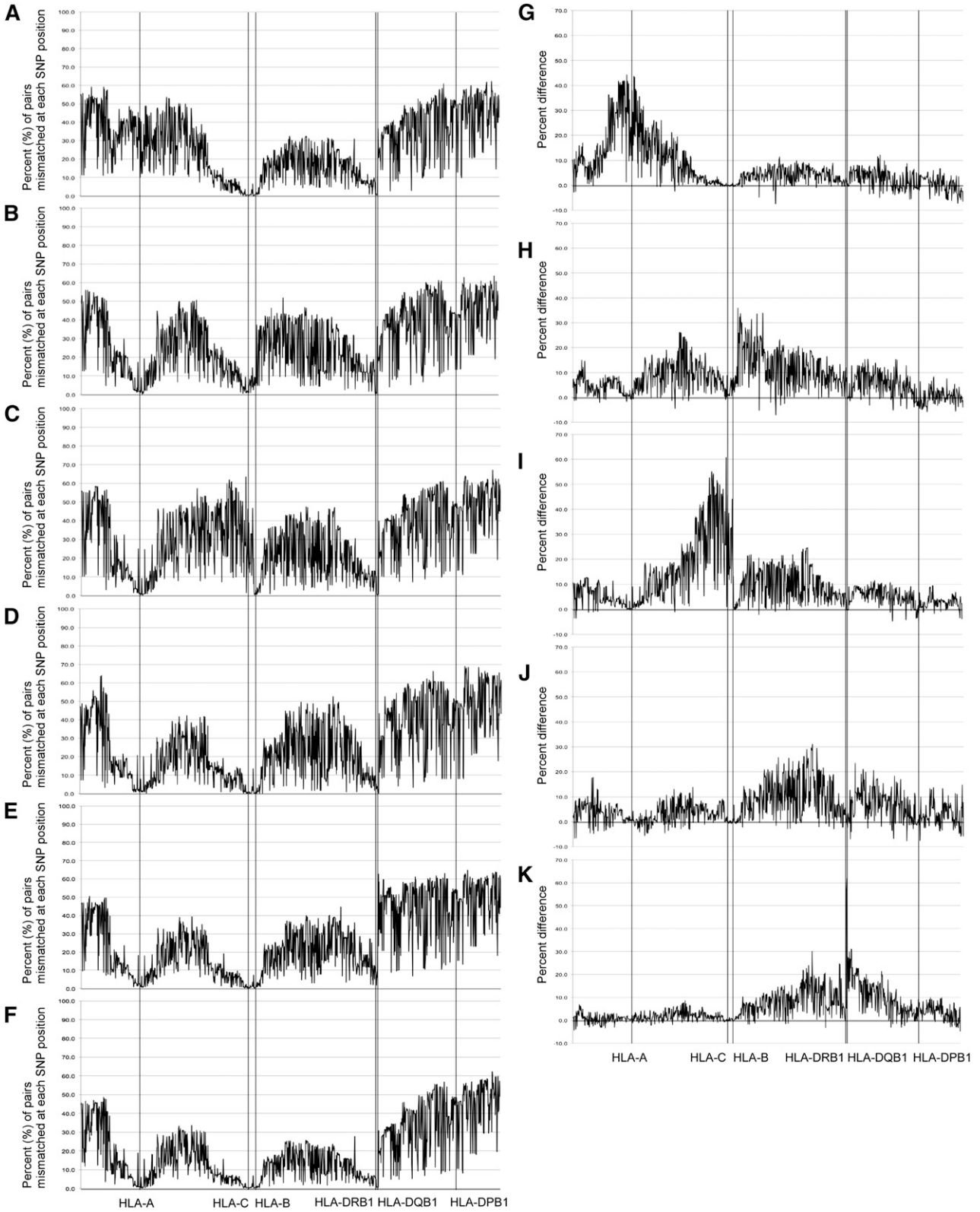

Figure 1. Extent of patient-donor SNP mismatching. The percent of pairs mismatched at each SNP position is shown for transplant pairs mismatched at (A) HLA-A,(B) HLA-B, (C) HLA-C, (D) HLA-DRB1, (E) HLA-DQB1, and (F) HLA-matched transplant pairs.[11] Of the 1078 SNPs common to both the HLA-mismatched and the HLA-matched population, there was a higher rate of patient-donor mismatching among HLA-mismatched pairs for 96% of the 1078 SNPs compared with the HLA-matched pairs. The difference between the percent of HLA-mismatched pairs and the percent of HLA-matched pairs[11] who were mismatched at each of 1078 SNP positions is graphically represented for (G) HLA-A, (H) HLA-B, (I) HLA-C, (J) HLA-DRB1, and (K) HLA-DQB1 mismatched pairs in this study. Percentiles >0 indicate a higher percent of HLA-mismatched transplants were mismatched at the SNP compared with HLA-matched pairs. Zero percentiles indicate the same percent of HLA-matched and -mismatched pairs were mismatched at the SNP position.

Table 2. Summary of SNP associations

Outcome	SNP	Gene/location*	Model	Overall *P*	Genotype or mismatch† group	Number of events	HR (95% CI)	*P*
Survival	rs429916	1.2 kb centromeric of HLA-DOA	Patient genotype	7.48×10^{-5}	**CC**	1254/1909	1	
					AC	201/296	1.11 (0.94-1.31)	.23
					AA	18/19	3.47 (1.95-6.16)	2.27×10^{-5}
Disease-free survival‡	rs429916	1.2 kb centromeric of HLA-DOA	Patient genotype	4.28×10^{-5}	**CC**	1147/1708	1	
					AC	188/268	1.06 (0.88-1.26)	.56
					AA	18/19	3.75 (2.10-6.68)	7.86×10^{-6}
Relapse‡	rs2244546	2.2 kb telomeric of HCP5	Donor genotype	6.92×10^{-4}	**CC**	373/1611	1	
					CG	117/435	1.19 (0.95-1.50)	.14
					GG	16/30	2.79 (1.60-4.85)	2.87×10^{-4}
	rs986522	COL11A2, intron	Donor genotype	3.20×10^{-5}	**CG**	209/1025	1	
					CC	136/478	1.46 (1.16-1.85)	1.45×10^{-3}
					GG	161/573	1.62 (1.30-2.03)	2.14×10^{-5}
Transplant-related mortality	rs915654	1.4 kb telomeric of LTA	Patient genotype	9.93×10^{-5}	**AT**	394/964	1	
					AA	142/300	1.45 (1.16-1.80)	1.16×10^{-3}
					TT	332/718	1.39 (1.17-1.64)	1.43×10^{-4}
	rs429916	1.2 kb centromeric of HLA-DOA	Patient genotype	2.82×10^{-5}	**CC**	720/1696	1	
					AC	134/267	1.20 (0.97-1.49)	9.67×10^{-2}
					AA	14/19	4.52 (2.31-8.86)	1.11×10^{-5}
Grades II-IV acute GVHD§	rs2242656	BAG6, intron	Mismatch	3.13×10^{-4}	**Matched**	865/1535	1	
					HVG	122/214	1.00 (0.82-1.22)	1
					GVH	136/203	1.46 (1.21-1.77)	6.92×10^{-5}
Grades III-IV acute GVHD	rs209130	3 kb telomeric of TRIM27	Mismatch	6.42×10^{-5}	**Matched**	251/920	1	
					HVG	154/488	1.22 (0.99-1.50)	6.11×10^{-2}
					GVH	124/420	1.19 (0.96-1.49)	.12
					Bi	45/103	2.17 (1.56-3.01)	3.70×10^{-6}
	rs2075800	HSPA1L, Glu602Lys	Patient genotype	8.37×10^{-4}	GG	289/848	1	
					AG	214/819	0.72 (0.60-0.86)	4.01×10^{-4}
					AA	71/264	0.73 (0.56-0.95)	2.07×10^{-2}
	rs394657	NOTCH4, intron	Donor genotype	4.15×10^{-4}	**AA**	150/626	1	
					AG	301/923	1.48 (1.21-1.81)	1.16×10^{-4}
					GG	123/382	1.43 (1.11-1.82)	4.75×10^{-3}
Chronic GVHD	rs2523957	26.6 kb centromeric of HLA-A	Mismatch	7.27×10^{-5}	**Matched**	637/1474	1	
					HVG	69/159	1.21 (0.92-1.59)	.18
					GVH	79/154	1.75 (1.36-2.26)	1.73×10^{-5}
	rs3830076	240 bp telomeric of FKBPL	Mismatch	1.24×10^{-4}	Matched	674/1499	1	
					HVG	59/152	0.68 (0.50-0.92)	1.22×10^{-2}
					GVH	52/136	0.56 (0.41-0.77)	3.07×10^{-4}
	rs2071479	HLA-DOB, intron	Mismatch	2.27×10^{-4}	**Matched**	724/1648	I	
					HVG	24/75	0.84 (0.53-1.31)	.43
					GVH	37/64	2.16 (1.48-3.16)	7.02×10^{-5}
	rs107822	711 bp telomeric of RING1	Mismatch	3.98×10^{-4}	**Matched**	378/911	1	
					HVG	183/391	1.35 (1.11-1.63)	2.17×10^{-3}
					GVH	176/400	1.15 (0.95-1.39)	.16
					Bi	48/85	1.81 (1.30-2.52)	4.95×10^{-4}

The table summarizes the 12 SNPs that met a $P < .001$ in multivariate models that adjusted for SNPs other than the one of interest.

Bi, bidirectional; GVH, graft-versus-host; HVG, host-versus-graft.

*Location of SNP according to the National Center for Biotechnology Information dbSNP database.[19]

†Bidirectional mismatches for SNPs rs2523957 (9 pairs), rs2242656 (10 pairs), rs3830076 (3 pairs), and rs2071479 (2 pairs) were excluded from analysis. Favorable SNP genotypes and favorable SNP (mis)matches are in bold.

‡Risks of relapse and disease-free survival were defined for patients with malignancies.

§The effect of the SNP for grades II-IV acute GVHD was significant among HLA-C antigen mismatches ($P = .005$) and remained significant after adjusting for patient KIR ligands and patient-donor KIR ligand mismatching.

grades III-IV acute GVHD (rs394657AA) is anticipated to lower risks of both complications. Among 331 patients transplanted from donors with rs2244546CC, rs986522CG, and rs394657AA, the HR of relapse was 0.66 (95% CI, 0.49-0.88; $P = 4.1 \times 10^{-3}$), and the HR of grades III-IV acute GVHD was 0.78 (95% CI, 0.61-1.0; $P = .05$) compared with transplants with all other donor genotypes. These data suggest that the selection of donors with this particular combination of favorable SNP genotypes may help to lower relapse and severe acute GVHD.

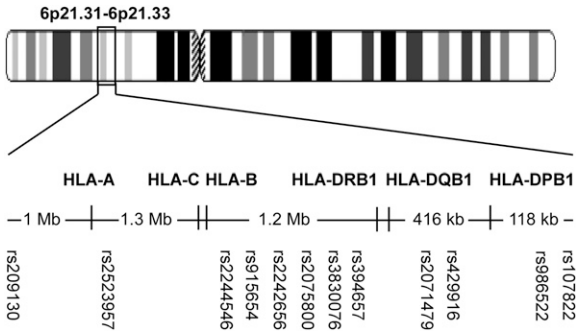

Figure 2. Twelve SNPs of clinical significance in HLA-mismatched unrelated donor transplantation. Each of the 12 SNPs having an association with grades II-IV or III-IV acute GVHD, chronic GVHD, relapse, transplant-related mortality, disease-free survival, or survival are shown on a map of the MHC on chromosome 6p21.3 (not to scale). SNPs are identified by their rs numbers. Chromosome 6 drawing modified from the National Library of Medicine, the National Center for Biotechnology Information public website.[21]

SNPs and HLA

All models were adjusted for HLA mismatching to identify SNPs that influence transplant outcome. In turn, patients transplanted from HLA-DQB1 antigen-mismatched donors had lower risks of grades III-IV acute GVHD and transplant-related mortality and improved disease-free survival and survival compared with HLA-C antigen-mismatched transplants (supplemental Table 1), consistent with previous observations.[4] Because HLA and SNPs each conferred independent risks, we tested the hypothesis that SNPs and HLA mismatching have additive effects. Among the 790 HLA-C antigen mismatches, the raw proportion of patients who developed grades III-IV acute GVHD was 22% with 0, 31% with 1, 40% with 2, and 45% with 3 unfavorable SNPs rs209130, rs2075800, and rs394657. Similar trends were observed among HLA-DQB1 antigen mismatches for rs209130, rs2075800, and rs394657: 10% of patients with 0, 20% with 1, 33% with 2, and 50% with 3 unfavorable SNPs developed grades III-IV acute GVHD. Therefore, the presence of ≥ 2 unfavorable SNPs for severe GVHD in HLA-DQB1–mismatched pairs confers risks that are as high as those in HLA-C–mismatched pairs with fewer unfavorable SNPs. In HLA-C– or -DQB1-mismatched transplantation, GVHD risks are conferred independently by the HLA mismatch and by SNP-associated effects. These data suggest that, at a minimum, information on HLA-C and -DQB1 matching and SNPs are needed to assess GVHD risks in individual patients.

Additive effects were most significant among HLA-B ($P = 8.58 \times 10^{-4}$) and HLA-DQB1 ($P = .005$) antigen mismatches for transplant-related mortality and for DRB1 alleles for grades III-IV acute GVHD ($P = .001$). The negative effects retained their significance regardless of patient KIR ligands or patient-donor KIR ligand mismatching.[13-15] Considering all 12 SNPs, we found cumulative negative effects on survival with each additional unfavorable SNP (Figure 3E, log-rank $P = .003$). Compared with 0 to 1 unfavorable SNP, the hazards of mortality associated with 2, 3, 4, 5, 6, and ≥ 7 unfavorable SNPs were as follows: HR, 1.28 (95% CI, 0.84−1.96; $P = .25$); HR, 1.43 (95% CI, 0.96-2.2; $P = .08$); HR, 1.68 (95% CI, 1.13-2.50; $P = 9.91 \times 10^{-3}$); HR, 1.86 (95% CI, 1.24-2.79; $P = 2.50 \times 10^{-3}$); HR, 1.63 (95% CI, 1.05-2.53; $P = .03$); and HR, 2.40 (95% CI, 1.39-4.14; $P = 1.7 \times 10^{-3}$), respectively. These data demonstrate that survival depends on the balance of favorable and unfavorable SNPs that affect relapse and nonrelapse complications and suggest that transplantation of HLA-mismatched donors with fewer unfavorable SNPs will lower risks after transplantation.

If specific patient-donor HLA mismatches are more likely to be associated with specific favorable SNPs, then this information could be used to select among HLA-mismatched donors. Patient-donor mismatching occurred most frequently at HLA-A, -C and -DQB1. The 2 most common HLA-A mismatches, A*02:01 vs A*02:05 and A*03:01 vs A*03:02, had similar frequencies of favorable SNPs at all 12 positions. The 2 most common HLA-C mismatches, C*03:03 vs C*03:04 (n = 96) and C*01:02 vs C*02:02 (n = 46), differed significantly for favorable SNP rs3830076 for chronic GVHD: 58.8% of C*03:03/03:04 mismatches had a favorable SNP rs3830076 in contrast to 26.1% of C*01:02/02:02 mismatches ($P = .0003$). The 2 most common HLA-DQB1 mismatches, DQB1*03:01 vs DQB1*03:02 (n = 104), and DQB1*02:02 vs DQB1*03:03 (n = 45), differed significantly for favorable SNP rs2075800 for grades III-IV acute GVHD: 74% of DQB1*03:01/03:02 mismatches had a favorable SNP rs2075800 compared with 35.6% of DQB1*02:02/03:03 mismatches ($P < .0001$). These observations suggest that HLA mismatches differ from one another for favorable SNPs carried on their haplotypes. A more complete understanding of HLA mismatches associated with favorable SNPs may offer a new approach for selecting HLA-mismatched donors in the future.

Acute and chronic GVHD

Chronic GVHD increases morbidity and lowers the quality of life after HLA-mismatched unrelated donor transplantation.[22] Although acute GVHD is a known risk factor, patients can develop chronic GVHD without prior acute GVHD; the absence of acute GVHD, therefore, is not a safeguard against chronic GVHD. Because acute and chronic GVHD have different SNP associations, we tested the hypothesis that the SNPs associated with chronic GVHD risk may depend on the presence or absence of acute GVHD. We examined the risk of chronic GVHD using a Cox proportional hazard model wherein grades III-IV acute GVHD was treated as a time-dependent variable. Patients who developed grades III-IV acute GVHD had a higher risk of developing chronic GVHD compared with patients with no acute GVHD (HR, 1.78; 95% CI, 1.49-2.12; $P = 1.23 \times 10^{-10}$). As expected, none of the risk SNPs for acute GVHD influenced the risk of chronic GVHD. However, among the 4 risk SNPs for chronic GVHD, patient-donor mismatching at SNPs rs2523957, rs2071479, and rs3830076 remained independent risk factors for chronic GVHD ($P = 3.5 \times 10^{-4}$, $P = 3.9 \times 10^{-4}$, and $P = 1.9 \times 10^{-3}$, respectively) regardless of grades III-IV acute GVHD. SNP rs107822 ($P = .019$) was no longer a significant risk factor for chronic GVHD after adjusting for acute GVHD (Table 2), suggesting that its effect on chronic GVHD may depend on the development of acute GVHD. A landmark analysis was also performed on 1630 patients who were evaluable at day 100 after transplant without chronic GVHD. SNPs rs2523957, rs2071479, and rs3830076 remained risk factors for chronic GVHD after adjustment for acute GVHD ($P = .013$, $P = .011$, and $P = .0043$, respectively), whereas SNP rs107822 was no longer significant ($P = .085$). These results suggest that efforts to match patients and donors at SNPs rs2523957, rs2071479, and rs3830076 may help to lower the risk of chronic GVHD independent of acute GVHD.

GVHD and relapse

GVHD is not always undesirable. The graft-versus-leukemia effect describes the lower risk of relapse in patients with clinical GVHD compared with patients without GVHD.[23-26] For individual patients, however, the development of clinical GVHD does not guarantee the patient will be relapse free, nor does the absence of GVHD necessarily

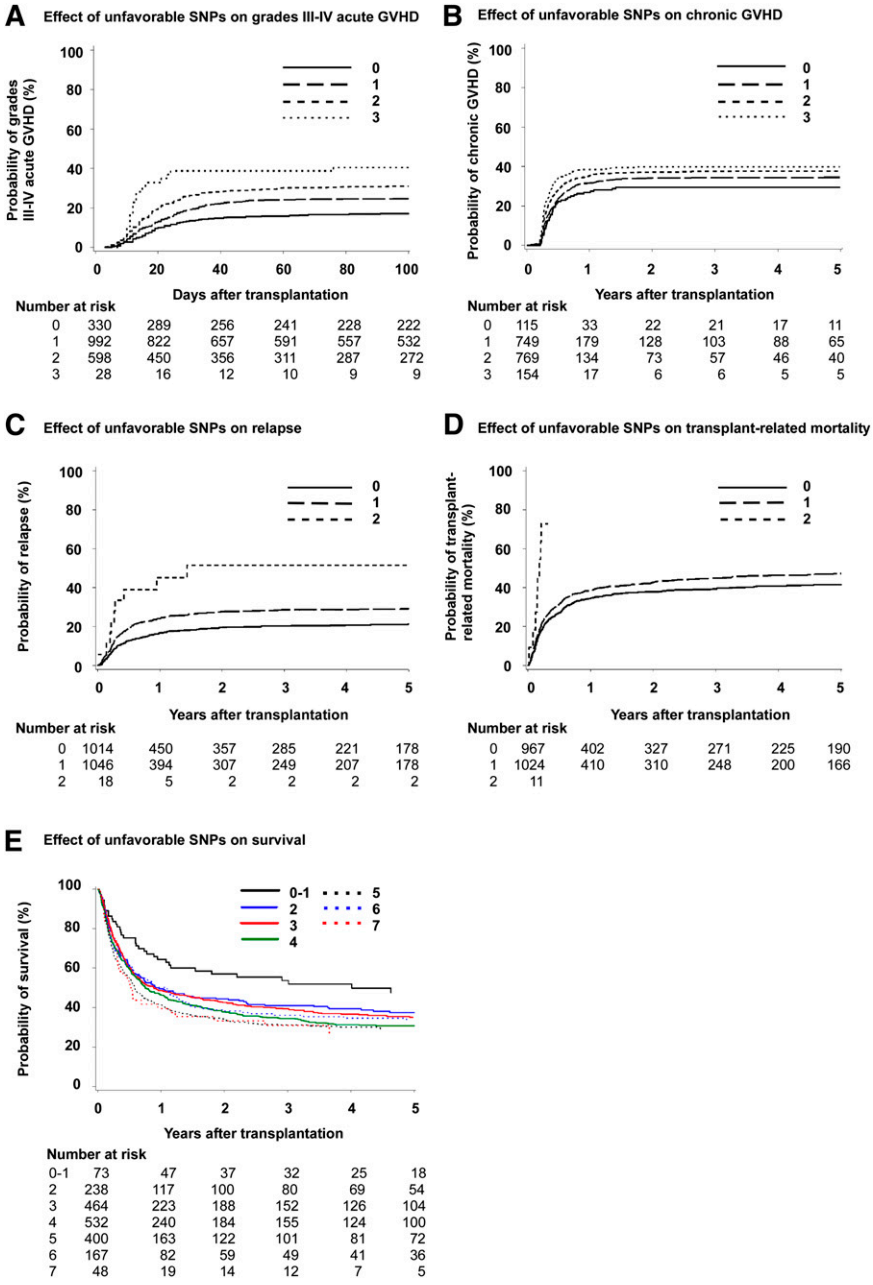

Figure 3. Impact of total number of unfavorable SNP genotypes on transplant outcome. The impact of the total number of unfavorable SNP genotypes was determined for patients and donors with complete SNP genotyping for all SNPs descriptive of the end point of interest. (A) Effect of unfavorable genotypes at SNPs rs209130, rs2075800, and rs394657 on grades III-IV acute GVHD. (B) Effect of unfavorable genotypes at SNPs rs2523957, rs3830076, rs2071479, and rs107822 on chronic GVHD. (C) Effect of unfavorable genotypes at SNPs rs2244546 and rs986522 on relapse. (D) Effect of unfavorable genotypes at SNPs rs915654 and rs429916 on transplant-related mortality. (E) Effect of 12 unfavorable SNP genotypes on survival.

predict that relapse is inevitable. To identify SNPs informative for relapse with or without GVHD, we used a Cox proportional hazard model for relapse with time-dependent variables for acute and chronic GVHD. Chronic but not acute GVHD was significantly associated with a lower risk for relapse (HR, 0.62; 95% CI, 0.47-0.82; $P < .0008$). None of the 8 risk SNPs associated with acute or chronic GVHD met a $P < .01$ significance level; however, SNPs rs2244546 and rs986522 remained predictive of relapse. Compared with transplants with 0 unfavorable SNPs for relapse, transplants with 1 or 2 unfavorable SNPs had an HR of 1.65 (95% CI, 1.36-2.00; $P < .0001$). A landmark analysis was performed on 1121 patients evaluable at day 100 without relapse. The effects of SNPs rs2244546 ($P = .0054$) and rs986522 ($P = .0022$) on relapse remained similar regardless of the presence or absence of acute GVHD. These results suggest that avoidance of donors with unfavorable SNPs for relapse may help to lower relapse independent of GVHD.

Haplotype content

Of the 12 SNPs, 3 (rs915654, rs2075800, and rs429916) were associated with transplant outcome through the patient's genotype. We hypothesized that these 3 SNPs define extended HLA haplotypes. Three biallelic SNPs yield 8 possible SNP haplotypes (Table 3). Some haplotypes displayed SNP haplotype diversity, whereas others were unique (supplemental Table 2). These data demonstrate that individuals with the same tissue type may have different extended haplotypes defined by their linked SNPs. Because the total number of favorable rs915654-rs2075800-rs429916 haplotypes depends on both the individual haplotype content and the combination of parental haplotypes, 2 individuals with the same HLA tissue type may have different numbers of favorable SNPs (Figure 4). Therefore, the consequences of HLA-SNP haplotype diversity leads to heterogeneity of transplant risks among patients with the same tissue type.

Table 3. HLA-SNP haplotype content

SNP haplotype				
rs915654*	rs2075800	rs429916	HLA haplotype	International Histocompatibility Workshop (IHW) reference cell number[10]
A	A	A	A*11:01-C*04:01-B*35:01-DRB1*01:01-DQB1*05:01	09006
A	A	C	A*02:01-C*05:01-B*44:02-DRB1*04:01-DQB1*03:01	09090
A	G	A	A*68:02-C*04:01-B*53:01-DRB1*15:03-DQB1*06:02	09010
A	G	C	A*02:01-C*01:02-B*27:05-DRB1*01:01-DQB1*05:01	09004
T	A	A	A*02:01-C*14:02-B*51:01-DRB1*08:03-DQB1*03:01	09070†
T	A	C	A*31:01-C*04:01-B*35:01-DRB1*04:01-DQB1*03:01	09025
T	G	A	A*01:01-C*07:01-B*08:01-DRB1*03:01-DQB1*02:01	09022, 09023, 09086†, 09088†
T	G	C	A*03:01-C*07:02-B*07:02-DRB1*15:01-DQB1*06:02	09013, 09017, 09081, 09318

A total of 163 homozygous individuals were characterized for 12 SNPs of clinical significance. The table shows one representative haplotype for each of the 3 SNP haplotypes defined by rs915654, rs2075800, and rs429916 which were each associated with transplant outcome through the patient's genotype. A full listing of the 12 SNPs among homozygous individuals is provided in supplemental Table 2.

*SNP rs915654 was PCR-amplified from 50 ng genomic DNA (0.42 μM of CAGCTCCAACCCCTCTAACA forward and CCTGCTGATACCCTCCAAAG reverse primers; 1× Apex Hot Start Master Mix [Genesee Scientific]) following 15 min at 95°C activation; 30 s at 95°C denaturation, 30 s at 60°C annealing, 2 min at 72°C extension for 30 cycles; 7 min at 72°C extension, and 4°C hold. Amplified products were sequenced with BigDye Terminator v.3.1 Cycle Sequencing kits and the 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

†IHW09070, IHW09086, and IHW09088 were heterozygous at rs429916.

Discussion

HLA-mismatched unrelated donor transplantation is a high-risk procedure. We found that clinical outcome after HLA-mismatched transplantation depends on undetected haplotype-linked SNPs that have synergistic effects with HLA mismatching. HLA haplotypes differ with respect to their SNP content. Genetic diversity of HLA-SNP haplotypes leads to heterogeneity of risks even among transplants with the same HLA mismatch. These observations indicate that the overall success of HLA-mismatched unrelated donor transplantation can be improved for future patients through a pretransplant definition of patients' haplotypes and patient and donor SNP genotypes. The avoidance of donors with multiple unfavorable SNPs may help to lower mortality. A patient's haplotypes cannot be modified, but if the patient carries high-risk variants, then these patients may benefit from alternative approaches for managing and preventing GVHD. Hence, a paradigm that considers the MHC region genetics of both the patient and the donor may aid in increasing the safety of HLA-mismatched unrelated donor transplantation.

We previously identified two SNPs, rs887464 and rs2281389, that play a role in HLA-matched unrelated donor transplantation.[11] In the current study, only rs2281389 showed a suggestive effect for acute GVHD ($P = .04$). There are several possible reasons for our findings. HLA-mismatched pairs were genetically different from HLA-matched pairs, in that they had higher frequencies of SNP mismatching (Figure 1) and were more ethnically and genetically diverse, encoding 159 unique HLA genotypes that were not present in the HLA-matched population. More importantly, the 2 populations differed significantly for demographic characteristics that influence transplant outcome: patient age, year of transplantation, patient-donor gender, disease diagnosis, GVHD prophylaxis, DPB1 mismatching, and patient-donor ethnicity ($P < .0001$; Table 1). These observations underscore the importance of the use of cohorts

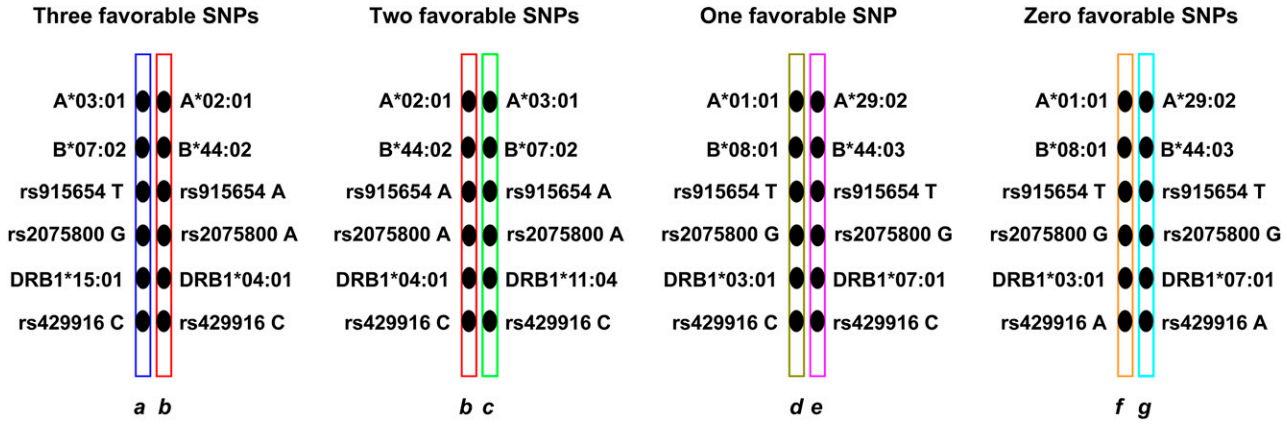

Figure 4. Patient-derived risks are defined by haplotypes. A patient's haplotypes can be used to assess risks associated with rs915654 (transplant-related mortality), rs2075800 (grades III-IV acute GVHD), and rs429916 (survival, disease-free survival, and transplant-related mortality). The number of favorable genotypes among these three positions depends on the combination of the patient's maternal and paternal haplotypes. Illustrated are patients whose haplotypes contribute 3, 2, 1, or 0 favorable genotypes at the 3 SNP positions. The patient with *ab* haplotypes has the TA-GA-CC haplotype at SNPs rs915654, rs2075800, and rs429916, respectively; these genotypes are all favorable (Table 2). The patient with *bc* haplotypes has 2 favorable (rs2075800AA and rs429916CC) and 1 unfavorable (rs915654AA) genotypes. The patient with *de* haplotypes has 1 favorable (rs429916CC) and 2 unfavorable (rs915654TT and rs2075800GG) genotypes. The patient with *fg* haplotypes has all unfavorable genotypes. The total number of favorable SNPs is defined as the combination of the maternal and paternal haplotypes. For example, the presence of HLA-A3-B7-DR15 (*a* haplotype) with HLA-A2-B44-DR4 (*b* haplotype) yields 3 favorable (rs915654, rs2075800, and rs429916) genotypes, but the same HLA-A2-B44-DR4 (*b* haplotype) with HLA-A3-B7-DR11 (*c* haplotype) yields 2 favorable genotypes. Two individuals with the same HLA tissue type may have different numbers of favorable SNPs because of haplotype diversity. For example, an individual with the HLA-A1,29-B8,44-DR3,7 tissue type can encode either TT-GG-CC (1 favorable SNP; haplotypes *de*) or TT-GG-AA (0 favorable SNPs; haplotypes *fg*) as a result of haplotypic diversity (Table 3; supplemental Table 2). Although a patient's haplotypes cannot be modified, knowledge of the haplotypes can be used for risk assessment, and risks can be lowered through the avoidance of donors with unfavorable genotypes or SNP mismatching.

that have similar HLA match status and other demographic variables for validation of the 12 SNPs in the future.

Delineation of the HLA-SNP haplotypes provides a first approximation of genetic diversity among several common North American white haplotypes and contrasts sharply with more conserved SNP haplotypes in other populations.[27] Two of the 1108 SNPs in this study were evaluated in Japanese transplants, where mismatching at SNP rs1799964 but not the recipient genotype at SNP rs2075800 was associated with grade IV acute GVHD.[28] In our data, mismatching at SNP rs1799964 yielded $P = .0054$ for grade IV acute GVHD, which did not reach significance after Bonferroni adjustment. The 2 studies evaluated transplants with different ethnicities and known HLA haplotype diversity (North American vs Japanese),[27] HLA match status (single mismatches vs all match grades) and study design (SNP discovery vs candidate gene approach). Future studies with sufficiently large numbers of ethnically diverse populations are required to fully evaluate the impact of SNPs in populations with different clinical and demographic characteristics.

SNP haplotype diversity has important clinical implications. Patients with the same tissue type may have different extended HLA-SNP haplotypes that give rise to different numbers of favorable SNPs and different risks (Figure 4). Although a patient's inherited HLA haplotypes might set the stage for variable degrees of protection or risk, transplant-associated risks can be lessened by the judicious avoidance of donors with unfavorable SNP genotypes or mismatching. Central to the global effort to increase the sharing of donors internationally[1] is the need for more complete information on haplotype content of clinically relevant MHC variation in ethnically diverse populations.

When the only option for transplantation is an HLA-mismatched unrelated donor, the criteria for selecting the donor with the least risky HLA mismatch include consideration of the specific HLA locus that is mismatched.[29] If certain HLA mismatches are associated with favorable SNPs, then both HLA and SNP haplotypes could aid clinicians in donor selection. We explored whether certain HLA mismatches are more likely to be associated with favorable SNPs. We found that the 2 most frequent HLA-C and HLA-DQB1 mismatches had significantly different frequencies of favorable SNPs for chronic GVHD and acute GVHD, respectively. Interestingly, C*03:03/03:04 is an "allele" mismatch, which, as a group, has historically been associated with lower posttransplant risks compared with "antigen" mismatches such as C*01:01/02:02.[3,4] Hence, the features that define a "permissible" HLA mismatch might be highly complex and involve polymorphisms that extend well beyond the physical boundaries of the HLA locus itself. These hypotheses should be amenable to examination in a much larger cohort of HLA-mismatched transplant pairs in the future.

The biological mechanisms that lead to the development of chronic GVHD in the absence of prior acute GVHD are unknown. We observed that patients mismatched at SNPs rs2523957 and rs107822 had a higher risk of chronic GVHD that did not depend on acute GVHD, suggesting that efforts to prospectively match patients and donors at these 2 SNPs will lower risks of chronic GVHD. Regardless of whether patients developed chronic GVHD or not, the risk of relapse depended on the presence of unfavorable SNPs for relapse. These observations may help to explain, in part, why some patients relapse despite clinical GVHD, whereas other patients are spared of both GVHD and relapse. The SNP associations permit further investigation into the genetic basis of GVHD-(in)dependent relapse and afford new possibilities for pretransplant risk assessment and for optimizing individualized treatment of patients.[25] Our data may also provide a means to explore GVHD that occurs after autologous or syngeneic transplantation,[30] particularly for SNP rs2075800, which was associated with GVHD through the patient's genotype.

SNPs provide clues to the candidate genes and mechanisms involved in transplant-associated complications. The identification of 2 SNPs within the HLA-DOA and -DOB genes provides strong evidence for a role for antigen processing and presentation in immune responses in HLA-mismatched transplantation.[31] The rs2075800G/A SNP defines a glutamic acid/lysine substitution at residue 602 of the heat shock protein-70 A1L molecule. We hypothesize a possible role for the differential binding of peptides by heat shock protein-A1L molecules and/or stimulation of cytokines in GVHD.[32] The second SNP marker for grades III-IV acute GVHD, rs394657, resides within the NOTCH4 gene intron and is in positive linkage disequilibrium with nonsynonymous substitutions. Sequence polymorphism of NOTCH4 receptors could influence the inflammatory nature of acute GVHD through altered ligand-receptor binding and production of TNF-α, IFN-γ, IL-4, and IL-17.[33] Alternatively, SNP rs394657 might influence GVHD through its role as a putative expression quantitative locus for HLA-DQA1,[34] the gene that encodes the DQα chain of the HLA-DQ heterodimer. Differential DQα expression may have consequences for alloantigen recognition in GVHD.

In conclusion, HLA and non-HLA MHC factors contribute to the high morbidity and mortality after HLA-mismatched unrelated donor transplantation. Knowledge of HLA-SNP haplotype-associated risks may provide clinicians with an approach for increasing the safety of HLA-mismatched transplantation. In the future, validation of the SNPs from this study will be feasible with a larger HLA-mismatched transplant experience. Systematic evaluation of HLA haplotypes in other HLA-mismatched settings including haploidentical related and cord blood transplantation may facilitate the investigation of the MHC barrier in transplantation.

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Authorship

Contribution: E.W.P. developed the hypotheses and designed the study; E.W.P., M.M., S.R.S., M.D.H., and M.M.H. provided HLA and clinical data; M.M. managed DNA genotyping and data; T.W. performed the statistical analysis; E.W.P., M.M., and T.W. analyzed the data; and all authors contributed to the preparation of the paper and approved the final manuscript.

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