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HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism

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The finding that the risk of relapse in hematologic malignancy decreases after allogeneic hematopoietic stem cell transplantation (HSCT) has lead to the concept of a graft-versus-leukemia (GVL) effect. However, this beneficial effect is considered to be frequently offset by graft-versus-host disease (GVHD). Thus, improving HSCT outcomes by separating GVL from GVHD is a key clinical issue. This cohort study registered 4643 patients with hematologic malignancies who received transplants from unrelated donors. Six major human leukocyte antigen (HLA) loci were retrospectively genotyped. We identified 4 HLA-Cw and 6 HLA-DPB1 mismatch combinations responsible for a decreased risk of relapse; of these, 8 of 10 combinations were different from those responsible for severe acute GVHD, including all 6 of the HLA-DPB1 combinations. Pairs with these combinations of HLA-DPB1 were associated with a significantly better overall survival than were completely matched pairs. Moreover, several amino acid substitutions on specific positions responsible for a decreased risk of relapse were identified in HLA-Cw, but not in HLA-DPB1. These findings might be crucial to elucidating the mechanism of the decreased risk of relapse on the basis of HLA molecule. Donor selection made in consideration of these results might allow the separation of GVL from acute GVHD, especially in HLA-DPB1 mismatch combinations. (Blood. 2009;113:2851-2858)

Introduction

The use of allogeneic hematopoietic stem cell transplantation (HSCT), an established treatment for hematologic malignancies, is associated with several immunologic events with contrary effects in the recipient. In graft-versus-host disease (GVHD), for example, graft immune cells attack host organs, whereas in the graft-versus-leukemia (GVL) effect, they eradicate residual leukemia cells.[1-3] GVL is likely to function not only in hematologic malignancies but also in solid tumors, particularly breast cancer and renal cell carcinoma,[4-6] in which it is referred to as the graft-versus-tumor (GVT) effect. Because both GVL and GVHD are caused by either or both major and minor histocompatibility antigen mismatches between donor and recipient, the beneficial effect of allogeneic HSCT due to GVL is thought to be frequently offset by GVHD. Thus, improving HSCT outcome by separating GVL from GVHD is a key clinical issue. Importantly, however, while most such efforts have been in the area of minor histocompatibility antigen,[7] few researchers have approached this problem in terms of the major histocompatibility antigen.

We recently identified 16 human leukocyte antigen (HLA) mismatch combinations associated with a high risk of severe acute GVHD. Results showed that the overall number of these high-risk mismatches was strongly associated with the occurrence of severe acute GVHD and poor overall survival (OS).[8] We speculated that the intensity of GVL and acute GVHD in any particular mismatch might not necessarily be parallel, and that among HLA mismatch combinations not inducing severe acute GVHD, those that induce strong GVL might occur. In other words, the hypotheses of this study were that particular mismatch combinations allow the separation of GVL from acute GVHD and that specific amino acid substitutions in HLA molecules contribute to this mechanism.

As part of efforts to improve donor selection and allogeneic HSCT outcomes, we identified HLA mismatch combinations that resulted in a decreased risk of relapse in all 6 major HLA loci and compared them with mismatch combinations carrying a high risk of severe acute GVHD. Further, we investigated specific amino acid substitution positions in the HLA molecule responsible for a decreased risk of relapse.

Methods

Patients

This study was conducted using clinical data that were collected prospectively at transplant centers participating in the Japan Marrow Donor Program. Patients who received a first transplant of T cell–replete marrow for a hematologic malignancy from a serologically HLA-A, -B, and -DR antigen-matched unrelated donor between January 1993 and December 2005 through the Japan Marrow Donor Program (n = 4643) were registered. Eligible diagnoses included acute lymphoblastic leukemia (ALL); acute myeloid leukemia (AML), which included only de novo AML;

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Table 1. Patient characteristics

		A locus		B locus		C locus		DRB1 locus		DQB1 locus		DPB1 locus	
	Total	Match	Mismatch	Match	Mismatch	Match	Mismatch	Match	Mismatch	Match	Mismatch	Match	Mismatch
	4643	4018	625	4351	292	3308	1335	3718	925	3597	1046	1584	3059
Median age, y	31.5	31.8	29.6	31.7	28.3	31.8	30.9	31.7	30.9	31.7	30.8	31.8	31.4
Sex, donor/patient													
Male/male	1904	1673	231	1769	135	1387	517	1551	353	1492	412	678	1226
Male/female	923	789	134	874	49	650	273	734	189	704	219	299	624
Female/male	894	747	147	843	51	634	260	693	201	672	222	268	626
Female/female	922	809	113	865	57	637	285	740	182	729	193	339	583
Disease													
ALL	1464	1267	197	1372	92	1051	413	161	303	1132	332	452	1012
AML	1571	1360	211	1478	93	1114	457	1255	316	1224	347	574	997
CML	979	827	152	905	74	682	297	779	200	746	233	343	636
ML	564	507	57	536	28	43	146	468	96	49	118	192	372
MM	65	57	8	60	5	418	22	55	10	446	16	23	42
Risk of leukemia relapse*													
Standard risk	1684	1485	199	1588	96	1184	500	1375	309	1322	362	572	1112
High risk	1909	1607	302	1772	137	1365	544	1485	424	1451	458	642	1267
Disease other than leukemia	1050	926	124	991	59	759	291	858	192	824	226	370	680
GVHD prophylaxis													
Cyclosporine-based	2503	2159	344	2346	157	1802	701	2107	396	2030	473	881	1622
Tacrolimus-based	2140	1859	281	2005	135	1506	634	1611	529	1567	573	703	1437
ATG													
ATG	152	112	40	135	17	102	50	110	42	118	34	51	101
Non-ATG	4491	3906	585	4216	275	3206	1285	3608	883	3479	1012	1533	2958
Preconditioning													
TBI regimen	3687	3175	512	3445	242	2623	1064	2933	754	2834	853	1242	2445
Non-TBI regimen	956	843	113	906	50	685	271	785	171	763	193	342	614

ATG indicates antithymocyte globulin; and TBI, total body irradiation.

*Standard risk for leukemia relapse was defined as the status of the first complete remission of AML and ALL and the first chronic phase of CML at transplant, while high risk was defined as a more advanced status than standard risk in AML, ALL, and CML. Disease other than leukemia was defined as other than ALL, AML, and CML.

chronic myeloid leukemia (CML); malignant lymphoma (ML); and multiple myeloma (MM).

Patient characteristics are shown in Table 1. A final clinical survey of the patients was completed by December 2006. Informed consent was obtained from patients and donors in accordance with the Declaration of Helsinki, and approval for the study was obtained from the Institutional Review Board of Aichi Cancer Center and the Japan Marrow Donor Program.

HLA typing of patients and donors

Alleles at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were identified by previously described methods in all 4643 pairs at the Japanese Red Cross Tokyo Metropolitan Blood Center.[8,9]

Matching of HLA allele between patient and donor

HLA allele mismatch among the donor-recipient pair was scored when the recipient's alleles were not shared by the donor (graft-versus-host vector) for all analyses.

Definition of relapse

Relapse was defined as the recurrence of malignancy as detected by the parameter by which the malignancy was first detected, namely marrow morphology; flow cytometry; cytogenetic studies, including fluorescence in situ hybridization; electrophoresis; immunofixation assays; polymerase chain reaction-based assays for disease markers; or imaging results. The day of relapse was defined as the day on which the respective clinical, hematologic, cytogenetic, or molecular relapse was recognized.

Definition of amino acid substitution

Amino acid sequences of HLA-Cw and -DPB1 molecules were obtained from the IMGT/HLA sequence database.[10] For example, Tyr99C-Phe99C indicated an amino acid substitution at position 99 in the HLA-C molecule in which the donor had tyrosine and the patient had phenylalanine. Substituted amino acids in HLA-Cw and -DPB1 are summarized in Tables S1 and S2 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Statistical analysis

OS rate was assessed using the Kaplan-Meier product limit method. To eliminate the effect of competing risk, the cumulative incidence of relapse was assessed using a previously described method.[11,12] The competing event for relapse was defined as death without relapse. Impact by the factor of interest was assessed using the log rank test. The impact of HLA allele mismatch combinations and the position and type of amino acid substitution (for example, alanine, arginine, and asparagine) in HLA molecules were evaluated using multivariable Cox regression analysis[13] for OS and the occurrence of acute GVHD, while the risk of relapse was evaluated using the multivariable proportional hazard modeling of subdistribution functions in competing risks.[14]

HLA mismatch combinations were evaluated for each locus separately. When the locus of interest was evaluated, we allowed the other loci to be mismatched, with the status of such mismatches adjusted for in the same way as other confounders. The HLA match and HLA one-allele mismatched in every locus were analyzed. For example, the A*0206-A*0201 mismatch combination meant that the donor had HLA-A*0206, the recipient had HLA-A*0201, while another HLA-A allele of the donor and recipient was identical. This mismatch was compared with the HLA-A allele match. Mismatch combinations that had 9 or fewer pairs were combined together as "other mismatch." The model was constructed with mismatch combinations, mismatch status in other loci (match, 1 allele mismatched, and 2 alleles mismatched, as an ordinal variable), and potential confounders. Confounders considered were sex (donor-recipient pair), patient age (linear), donor age (linear), transplant year, type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (cyclosporine [CSP] vs tacrolimus [FK]), ATG (vs no ATG), and

preconditioning (TBI vs non-TBI). These confounders were used in all analyses to maintain the comparability of results.

The impact of position and type of amino acid substitutions in HLA molecules was evaluated in pairs with oneallele mismatched in HLA-Cw and -DPB1 separately. The amino acid positions we analyzed were all positions at which an amino acid was substituted in the respective locus. We analyzed the impact of each amino acid substitution on each position separately. Multivariable models were constructed to include the position and type of amino acid substitution, mismatch status in other loci (match, 1 allele mismatched, and 2 alleles mismatched as an ordinal variable) and the confounders described above. A P value less than .05 was considered statistically significant. All statistical tests were 2-sided. All analyses were performed using STATA version 10.0 (StataCorp, College Station, TX) and R version 2.5.1 (The R Foundation for Statistical Computing, www.r-project.org).

Validation of statistical analysis

Statistical analyses were validated using the bootstrap resampling method.[15] Briefly, we estimated the measure of association with resampled data drawn repeatedly from the original data. Although approximately 100 to 200 bootstrapped samples are generally sufficient,[16] we used 1 000 bootstrap samples for all analysis validations. Further, we judged the results of analysis as statistically significant only when the results of both base analysis and analysis validation using bootstrap resampling were significant; cases in which the result of base analysis was significant but that of analysis validation using bootstrap resampling was not are indicated by an asterisk next to the P value of the base analysis.

Results

Impact of HLA allele mismatches in locus level on relapse

The number of mismatched alleles of HLA-Cw (1 allele mismatched: hazard ratio [HR], 0.68; 95% confidence interval [CI], 0.58-0.80; 2 alleles mismatched: HR, 0.43;95% CI, 0.24-0.75) and HLA-DPB1 (1 allele mismatched: HR, 0.80; 95% CI, 0.70-0.92; 2 alleles mismatched: HR, 0.62; 95% CI, 0.51-0.75) was strongly associated with a decreased risk of relapse. In contrast, no associations were seen for HLA-A (1 allele mismatched: HR, 1.00; 95% CI: 0.82-1.22; 2 alleles mismatched: HR, 0.79; 95% CI, 0.28-2.28), HLA-B (1 allele mismatched: HR, 1.06; 95% CI, 0.79-1.41; 2 alleles mismatched: not applicable), HLA-DRB1 (1 allele mismatched: HR, 0.93; 95% CI, 0.74-1.18; 2 alleles mismatched: HR, 1.18, 95% CI: 0.53-2.63) or HLA-DQB1 (1 allele mismatched: HR, 1.12; 95% CI, 0.90-1.40; 2 alleles mismatched: HR, 0.73; 95% CI, 0.35-1.52; Figure 1; Table 2).

Impact of HLA mismatch combinations on relapse

Four mismatch combinations in HLA-Cw and 6 in HLA-DPB1 were significantly associated with a decreased risk of relapse (Tables 3 and S3). In contrast, mismatch combinations in HLA-A, -B, -DRB1, and -DQB1 were not significantly associated with differences in risk of relapse (data not shown). The 10 HLA mismatch combinations associated with lower risks of relapse were Cw*0102-Cw*1402 (HR not estimated due to no event), Cw*0801-Cw*0102 (HR not estimated), Cw*1402-Cw*0304 (HR not estimated), Cw*1502-Cw*1402 (HR, 0.28; 95% CI, 0.09-0.88), DPB1*0402-DPB1*0201 (HR, 0.32, 95% CI, 0.12-0.87), DPB1*0501-DPB1*0201 (HR, 0.67; 95% CI:0.50-0.91), DPB1*0501-DPB1*0401 (HR, 0.36; 95% CI, 0.13-0.98), DPB1*0501-DPB1*0402 (HR, 0.55; 95% CI, 0.33-0.93), DPB1*0901-DPB1*0201 (HR, 0.37; 95% CI, 0.14-0.96), and DPB1*1301-DPB1*0201 (HR not estimated; Tables 3 and S3). All 10 HLA mismatch combinations were also significant on validation analysis using the bootstrap resampling method. We speculated that these mismatch combinations would mainly decrease the risk of relapse due to GVL, so we tentatively call them GVL mismatch combinations.

Evaluation of clinical importance of GVL mismatch combinations

We evaluated the clinical importance of GVL mismatch combinations in HLA-Cw and -DPB1. All analyses in this section were conducted in matched pairs other than the evaluated locus. In HLA-C mismatch, the small number of patients with GVL mismatch combinations (n = 13) in matched pairs at the allele level for HLA-A, -B, -DRB1, -DQB1, and -DPB1 prevented comprehensive analysis. We evaluated the GVL mismatch combinations of HLA-DPB1 in matched pairs for HLA-A, -B, -Cw, -DRB1, and -DQB1. Pairs with HLA-DPB1 mismatch were divided into 2 groups, those with a GVL mismatch combination and those with mismatch combinations other than GVL mismatch combinations. These were then compared with 12/12 matched pairs for association with severe acute GVHD, relapse, and OS (Table 4). The curve of the cumulative incidence of OS is shown in Figure 2. Multivariable analysis revealed that although OS was similar between the 12/12 matched pairs and the pairs with mismatch combinations other than GVL mismatch combinations, it was significantly improved in pairs with a GVL mismatch combination (Table 4). In terms of mortality due to relapse according to HLA-DPB1 matching status and whether the mismatch combinations were GVL mismatch combinations, the HLA-DPB1 matched group, HLA-DPB1 1 allele mismatched group, and GVL mismatch combination group showed an expected decreased mortality due to relapse (20.0%, 15.3%, and 10.5%, respectively). Further, mortality due to relapse in the GVL mismatch combination group was significantly lower than that in the HLA-DPB1 1 allele mismatched group (P = .049). We conducted the same analyses with stratification by leukemia type (ALL, AML, or CML) and found that the myeloid malignancies (AML and CML) had the same tendency (Table 4). In particular, in CML, GVL mismatch combinations in HLA-DPB1 were associated with a significantly reduced risk of relapse (HR, 0.14; 95% CI, 0.03-0.55) and significantly improved OS relapse (HR, 0.50; 95% CI, 0.25-0.98).

Impact of position and type of amino acid substitutions of HLA molecules on relapse

We surveyed all substituted positions in HLA-Cw and -DPB1 and found 159 specific amino acid substitutions at 55 positions in HLA-Cw and 55 specific amino acid substitutions at 19 positions in HLA-DPB1 (Tables S1,S2). Analysis revealed 3 specific amino acid substitutions responsible for a decreased risk of relapse in HLA-C, namely Ser9C-Tyr9C (HR, 0.53; 95% CI, 0.30-0.92), Phe99C-Tyr99C (HR, 0.52, 95% CI, 0.30-0.91), and Arg156C-Leu156C (HR, 0.59; 95% CI, 0.37-0.92). In contrast, no decrease in the risk of relapse was seen for substitutions in HLA-DPB1 (Table 5). However, Tyr9C-Ser9C and Tyr99C-Phe99C were strongly linked (see "Discussion"). These specific amino acid substitutions were all significant on validation analysis using the bootstrap resampling method.

Discussion

Improving outcomes in allogeneic HSCT for hematologic malignancies by separating GVL from GVHD is considered a key clinical

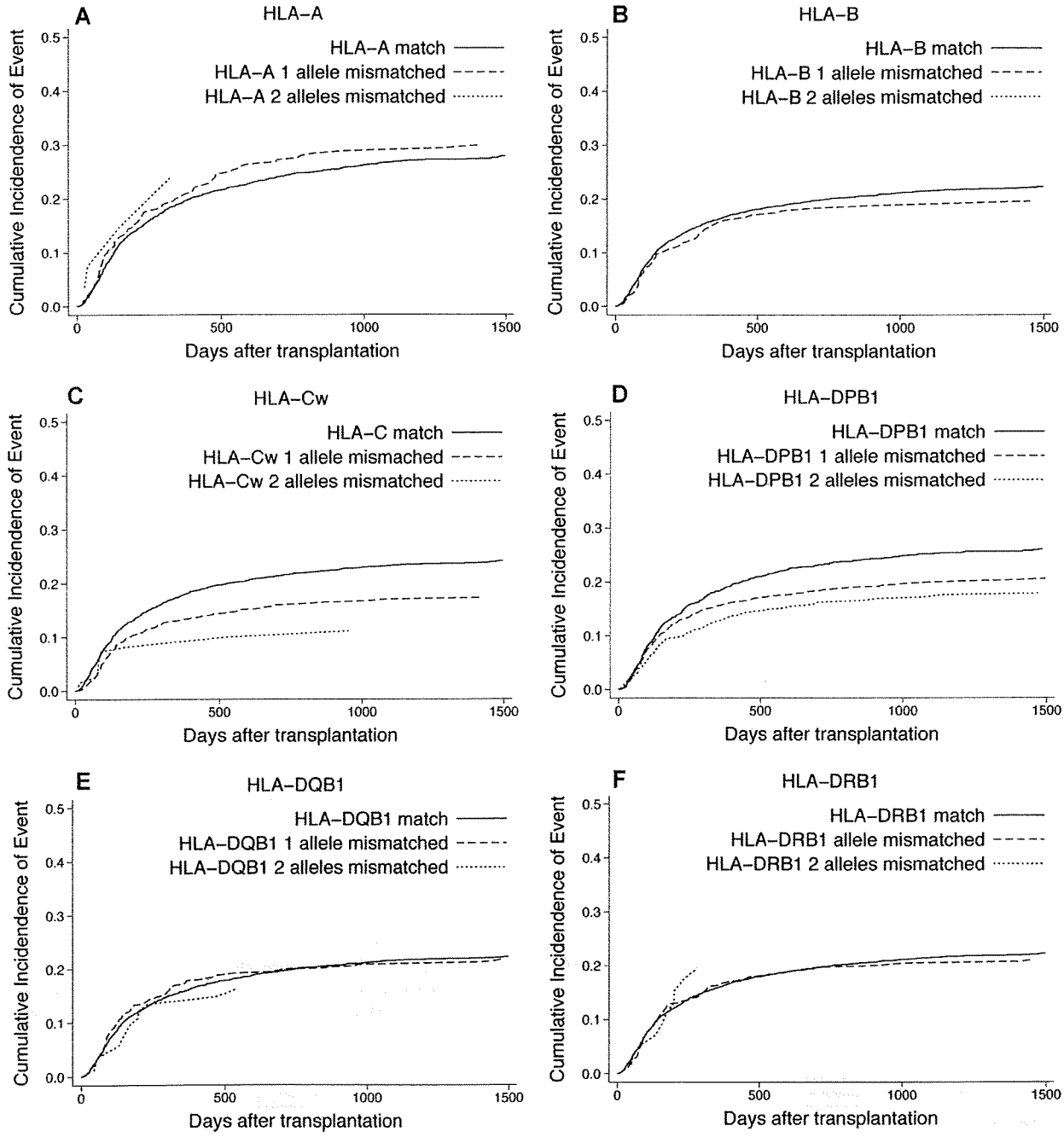

Figure 1. Impact of individual HLA locus mismatches on relapse. Cumulative incidence of relapse for each HLA locus. [—] indicates matched pairs in each locus; [····], 1-allele mismatched pairs in each locus; and [...], 2-allele mismatched pairs in each locus.

challenge. Here, our analysis demonstrated that several donor-recipient HLA mismatch combinations and specific amino acid substitutions in HLA molecules were associated with a decreased risk of relapse, and, in some cases, no significant increase in the risk of severe acute GVHD. These findings suggest that GVL might be separated from severe acute GVHD by selection of suitable HLA mismatch combinations.

We recently reported 16 significant high-risk HLA allele mismatch combinations for severe acute GVHD in 6 HLA loci, a number of which were highly associated with the occurrence of severe acute GVHD and worse OS.[8] Of note, a group of pairs with mismatches other than severe acute GVHD high-risk mismatches showed an incidence of severe acute GVHD and OS rates almost equal to those of 12/12 matched pairs. In the present study, we elucidated a total of 10 mismatch combinations that were significantly associated with a decreased risk of relapse, which we termed GVL mismatch combinations. Of course, it is possible that some mismatch combinations not classified as GVL mismatch combinations might actually induce strong GVL. Misclassification might have occurred as a result of insufficient statistical power due to the relatively small number of patients in the subcategories. Among these mismatch combinations, 2 of 4 in HLA-Cw were identical to the severe acute GVHD high-risk combinations; a third had a marginal effect on the occurrence of severe acute GVHD, while the

Table 2. Impact of HLA mismatches in allele level on relapse

	All diseases		
	n	HR (95% CI)	P
HLA-A matched	4018	1.00 (ref)	
HLA-A 1 allele mismatched	597	1.00 (0.82-1.22)	.99
HLA-A 2 alleles mismatched	28	0.79 (0.28-2.28)	.67
HLA-B matched	4351	1.00 (ref)	
HLA-B 1 allele mismatched	288	1.06 (0.79-1.41)	.7
HLA-B 2 alleles mismatched*	4	ND	ND
HLA-C matched	3308	1.00 (ref)	
HLA-C 1 allele mismatched	1212	0.68 (0.58-0.80)	<.001
HLA-C 2 alleles mismatched	123	0.43 (0.24-0.75)	.003
HLA-DRB1 matched	3718	1.00 (ref)	
HLA-DRB1 1 allele mismatched	866	0.93 (0.74-1.18)	.56
HLA-DRB1 2 alleles mismatched	59	1.18 (0.53-2.63)	.68
HLA-DQB1 matched	3597	1.00 (ref)	
HLA-DQB1 1 allele mismatched	958	1.12 (0.90-1.40)	.30
HLA-DQB1 2 alleles mismatched	88	0.73 (0.35-1.52)	.40
HLA-DPB1 matched	1584	1.00 (ref)	
HLA-DPB1 1 allele mismatched	2190	0.80 (0.70-0.92)	.002
HLA-DPB1 2 alleles mismatched	869	0.62 (0.51-0.75)	<.001

Each group was compared with the matched group in each locus after adjusting for other matching status of HLA, sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (CSP vs FK), ATG vs no ATG), and preconditioning (TBI vs non-TBI).

ref indicates reference; and ND, not determined.

*Comprehensive analysis could not be performed due to the small number of cases.

fourth combination was different from acute GVHD high-risk mismatch combinations. In contrast, all 6 mismatch combinations in HLA-DPB1 were different from acute GVHD high-risk mismatch combinations (Table 3). As expected, HLA-A, -B, -Cw, -DRB1, and -DQB1 matched pairs with GVL mismatch combinations of HLA-DPB1 were associated with significantly better OS than 12/12 matched pairs (Table 4; Figure 2), indicating that the beneficial antitumor effect of GVL mismatch combinations in HLA-DPB1 would not be offset by the effect of severe acute GVHD. We speculate that conformational changes of HLA molecules in each mismatch combination control the intensity of the acute GVHD and GVL effect, as described later in "Discussion" and in our previous report[8]; namely, conformational changes of HLA molecules in GVL mismatch combinations in HLA-DPB1 induce strong GVL with mild or no acute GVHD. These findings suggest that HLA mismatch selection according to these results might improve HSCT outcomes over those obtained with a complete match. The same tendency was seen for AML and CML, whereas the effect of GVL mismatch combination in the HLA-DPB1 allele in ALL patients would be weaker than in the other leukemia types (Table 4). Comprehensive analyses for ML and MM could not be done because of the small number in each group. Thus, the effects of GVL mismatch combination vary according to disease type and may also change according to other factors, including particular cytogenetic abnormalities.

Recent research has shown that HLA-Cw and -DPB1 mismatch at the allele level is strongly associated with a decreased risk of relapse.[17,18] These findings were confirmed in the present large cohort. In addition, the present study also clarified that the mismatching of 2 alleles in either the HLA-Cw or -DPB1 locus had a stronger association with decreased risk than respective mismatching of one allele. Moreover, no association whatsoever was seen for

Table 3. GVL mismatch combinations

Mismatch combination, donor-recipient	n	HR (95% CI)	P
Cw*0102-Cw*1402*†	13	ND	ND
Cw*0801-Cw*0102*†	10	ND	ND
Cw*1402-Cw*0304†	20	ND	ND
Cw*1502-Cw*1402	43	0.28 (0.09-0.88)	.030
DPB1*0402-DPB1*0201*	54	0.32 (0.12-0.87)	.026
DPB1*0501-DPB1*0201*	301	0.67 (0.50-0.91)	.009
DPB1*0501-DPB1*0401*	48	0.36 (0.13-0.98)	.046
DPB1*0501-DPB1*0402*	112	0.55 (0.33-0.93)	.026
DPB1*0901-DPB1*0201*	43	0.37 (0.14-0.96)	.042
DPB1*1301-DPB1*0201*†	20	ND	ND

As an example of the mismatch combination analysis, the Cw*0102-Cw*1402 mismatch combination meant that the donor has HLA-Cw*0102, the recipient has HLA-Cw*1402 and another HLA-Cw allele of each donor and recipient was identical. Each mismatch pair in HLA-Cw was compared with the HLA-Cw allele match, and each mismatch pair in HLA-DPB1 was compared with the HLA-DPB1 allele match. All indicated results were concurrently significant in both the base analysis and validation analysis using bootstrap resampling.

ND indicates not determined.

*Mismatch combinations that were not significantly associated with a higher occurrence of severe acute GVHD in our previous study.[8] However, the Cw*0102-Cw*1402 mismatch combination has a marginal effect on the occurrence of severe acute GVHD; that is, Cw*0102-Cw*1402 was significantly associated with a higher occurrence of severe acute GVHD in base analysis, but not in validation analysis.

†HR was not estimated due to the lack of an event in this group.

Table 4. Clinical importance of GVL mismatch combinations in HLA-DPB1 mismatch

		Acute GVHD		Relapse		OS*	
All diseases	**n**	**HR (95% CI)**		**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
HLA-DPB1 matched	864	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	808	1.34 (1.03-1.74)	.028	0.83 (0.68-1.01)	0068	0.96 (0.83-1.12)	.62
GVL mismatch combination	258	1.18 (0.81-1.73)	.375	0.47 (0.33-0.67)	<.001	0.75 (0.59-0.94)	.012
ALL							
HLA-DPB1 matched	250	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	263	1.56 (0.96-2.54)	.067	0.85 (0.6-1.19)	.33	1.10 (0.85-1.43)	.48
GVL mismatch combination	80	1.27 (0.63-2.57)	.5	0.75 (0.45-1.26)	.28	0.95 (0.65-1.39)	.8
AML							
HLA-DPB1 matched	308	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	264	1.47 (0.9-2.39)	.13	0.83 (0.61-1.14)	.26	0.95 (0.74-1.23)	.72
GVL mismatch combination	89	1.25 (0.62-2.5)	.54	0.44 (0.24-0.78)	.006	0.71 (0.48-1.06)	.1
CML							
HLA-DPB1 matched	176	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	162	1.25 (0.74-2.14)	.41	0.69 (0.40-1.20)	.19	0.93 (0.65-1.33)	.69
GVL mismatch combination	54	1.13 (0.51-2.47)	.66	0.14 (0.03-0.55)	.005	0.50 (0.25-0.98)	.041

Each group was compared with the HLA-DPB1 matched group. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (CSP vs FK), ATG vs no ATG), and preconditioning (TBI vs non-TBI).
ref indicates reference.
*The HR indicates the likelihood that OS will be shorter (if HR > 1) or longer (HR < 1) than when the HLA type matches (ie, the Ref condition).

HLA-A, -B, -DRB1, or -DQB1 (Figure 1; Table 2). Furthermore, all 10 GVL mismatch combinations were elucidated from mismatch combinations of HLA-Cw and HLA-DPB1 (Tables 3 and S3), although we also analyzed HLA-A, -B, -DRB1, and -DQB1. These findings indicate that GVL after allogeneic HSCT is mainly induced by HLA-Cw and -DPB1, not HLA-A, -B, -DRB1 or -DQB1, although the role of each HLA locus might vary with the type of disease.[18] There are 3 possible explanations for this. First, the relative expression of HLA-Cw and -DPB1 on malignant cells may be higher than that on normal hematopoietic cells; second, HLA-Cw and -DPB1 may be preferentially expressed on malignant stem cells; and third, surface expression of a few key molecules—such as major histocompatibility complex (MHC), adhesion, and costimulatory molecules—on malignant cells may determine the effect of each HLA locus on GVL.[19-21] In other words, some molecules might stimulate GVL of HLA-Cw or -DPB1, and other molecules might block GVL of other than HLA-Cw and -DPB1. Further investigation of this question is warranted.

In this study, 3 specific amino acid substitutions responsible for GVL at positions 9, 99, and 156 were identified in HLA-Cw, of which only 2, Ser9C-Tyr9C and Phe99C-Tyr99C, were strongly linked in our sample. We were therefore unable to determine which substitutions are the main contributors to the effect of interest (Table 5). These amino acid positions, 9, 99, and 156, were identical to those we elucidated in our previous study as responsible for severe acute GVHD.[8] These findings suggest that these 3 amino acid positions are important determinants of alloreactivity. Although position 156 of the HLA molecule has been shown to modify T-cell alloreactivity in vitro in HLA-A2,[22-24] -B35,[25] and -B44,[26] to our knowledge, the present study is the first to identify positions 9 and 99. On the other hand, substituted amino acids were not necessarily identical. In Ser9C-Tyr9C and Phe99C-Tyr99C substitutions, for example, the substituted amino acid position was identical with that responsible for severe acute GVHD, whereas the substituted amino acids were inverse between donor and recipient, even though both substituted position and amino acids were identical in the Arg156C-Leu156C substitution. These findings suggest that Ser9C-Tyr9C and Phe99C-Tyr99C might play an important role in separating GVL from acute GVHD in HLA-Cw mismatch, although the mechanism requires further molecular clarification.

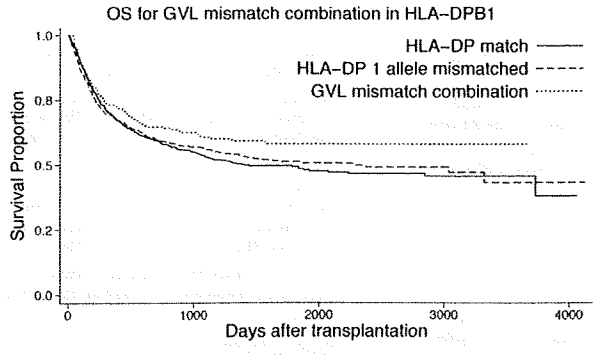

Figure 2. Clinical importance of GVL mismatch combinations in HLA-DP mismatch. Kaplan-Meier estimates of survival according to HLA-DPB1 mismatch status. The solid line indicates HLA-DPB1 matched pairs; the short broken line, HLA-DPB1 1 allele mismatched but not GVL mismatch combinations; and the dotted line, HLA-DPB1 1 allele mismatched (GVL mismatch combinations). All groups are HLA-A, -B, -C, -DRB1, and -DQB1 matched pairs.

Table 5. Impact of position and type of amino acid substitution of HLA molecules on relapse

Position and amino acid substitution in HLA-C (donor-recipient)	n	HR (95% CI)	*P*
Ser9C-Tyr9C	152	0.53 (0.30-0.92)	.024
Phe99C-Tyr99C	153	0.52 (0.30-0.91)	.022
Arg156C-Leu156C*	225	0.59 (0.37-0.92)	.020

The impact of position and type of amino acid substitution in HLA molecules was evaluated in pairs with HLA one-locus mismatch in HLA-C and -DPB1 separately. For example, Tyr9C-Ser9C indicated amino acid substitutions of position 9 in the HLA-C molecule in which the donor had tyrosine and the patient serine. The impact of position and kind of amino acid substitution in each HLA molecule was evaluated in pairs with HLA one locus mismatch in each HLA locus separately. Pairs that substituted a specific amino acid at each position were compared with amino acid matched pairs at that position.

No significant amino acid substitutions were found in HLA-DPB1.

All indicated results were concurrently significant in both base analysis and validation analysis using bootstrap resampling.

The 2 specific amino acid substitutions Tyr9C-Ser9C and Tyr99C-Phe99C were strongly linked in our sample.

*An amino acid substitution that was significantly associated with a higher occurrence of severe acute GVHD in our previous study.[8]

With regard to specific amino acid substitutions of HLA-DPB1, we found no significant association among these with a decreased risk of relapse. Shaw et al[27] reported that mismatches at position 57 and 65 in the HLA-DPB1 molecule were associated with transplant complications, but not with GVHD or relapse, which is consistent with our present data. We speculate that, compared with MHC class I, the conformational diversity of MHC class II and peptide complex hampers the identification of strict rules of association between specific amino acid substitutions in MHC class II molecules and the occurrence of alloreaction such as GVHD and GVL. In HLA class I, binding peptides are held by their ends, whereas peptides bind to HLA class II by attachment in the middle, allowing them to vary greatly in length.[28]

Given that this analysis was conducted using a Japanese cohort of patients who received transplants through the Japan Marrow Donor Program, the applicability of our data to other ethnic groups warrants discussion. We speculate that the effect of alloreaction is a reflection and summation of HLA allele mismatch combinations. Discrepancies in the effect of HLA locus on alloreactions between ethnically diverse transplantation might be explained by the proportions of each HLA mismatch combination in each HLA locus. In HLA-DPB1, on the other hand, the allele variations between white and Japanese populations are relatively close, hence our findings in HLA-DPB1 might also be useful for white populations. Regarding HLA-Cw and killer immunoglobulin-like receptor (KIR) incompatibility, we previously reported adverse effects in unrelated T cell–replete HSCT through the Japan Marrow Donor Program,[18] although Ruggieri et al[29] demonstrated that beneficial effects were shown in T-cell depleted haploidentical transplantation. We speculated that in vivo and/or in vitro T-cell depletion could account for this discrepancy.[30] Therefore, results for mismatch combinations in HLA-Cw obtained in other populations treated in other settings may differ from our results. Nevertheless, clarification of these questions would require the same study in other ethnic populations.

Given the general acceptance that GVL is more closely correlated with chronic GVHD than acute GVHD,[3] separating GVL from chronic GVHD may be more difficult than separating it from acute GVHD. On this basis, our results suggest that GVL could be separated from acute GVHD in HSCT from a specific HLA partially mismatched donor. Clarification of whether GVL can also be separated from chronic GVHD requires further study.

In conclusion, we identified 4 HLA-C and 6 HLA-DPB1 mismatch combinations that decrease the risk of relapse in patients after HSCT. Eight of 10 GVL combinations were different from those responsible for severe acute GVHD. In particular, all 6 GVL combinations in HLA-DPB1 were different. Further, pairs with these GVL combinations of HLA-DPB1 were associated with significantly better OS than completely matched pairs. These findings suggest that donor selection according to these results could separate the occurrence of GVL from acute GVHD, especially in HLA-DPB1. Further, amino acid substitutions on specific positions responsible for this decreased risk of relapse were also elucidated in HLA-C, but not in HLA-DPB1. Our finding that specific amino acid substitutions decrease the risk of relapse might be key to revealing the mechanism of the decreased risk of relapse due to GVL with regard to the HLA molecule.

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Authorship

Contribution: T.K., Y.M., T.S., S.O., and Y.K. participated in the conception of this study; K.K., H.I., and H.S. participated in the assessment of histocompatibility; Y.M. and S.K. participated in the execution of transplantation; T.K. and K.M. participated in the statistical data analysis; T.K. and Y.M. wrote the paper; and all authors checked the final version of the manuscript.

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ORIGINAL ARTICLE

Donor single nucleotide polymorphism in the *CCR9* gene affects the incidence of skin GVHD

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The interactions between chemokines and their receptors may have an important role in initiating GVHD after allogeneic hematopoietic SCT (allo-HSCT). CCL25 and CCR9 are unique because they are exclusively expressed in epithelial cells and in Peyer's patches of the small intestine. We focused on rs12721497 (G926A), one of the non-synonymous single nucleotide polymorphisms (SNPs) in the *CCR9* gene, and analyzed the SNP of donors in 167 consecutive patients who received allo-HSCT from an HLA-identical sibling donor. Genotypes were tested for associations with acute and chronic GVHD in each organ and transplant outcome. Multivariate analyses showed that the genotype 926AG was significantly associated with the incidence of acute stage $\geqslant 2$ skin GVHD (hazard ratio: 3.2; 95% confidence interval (95% CI): 1.1–9.1; $P = 0.032$) and chronic skin GVHD (hazard ratio: 4.1; 95% CI: 1.1–15; $P = 0.036$), but not with GVHD in other organs or with relapse, non-relapse mortality or OS. To clarify the functional differences between genotypes, each SNP in retroviral vectors was transfected into Jurkat cells. In chemotaxis assays, the 926G transfectant showed greater response to CCL25 than the 926A transfectant. In conclusion, more active homing of *CCR9*-926AG T cells to Peyer's patches may produce changes in Ag presentation and result in increased incidence of skin GVHD.
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Introduction

Acute GVHD is a severe complication of allogeneic hematopoietic SCT (allo-HSCT).[1] After Ag presentation in secondary lymphoid tissues, migration of activated donor T lymphocytes to target organs has a central role in its induction. Recent studies have shown that the migration of lymphocytes to secondary lymphoid tissues or target organs, such as the skin, liver and gut, is regulated by specific chemokines.[2,3] Chemokines are a group of small molecules that regulate the trafficking of leukocytes through interactions with a subset of seven transmembrane, G protein-coupled receptors (chemokine receptors).[4,5] Their interactions may have an important role in initiating organ-specific GVHD.

Sites of expression are ubiquitous in many chemokines. For example, CCL17, which is well known as a skin-homing chemokine, is also expressed in many other organs, including the adrenal gland, bronchus, cerebellum, colon, heart and liver.[4] CCL28 is expressed by epithelial cells in several mucosal tissues, including the trachea, small intestine, colon, rectum, salivary gland and mammary gland.[6,7] By contrast, CCL25 (thymus-expressed chemokine) and its receptor CCR9 are unique because, outside the thymus, they are almost exclusively expressed by epithelial cells and Peyer's patches in the small intestine.[8–10] Therefore, we focused on CCR9 because it may influence the onset of intestinal GVHD or Ag presentation in Peyer's patches.

The *CCR9* gene is located on chromosome 3p21.3. A variety of single nucleotide polymorphisms (SNPs) in the *CCR9* gene have been reported, although their functional differences are not yet known. Within these SNPs, rs12721497 (G926A) is non-synonymous in exons, and it is the sole SNP whose frequency and linkage disequilibrium have been reported. This SNP alters the CCR9 amino acid sequence of the third exoloop from Val272 to Met272. We hypothesize that this SNP may have an effect on the onset of GVHD and transplant outcome because of the differences in the tissue-specific migration of T lymphocytes.

Materials and methods

Patients

A total of 186 consecutive patients received allogeneic BM or PBSC transplantation from an HLA-identical sibling donor at the Nagoya University Hospital and the Japanese Red Cross Nagoya First Hospital between 1987 and 2006. HLA matching among donor–recipient pairs was confirmed by either family study or genotyping in all patients. Of these 186 patients, 167 who received T-cell-replete transplantation and CYA in combination with short-term MTX as a GVHD prophylaxis were selected to participate in the study. CYA was administered daily at 3.0 mg/kg from day 1 as an i.v. infusion, and then switched to an oral dose at twice the i.v. dose when oral intake resumed. MTX was administered at 10 mg/m^2 on day 1 and, on days 3 and 6, was administered at 7 mg/m^2. Informed consent was obtained from all patients and donors, and the study was approved by the ethics committees at the Nagoya University Hospital and Japanese Red Cross Nagoya First Hospital.

Allelic discrimination of the polymorphism G926A in the CCR9 gene

The *CCR9*-G926A polymorphism (rs12721497) was determined by the PCR-RFLP method using genomic DNA obtained from donor PBMCs. The primers used for PCR were 5′-CACACCCTGATACAAGCCAA (forward) and 5′-CTCCAGCAACATAGACGACA (reverse). Sequences of interest were amplified by PCR, using Advantage II Polymerase Mix (Clontech Laboratories, Mountain View, CA, USA) in reaction mixtures containing 0.5 μl of genomic DNA and 10 pmol of each primer in a volume of 20 μl. Amplifications were performed using 35 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 15 s and elongation at 72 °C for 30 s on a model 9600 thermocycler (Perkin-Elmer, Norwalk, CT, USA). After amplification, the 369-bp *CCR9* fragment was digested for 2 h at 37 °C with 5 U of *NLA*III (New England BioLabs, Ipswich, MA, USA) in a 20 μl reaction mixture. The digested products were analyzed by electrophoresis on a 1.5% agarose gel. Wild-type (AA) individuals were identified by the presence of only a 369-bp fragment, heterozygotes (AG) by the presence of both 231/138- and 369-bp fragments and homozygotes (GG) by the presence of only the 231- and 138-bp fragments. To rule out the incomplete digestion of the AG genotype, PCR products of this genotype were directly sequenced using the Applied Biosystems 310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

Site-directed mutagenesis and construction of CCR9-926A and 926G expression vectors

Site-directed mutagenesis of the human wild-type *CCR9* cDNA in pF1K vector (purchased from Kazusa DNA Research Institute, Kisarazu, Chiba, Japan) was carried out using the Quickchange Kit (Stratagene, La Jolla, CA, USA). Synthetic oligonucleotide primers containing the corresponding 926G point mutation had the following sequences: 5′-CCATTGACGCCTATGCCGTGTTCATC TCCAACTGT (forward) and 5′-ACAGTTGGAGA TGAACACGGCATAGGCGTCAATGG (reverse). The oligonucleotide was amplified with Pfu turbo DNA polymerase (Stratagene), and the template plasmid was digested by *Dpn*I. Each sequence of *CCR9* cDNA was amplified by PCR with primers containing the following *Eco*RI/*Not*I sites: 5′-CGCGGAATTCATGACACCCAC AGACTTCACA (forward) and 5′-ATCGGCGGCCGC TCAGAGGGAGAGTGCTCCTGAGGT (reverse). Each product was cut at *Eco*RI/*Not*I sites, and ligated into pMX-IRES-Puro (a kind gift from Dr Toshio Kitamura, University of Tokyo), which had been digested with *Eco*RI and *Not*I. The final construct used for cell transfection was sequenced entirely to verify the presence of the mutation and to ensure that no other variant was accidentally introduced during DNA amplification.

Retrovirus transfection

PLAT-A packaging cells (a kind gift from Dr Toshio Kitamura, University of Tokyo) were used to produce recombinant retrovirus particles.[11] PLAT-A cells were transfected with retroviral vectors using FuGENE6 (Roche, Indianapolis, MN, USA). Jurkat cells were infected with each of the pMX-CCR9-926A-IRES-Puro, pMX-CCR9-926G-IRES-Puro and pMX-IRES-Puro (control) retroviruses. The cells were washed once and resuspended in the fresh selection medium containing 500 ng/ml of puromycin (Cayla, Toulouse, France), 48 h after transfection .

Flow cytometric analysis

Phycoerythrin-labeled monoclonal anti-CCR9 (112509) was purchased from R&D Systems (Minneapolis, MN, USA). Analyses were carried out on FACSAria (BD Biosciences, San Jose, CA, USA) using the FlowJo software (Treestar, San Carlos, CA, USA).

Chemotaxis assays

Chemotaxis assays were carried out as previously described[12] using 6.5-mm-thick Transwell tissue culture inserts with a 5-μm pore size (Corning, Corning, NY, USA). The transfected cell lines were starved overnight in the plain RPMI 1640 medium, suspended at 1×10^7 cells per ml in this medium with 0.1% of BSA, and 100 μl of cell suspension was added to an upper insert in a lower well with 600 μl of the medium. After equilibration at 37 °C for 2 h, various concentrations (0–2000 ng/ml) of recombinant human CCL25 (R&D Systems) were added to the lower wells, and the plates were incubated for an additional 90 min before migrated cells in the lower well were counted.

Statistical analysis

OS was calculated from the date of transplantation to the date of death from any cause using the Kaplan–Meier method, and *P*-values were calculated using a log-rank test. Non-relapse mortality (NRM) was defined as mortality due to any cause other than relapse or disease progression. Cumulative incidences of NRM and relapse were estimated

using Gray's method, with relapse and NRM, respectively, as a competing risk. Acute GVHD was graded by established criteria.[13] Chronic GVHD was evaluated in patients who survived beyond day +100, and was classified as limited or extensive according to the Seattle criteria.[14] A multivariate Cox model was created for grade II–IV acute GVHD, organ stages of acute GVHD, chronic GVHD, OS, NRM and relapse using stepwise selection at a significance level of 5%. Age, conditioning, disease risk, remission state, donor–recipient sex combination and graft source were used as covariates. For chronic GVHD analysis, a history of acute GVHD was included as covariates. Hazard ratios of the *CCR9* genotype were adjusted by these models. In acute GVHD analysis, patients who died before day +30 were censored. Analysis was carried out using STATA (StataCorp. 2007; Stata Statistical Software: Release 10.0. Special Edition. Stata Corporation, College Station, TX, USA). Data analyses were completed as of January 2007 using the most updated database at each institute.

Results

Patient characteristics

Patient characteristics are summarized in Table 1. Ninety-four male patients and 73 female patients, with a median age of 38 years, were included in the study. Our patients were afflicted with various diseases, including myeloid malignancies ($n=106$), lymphoid malignancies ($n=42$) and benign diseases ($n=19$). Disease risk was standard in 97 patients and high in 70 patients. Standard risk included malignancies in the first and second remission, chronic myelogenous leukemia in the chronic phase, myelodysplastic syndrome with refractory anemia with or without ringed sideroblasts and benign diseases. High risk included all others. Ninety-eight patients with malignant disease received transplantation with their disease in remission. The graft source was BM in 130 patients and PBSCs in 37 patients. The conditioning regimen was myeloablative in 147 patients and reduced-intensity conditioning in 20 patients. TBI was used as part of the conditioning in 98 patients. Myeloablative conditioning regimens for malignancy included BU 16 mg/kg + CY 120 mg/kg ($n=32$), CY 120 mg/kg + TBI 12 Gy ($n=9$), CY 120 mg/kg + TBI 10 Gy + another agent of BU 8 mg/kg, cytarabine 8 g/m^2, etoposide 50 mg/kg or melphalan 140 mg/m^2 ($n=62$), BU 8 mg/kg + melphalan 180 mg/m^2 + TBI 10 Gy ($n=12$) and melphalan 180 mg/m^2 + TBI 10 Gy ($n=13$). Reduced-intensity conditioning regimens for malignancy included fludarabine 125 mg/m^2 + melphalan 100–180 mg/m^2 ($n=20$). Conditioning regimens for aplastic anemia included CY 200 mg/kg + TLI 7.5 Gy ($n=17$) and CY 200 mg/kg + TLI 5 Gy + TBI 5 Gy ($n=1$) as previously described.[15] One patient with paroxysmal nocturnal hemoglobinuria was conditioned with CY 120 mg/kg + TBI 12 Gy. At a median follow-up of 42 months (range: 2–220 months), 104 patients were still alive. The estimated 4-year OS, NRM and relapse rates were 55, 18 and 30%, respectively. Causes of 26 non-relapse mortalities included bronchiolitis obliterans ($n=2$), idiopathic pneumonia syndrome ($n=8$), hepatic failure ($n=1$), veno-occlusive

Table 1 Patient characteristics

No. of patients	167
Median age in years (range)	38 (15–62)
Sex (M/F)	94/73
Race (Japanese/other)	167/0
Disease	
AML	50
ALL	30
CML	40
MDS	16
ML	6
ATL	1
MM	5
AA	18
PNH	1
Disease risk	
Standard	97
High	70
Status at transplant among patients with malignant disease	
Remission	98
Non-remission	50
Graft source	
Bone marrow	130
PBSC	37
Gender compatibility	
Female donor in male recipient	45
Others	122
Conditioning	
TBI-containing conditioning	98
Myeloablative conditioning for malignancy	147
BU + CY	32
CY + TBI	9
CY + TBI + another agent	62
BU + melphalan + TBI	12
Melphalan + TBI	13
Reduced-intensity conditioning for malignancy	20
Fludarabine + melphalan	20
Conditioning for aplastic anemia/PNH	
CY + TLI	17
CY + TLI + TBI	1
CY + TBI	1
GVHD prophylaxis	
Cyclosporine + MTX	167
Overall survival at 4 years	55%
Non-relapse mortality at 4 years	18%
Relapse rate at 4 years	30%
Acute GVHD	
Grade (0/I/II/III/IV)	99/37/20/8/3
Skin stage (0/1/2/3/4)	104/33/6/22/2
Liver stage (0/1/2/3/4)	159/3/1/3/1
Gut stage (0/1/2/3/4)	153/4/2/5/3
Chronic GVHD (n = 155 evaluable)	
None	91
Limited/extensive	13/51
Organ (eye/oral/skin/lung/liver)	21/55/31/2/29

Abbreviations: AA = aplastic anemia; ATL = adult T-cell leukemia/lymphoma; F = female; M = male; MDS = myelodysplastic syndrome; ML = malignant lymphoma; MM = multiple myeloma; PNH = paroxysmal nocturnal hemoglobinuria.

Table 2 Proportion of patients who developed GVHD in each genotype

Events	*Genotype 926AG*	*Genotype 926AA*
Acute GVHD		
Grade II–IV	2/10 (20%)	29/157 (18%)
Skin stage 2–4	4/10 (40%)	26/157 (17%)
Liver stage 2–4	1/10 (10%)	4/157 (2.5%)
Gut stage 2–4	1/10 (10%)	9/157 (5.7%)
Chronic GVHD		
Limited/extensive	4/10 (40%)	60/145 (41%)
Eye	2/10 (20%)	19/145 (13%)
Oral	4/10 (40%)	51/145 (35%)
Skin	3/10 (30%)	28/145 (19%)
Lung	1/10 (10%)	1/145 (0.6%)
Liver	2/10 (20%)	27/145 (19%)

disease ($n=2$), hepatitis ($n=2$), intestinal bleeding ($n=1$), transplant-associated microangiopathy ($n=3$), acute GVHD ($n=3$), scleroderma ($n=1$) and bacterial or fungal pneumonia ($n=3$). The incidence rates of grade II–IV, III–IV acute GVHD and chronic limited/extensive GVHD were 18.6, 6.6 and 41%, respectively. The incidence rates of stage 2–4 skin, liver and gut involvement were 18, 3 and 6%, respectively.

Frequency of CCR9 genotypes

Ten donors had genotype 926AG by the RFLP method, which was subsequently confirmed using direct sequence. The frequencies of the 926AA, 926AG and 926GG genotypes among the donors were 94, 6 and 0%, respectively, which were comparable with those reported in the HapMap-JPT database (http://www.hapmap.org).

Hazard analysis and the effect of CCR9 genotypes on transplant outcome

The proportion of patients who developed GVHD in each genotype is summarized in Table 2. Grade II–IV GVHD, stage 2–4 skin GVHD, stage 2–4 liver GVHD, stage 2–4 gut GVHD and chronic limited/extensive GVHD developed in 2, 4, 1, 1 and 4 patients, respectively, among patients whose donor had the genotype 926AG, whereas they developed in 29, 26, 4, 9 and 60 patients, respectively, among patients whose donor had the genotype 926AA. The estimated 4-year OS, NRM and relapse rates were not significantly different between G926A genotypes (56 vs 55%, $P=0.78$; 33 vs 17%, $P=0.32$; 10 vs 32%, $P=0.19$, respectively) (Figure 1). Multivariate analyses showed that PBSC transplantation was a risk factor for grade II–IV GVHD, stage 2–4 skin GVHD and stage 2–4 gut GVHD; high risk disease was a risk factor for OS and relapse; age of more than 40 years was a risk factor for OS and NRM; and female-to-male transplantation was a risk factor for chronic liver GVHD (Table 3, middle column). Hazard ratios of the genotype 926AG, adjusted by these factors, are listed in Table 3 (right column). The genotype 926AG was significantly associated with acute stage 2–4 skin GVHD (hazard ratio: 3.2; 95% confidence interval (95% CI): 1.1–9.1; $P=0.032$) and chronic skin GVHD (hazard ratio: 4.1; 95% CI: 1.1–15; $P=0.036$), whereas it was not

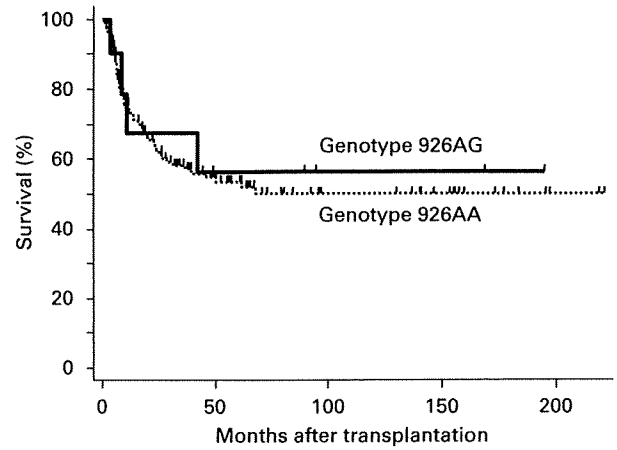

Figure 1 Overall survival between G926A genotypes. At a median follow-up of 42 months (range: 2–220 months), the estimated 4-year overall survival was not statistically different between G926A genotypes (56 vs 55%, $P=0.78$).

associated with grade II–IV GVHD or with stage 2–4 liver GVHD, stage 2–4 gut GVHD, limited or extensive chronic GVHD, chronic GVHD in organs other than skin, OS, NRM or relapse. PBSCs were used in only 1 of 10 patients who received transplantation from 926AG donors.

Functional comparison between CCR9-926A and 926G

To clarify the functional differences between genotypes of 926A and 926G, we created cDNA constructs with each genotype using the Quickchange Kit. Each construct was transfected into Jurkat cells with retroviral vectors. After 3 weeks of selection with puromycin, we analyzed the expression of CCR9 in each of the stably transfected cells. Cells were stained with phycoerythrin-labeled monoclonal anti-CCR9. The level of CCR9 expression was higher in CCR9-transfected cells compared with that in control-transfected cells, and equivalent between the 926A and 926G genotypes (Figure 2a).

We next analyzed the migration of Jurkat cells transfected with control vectors, 926A and 926G, as well as plain Jurkat cells in response to varying concentrations of CCL25, using porous Transwell tissue culture inserts to separate the cells in the upper chambers from the chemokine-containing medium in the lower chambers. As shown in Figure 2b, *CCR9*-transfected cells, but not the control-transfected or plain Jurkat cells, migrated in response to CCL25 in a dose-dependent manner, producing a bell-shaped curve. It is noted that *CCR9*-926G-expressing cells were more responsive to CCL25 compared with those expressing *CCR9*-926A.

Discussion

Several genetic polymorphisms of inflammatory cytokine genes are reported to affect the outcome of allo-HSCT.[16–21] Chemokines are another group of cytokines that control the trafficking of leukocytes through interactions with chemokine receptors. We hope to clarify the role of these

Table 3 Effect of the *CCR9* genotype on transplant outcome

Events	*Risk factor(s)*	*Multivariate*[a]		*Genotype 926AG*[b]	
		Hazard ratio (CI)	P-*value*	*Hazard ratio (CI)*	P-*value*
Acute GVHD					
Grade II–IV	PBSCT	3.4 (1.7–6.9)	0.001	1.2 (0.30–5.3)	0.76
Skin stage 2–4	PBSCT	2.7 (1.3–5.5)	0.008	3.2 (1.1–9.1)	0.032
Liver stage 2–4	—	—	—	4.2 (0.47–37)	0.20
Gut stage 2–4	PBSCT	5.7 (1.6–20)	0.007	2.4 (0.30–19)	0.41
Chronic GVHD					
Limited/extensive	—	—	—	1.7 (0.52–5.8)	0.37
Eye	—	—	—	6.2 (0.56–68)	0.14
Oral	—	—	—	2.4 (0.71–8.4)	0.16
Skin	—	—	—	4.1 (1.1–15)	0.036
Lung	—	—	—	12 (0.76–196)	0.077
Liver	Female to male	3.5 (1.0–12)	0.05	1.7 (0.21–13)	0.63
Overall survival	Age > 40	2.1 (1.3–3.6)	0.004	0.84 (0.30–2.3)	0.73
	High risk	2.1 (1.3–3.6)	0.004		
Non-relapse mortality	Age > 40	2.7 (1.2–6.1)	0.015	1.3 (0.40–4.5)	0.64
Relapse	High risk	4.0 (2.0–7.8)	<0.001	0.36 (0.05–2.6)	0.31

Abbreviations: CI = confidence interval; PBSCT = peripheral blood stem cell transplantation.
[a]Covariates used were age, conditioning, disease risk, remission state, donor–recipient sex combination and graft source. For chronic GVHD analysis, history of acute GVHD was included in the covariates. Only significant factors were listed.
[b]Adjusted by significant factors.

chemokines in initiating GVHD. Specifically, we address the association of polymorphism in the tissue-specific chemokine receptor gene with acute and chronic GVHD and the regulation of leukocyte trafficking.

CCL25 and CCR9 (as chemokine and chemokine receptor) are selectively expressed in both the thymus and the small intestine.[22,23] One of their important functions is the selective homing and retention of CCR9-positive T cells and B cells to the small intestine rather than to the colon, which provides a mechanism for regional specialization of the mucosal immune system.[9,24] Another function is regulating intrathymic T-cell development, particularly double-negative to double-positive transition,[25,26] which may be associated with T-cell recovery after allo-HSCT. Therefore, the effect of the *CCR9* genotype on acute GVHD is hypothesized to result from its function in the small intestine because T cells educated in the thymus will appear at least 6 months after transplantation.[27]

Interestingly, donor *CCR9* SNPs affected the incidence of skin GVHD, but did not affect the incidence of intestinal GVHD. This observation may be partially explained by the findings of Beilhack *et al.*,[28] who recently reported the redundancy of secondary lymphoid organs at different anatomical sites in GVHD initiation. They suggested that primed T cells could initiate GVHD at sites other than their original priming sites. As Peyer's patches are important sites of Ag presentation,[29] differences in T cell homing to Peyer's patches between each *CCR9* genotype may produce changes in Ag presentation and result in varying incidences of skin GVHD.

Our results suggest the possibility of CCL25/CCR9-targeting modalities for GVHD. CCL25 and CCR9 have an important role in the adherence of T lymphocytes to the intestinal endothelium under inflammatory and normal conditions, and anti-CCL25 Ab attenuates the TNF-α-induced T-cell adhesion in the small intestine.[30] Although blocking the interactions of CCL25 and CCR9 may delay immunological reconstitution in the thymus, CCR9-deficient mice showed no major effect on intrathymic T-cell development despite a 1-day lag in the appearance of double-positive cells and a diminution of γδ-T cells.[31]

One possible limitation of this study is that genetic associations can be biased by population stratification,[32] and there is also the chance of false-positive associations with the *CCR9* genotype on the basis of multiple statistical tests. Confirmation of the results with another separate cohort is needed for eliminating a possible confounding effect. Another limitation is that this SNP might be in linkage disequilibrium with SNPs in the *CCR9* gene or in the other genes located nearby. Linkage disequilibrium mapping of *CCR9* using the HapMap-JPT database showed one small block in introns of the *CCR9* gene, but G926A was outside the block with no known associations with other SNPs in the *CCR9* gene or with genes located around chromosome 3p21.3. In addition, this SNP alters CCR9 amino acid sequences of the third exoloop, which is an important site for chemokine binding and specificity.[33] Therefore, this SNP can affect biological functions due to altered efficiencies of the receptor or signal transduction from the receptor. Although Transwell assays using SNP-transfected cells showed that biological functions varied according to this SNP, the elucidation of additional mechanisms are matters for future research.

In summary, this study suggests that donor 926AG is associated with an increased incidence of acute and chronic skin GVHD in related HSCT recipients. CCL25 and CCR9

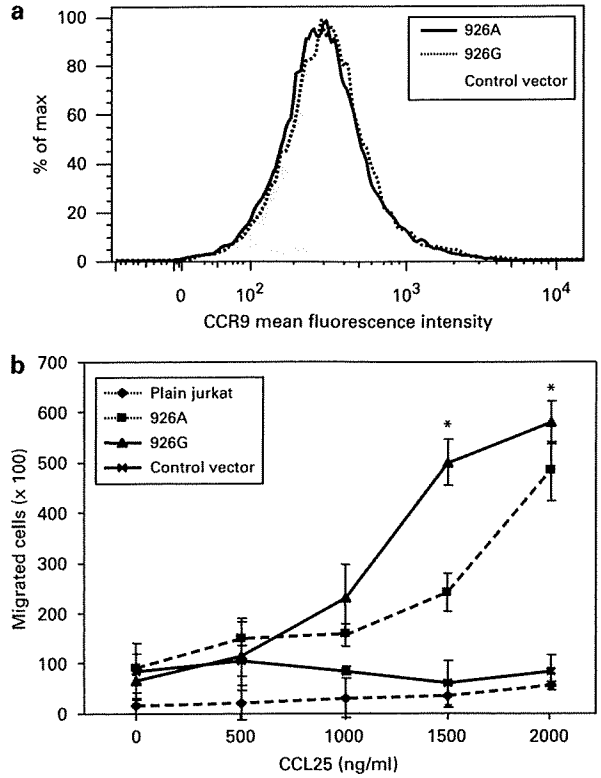

Figure 2 Chemotaxis assays with *CCR9*-polymorphism-transfected Jurkat cells: (**a**) Flow cytometric analysis of CCR9 expression. Transfected Jurkat cells were stained with phycoerythrin (PE)-labeled monoclonal anti-CCR9. Control staining with control-transfected Jurkat cells is also shown (shadow); (**b**) Jurkat cells transfected with cDNAs encoding *CCR9* migrated in response to CCL25. After puromycin selection, 1×10^6 transfected cells and the same number of Jurkat cells were added to porous Transwell tissue culture inserts and placed in wells containing various concentrations of CCL25. After 90-min of incubation, cells migrating through the membranes into the lower wells were counted. Results are expressed as cells migrating per 10^6 input cells. Assays were carried out in triplicate and error bars represent s.d. $*P<0.05$

may be candidates for future therapeutic targets that alter the quality and incidence of GVHD.

Conflict of interest

The authors declare no conflict of interest.

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