Biology of Blood and Marrow Transplantation 13:315-328 (2007)

1083-8791/07/1303-0001$32.00/0
doi:10.1016/j.bbmt.2006.10.027

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American Society for Blood and Marrow Transplantation

Effects of HLA Allele and Killer Immunoglobulin-Like Receptor Ligand Matching on Clinical Outcome in Leukemia Patients Undergoing Transplantation With T-cell–Replete Marrow From an Unrelated Donor

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Received June 4, 2006; accepted October 26, 2006

ABSTRACT

The responsible human leukocyte antigen (HLA) locus and the role of killer immunoglobulin-like receptor (KIR) ligand matching on transplantation outcome were simultaneously identified by multivariate analysis in 1790 patients with leukemia who underwent transplantation with T-cell–replete marrow from an unrelated donor (UR-BMT) through the Japan Marrow Donor Program. The graft-versus-leukemia (GVL) effect depended on leukemia cell type. HLA-C mismatch reduced the relapse rate in acute lymphoblastic leukemia (ALL) (hazard ratio [HR] = 0.47; $P = .003$), and HLA-DPB1 mismatch reduced it in chronic myeloid leukemia (CML) (HR = 0.35; $P < .001$). In contrast, KIR2DL ligand mismatch in the graft-versus-host (GVH) direction (KIR-L-MM-G) increased in ALL (HR = 2.55; $P = .017$). An increased rejection rate was observed in KIR2DL ligand mismatch in the host-versus-graft direction (HR = 4.39; $P = .012$). Acute GVH disease (GVHD) was increased not only in the mismatch of HLA-A, -B, -C, and -DPB1, but also in KIR-L-MM-G. As a whole, the mismatch of HLA-A, -B, and -DQB1 locus and KIR-L-MM-G resulted in increased mortality. In conclusion, not only the mismatch of HLA-C and -DPB1, but also KIR-L-MM-G affected leukemia relapse, which should be considered based on leukemia cell type. Furthermore, KIR-L-MM induced adverse effects on acute GVHD (aGVHD) and rejection, and brought no survival benefits to patients with T-cell–replete UR-BMT.

KEY WORDS
KIR ligand incompatibility • HLA • Leukemia • Unrelated bone marrow transplantation

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)-matched unrelated (UR) donor has been established as one mode of curative therapy for hematologic malignancies and other hematologic or immunologic disorders [1,2]. Extensive research on genetic factors such

as HLA has produced mounting evidence of the presence of HLA alleles that drastically affect HSCT outcome through T cells. Induction of the graft-versus-leukemia (GVL) effect to reduce relapse of leukemia is considered an advantage of allogeneic HSCT [3]. There have been several large-scale analyses of UR-HSCT. The Japan Marrow Donor Program (JMDP) demonstrated the effect of matching of HLA class I alleles (HLA-A, -B, and -C) on the development of severe acute graft-versus-host disease (aGVHD) and the importance of HLA-A and -B allele matching for better survival in T-cell–replete UR-HSCT [4,5]. The Fred Hutchinson Cancer Research Center and the US National Marrow Donor Program (NMDP) reported the importance of HLA class II matching in GVHD and survival [6,7]. Updated analysis of the NMDP indicated that HLA-A allele-level mismatching, HLA-B serologic mismatching, and HLA-DRB1 mismatching are significant risk factors for severe aGVHD, and that disparity in HLA class I (HLA-A, -B, or -C) and/or HLA-DRB1 increased the mortality [8]. Furthermore, the role of HLA-DPB1 matching has been elucidated for aGVHD [9-11] and leukemia relapse [12]. However, the aforementioned reports have produced considerable conflicting results.

It has become evident that natural killer (NK) cells and the subpopulation of T cells express NK cell receptors, and that the activity of NK cells is controlled by the recognition of HLA class I molecules on the target cells by NK cell inhibitory and activating receptors [13,14]. The genotype and haplotype of the killer immunoglobulin-like receptors (KIRs) have been identified, and ligand specificities of KIRs have been characterized. C1 specificity of the HLA-C epitope (Asp80) is the ligand of inhibitory KIR2DL2/3, C2 specificity (Lys80) is the ligand of inhibitory KIR2DL1, and HLA-Bw4 is the ligand of KIR3DL1. With allogeneic HSCT, the disparities of these receptors between donor and recipient are suspected to induce transplant-related immunologic events through activation of NK cells, and evidence of the clinical outcome of HSCT in relation to KIR disparities has been accumulated [15]. However, reports of KIR ligand matching in UR-HSCT have shown contradictory results [16]. Limited patient numbers, different diseases, and various GVHD prophylaxes make it difficult to draw definite conclusions from these studies.

In the present study, the effects of HLA locus and KIR ligand matching were simultaneously analyzed in leukemia patients receiving T-cell–replete marrow from unrelated donors through the JMDP after a myeloablative conditioning regimen, focusing in particular on the influence of leukemia cell type on the GVL effect.

PATIENTS AND METHODS

Patients

A total of 1790 consecutive leukemia patients who underwent transplantation with marrow from a serologically HLA-A, -B, and -DR antigen–matched donor in Japan between January 1993 and March 2000 through the JMDP were analyzed. No patients receiving T-cell–depleted marrow and/or antithymocyte globulin (ATG) as GVHD prophylaxis were eligible for this study. Partial HLA-A and -B alleles and complete HLA-DRB1 alleles were identified as confirmatory HLA typing during the coordination process, and HLA-A, -B, -C, -DQB1, and -DPB1 alleles were retrospectively reconfirmed or identified after transplantation. The final clinical survey of these patients was completed as of June 1, 2005. Informed consent was obtained from patient and donor according to the Declaration of Helsinki, and approval was obtained from the JMDP and the Institutional Review Board of the Aichi Cancer Center.

Characteristics of patients and donors are listed in Table 1. The patients' age ranged from 0 to 59 years (median, 27 years), and donors' age ranged from 20 to 51 years (median, 35 years). There were 577 patients with acute myeloblastic leukemia (AML), of whom 186 underwent transplantation while in first complete remission (CR), 191 who did so while in second or further CR, and 200 who did so while in non-CR; 617 patients with acute lymphoblastic leukemia (ALL), of whom 236 underwent transplantation while in first CR, 207 who did so while in second or further CR, and 174 who did so while in non-CR; and 596 patients with chronic myeloid leukemia (CML), of whom 417 were in the first chronic phase (CP), 34 were in the second or further CP, 90 were in the accelerated phase, and 55 were in the blastic phase. Standard risk for leukemia relapse was defined as the status of the first CR of AML and ALL and the first CP of CML at transplantation, whereas high risk was defined as a more advanced status than standard risk in AML, ALL, and CML.

HLA Typing of Patients and Donors

Alleles at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were identified as described previously [4,5]. HLA 6 locus alleles were typed in 1773 pairs, and HLA 5 locus alleles except HLA-DPB1 were typed in 17 pairs. HLA genotypes of HLA-A, -B, -C, -DQB1, and -DPB1 alleles of patient and donor were reconfirmed by the Luminex microbead method (100 System; Luminex, Austin, TX) adjusted for the JMDP [17] and in part by the sequencing-based typing method in 2004 and 2005. As a result, all HLA alleles that were observed with > 0.1% frequency among Japanese were identified. The numbers of

Table 1. *Patient characteristics and matching status of HLA allele and KIR2DL ligand*

	Patient Number (%) M/MM*	Patient Age Median (years) M/MM*	Patient Sex Female (%) M/MM*	Donor Age Median (years) M/MM*	Donor Sex Female (%) M/MM*	Sex Match (%) M/MM*	Stage at Transplant High (%) M/MM*	GVHD Prophylaxis Cyclosporine (%) M/MM*	Total Body Irradiation (%) M/MM*
All leukemia (n = 1790)									
HLA-A	1484/306	27/26	39/37	34/33	38/40	57/55	52/57	73/73	83/72
HLA-B	1645/145	27/26	40/34	34/35	39/36	56/63	52/51	72/76	83/84
HLA-C	1256/534	27/26	39/41	34/33	38/40	56/58	52/55	74/70	83/82
HLA-DRB1	1434/356	27/26	40/38	34/34	38/41	57/57	51/60	74/66	83/82
HLA-DQB1	1391/399	27/26	40/38	34/33	38/41	57/57	52/56	74/67	83/83
HLA-DPB1	612/1163	26/27	42/39	34/34	39/39	60/56	50/55	75/71	81/84
KIR2DL-G†	1693/97	26/27	39/35	34/34	39/43	57/74	53/63	73/64	83/84
KIR2DL-R‡	1679/111	27/25	39/40	34/32	39/60	57/51	53/59	73/67	83/84
Acute myeloblastic leukemia (n = 577)									
HLA-A	486/91	28/27	44/44	33/33	38/39	58/55	67/71	72/60	81/89
HLA-B	537/40	27/31	45/33	33/35	39/30	56/73	67/83	71/68	83/80
HLA-C	405/172	28/28	43/45	33/34	39/37	56/61	66/73	74/63	82/83
HLA-DRB1	474/103	28/27	44/43	33/33	37/47	58/55	66/77	72/63	82/86
HLA-DQB1	469/108	27/29	45/40	33/33	38/43	57/56	67/72	72/64	83/81
HLA-DPB1	206/366	27/28	48/42	34/33	40/38	58/57	65/70	71/70	81/84
KIR2DL-G†	546/31	28/28	43/55	33/33	38/39	57/65	67/71	72/52	82/83
KIR2DL-R‡	546/31	28/28	43/55	33/35	38/39	59/32	68/68	71/58	82/83
Acute lymphoblastic leukemia (n = 617)									
HLA-A	515/102	20/19	41/40	34/32	42/42	55/50	60/69	73/74	91/88
HLA-B	567/50	19/20	41/42	33/36	42/38	54/60	61/70	72/80	91/86
HLA-C	437/180	19/19	41/41	34/32	41/42	54/57	61/63	73/72	91/89
HLA-DRB1	485/132	19/19	41/42	33/33	43/36	55/52	61/64	74/70	90/90
HLA-DQB1	467/150	19/20	41/41	34/33	42/41	55/51	61/63	75/68	90/92
HLA-DPB1	190/425	19/29	43/40	34/33	38/43	61/52	61/62	77/71	89/91
KIR2DL-G†	587/30	20/17	42/20	33/35	42/40	55/53	61/73	73/73	91/83
KIR2DL-R‡	577/40	19/19	39/40	34/30	42/43	54/53	61/73	73/70	90/93
Chronic myelocytic leukemia (n = 596)									
HLA-A	483/113	32/31	33/35	34/34	35/40	59/60	29/35	73/81	76/72
HLA-B	541/55	32/29	34/27	34/37	36/38	56/60	29/36	74/78	74/85
HLA-C	414/182	32/31	33/36	35/34	35/39	60/58	30/31	74/76	75/74
HLA-DRB1	475/121	32/33	34/31	34/36	35/40	58/63	27/41	77/64	76/70
HLA-DQB1	455/141	32/31	34/33	35/33	35/39	57/65	28/35	76/69	75/74
HLA-DPB1	216/372	31/33	35/33	34/35	38/34	60/59	28/31	76/73	73/76
KIR2DL-G†	560/36	32/32	34/31	35/32	35/50	59/53	29/44	75/67	71/83
KIR2DL-R‡	556/40	32/27	34/28	35/31	36/38	59/65	29/38	75/68	75/75

Standard-first complete remission or first chronic phase; high more advanced stage than standard.
*M/MM match/mismatch in GVH direction for HLA matching.
†KIR2DL ligand mismatching in GVH direction.
‡KIR2DL ligand mismatching in HVG direction.

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identified alleles in this study were 25 in HLA-A, 43 in HLA-B, 20 in HLA-C, 33 in HLA-DRB1, 14 in HLA-DQB1, and 21 in HLA-DPB1.

Matching of HLA Allele and KIR2DL Ligand

For the analysis of GVHD and leukemia relapse, HLA allele mismatch among the donor–recipient pair was scored when the recipient's alleles were not shared by the donor (graft-versus-host [GVH] direction). For graft rejection, HLA allele mismatch among the donor–recipient pair was scored when the donor's alleles were not shared by the patient (host-versus-graft [HVG] direction). For survival, the mismatch was defined as that of either the GVH direction or the HVG direction.

KIR2DL ligand specificity of HLA-C antigen was determined according to the HLA-C allele. The epitope of HLA-Cw3 group (C1 specificity) consists of Asn80, and that of the HLA-Cw4 group (C2 specificity) consists of Lys80.

KIR ligand mismatch in the GVH direction (KIR-L-MM-G) was scored when the donor's KIR2DL epitope of HLA-C was not shared by the patient epitope. This mismatch occurred when KIR2DL2/3- or KIR2DL1-positive effector cells were activated without the expression of corresponding HLA-C ligand (C1 or C2, respectively) on the patient's target cells. KIR ligand mismatch in HVG direction (KIR-L-MM-R) was scored when the patient's KIR2DL epitope of HLA-C was not shared by the donor. This mismatch occurred when patient KIR2DL2/3- or KIR2DL1-positive effector cells were activated without the expression of corresponding HLA-C ligand (C1 or C2, respectively) on donor cells.

Matching Status of HLA Locus in Allele Level and KIR2DL Ligand

The matching status of HLA allele matching in the GVH direction in each HLA locus and KIR ligand matching in both directions are given in Table 1. The HLA-C epitope of KIR2DL was estimated from HLA-C allele type, with 92.4% of the HLA-C allele belonging to the Cw3 group (C1 specificity) and 7.6% belonging to the Cw4 group (C2 specificity). KIR2DL ligand match in both directions occurred in 1583 pairs (88.4%). KIR-L-MM-G, which occurred in the combination of KIR2DL ligand in patient–donor pairs, was found in 97 pairs (5.4%): C1/C1 and C1/C2 in 92 pairs, C2/C2 and C1/C2 in 4 pairs, and C1/C1 and C2/C2 in 1 pair. KIR-L-MM-R, which occurred in the combination of KIR2DL ligand in patient and donor pairs, was found in 111 pairs (6.2%): C1/C2 and C1/C1 in 105 pairs, C1/C2 and C2/C2 in 5 pairs, and C1/C1 and C2/C2 in 1 pair. Mismatches in both directions were found in only 1 pair. Because all pairs were a serologic HLA-B match in this study, the combination of KIR3DL1 and its ligand of Bw4 matched in all pairs.

Definition of Transplantation-Related Events

The occurrence of aGVHD was evaluated according to grading criteria in patients who survived more than 8 days after transplantation, and chronic GVHD (cGVHD) according to the criteria in patients who survived more than 100 days after transplantation as described previously [5]. Rejection was defined as when the peripheral granulocyte count became $< 500/\mu L$ with the finding of severe hypoplastic marrow in engrafted patients. Engraftment was defined as a peripheral granulocyte count of $> 500/\mu L$ for 3 successive days in patients surviving > 21 days after transplantation.

GVHD Prophylaxis

Among the 1790 patients transplanted with T-cell–replete marrow, 1302 received a cyclosporine-based regimen and 488 received a tacrolimus-based regimen for GVHD prophylaxis. Anti-thymocyte globuline (ATG) was not given for GVHD prophylaxis.

Preconditioning Regimen

All patients were preconditioned with a myeloablative regimen, with 1480 receiving total body irradiation (TBI)-containing regimens and 310 receiving non-TBI regimens.

Statistical Analysis

All of the analyses were conducted using STATA version 8.2 (STATA Corp, College Station, TX). Overall survival rate was assessed by the Kaplan-Meier product limit method [18]. Cumulative incidences of aGVHD, cGVHD, rejection, and leukemia relapse were assessed as described previously to eliminate the effect of competing risk [19,20]. The competing events regarding aGVHD, cGVHD, rejection, and relapse were defined as death without aGVHD, cGVHD, rejection, and relapse, respectively. For each endpoint, a log-rank test was applied to assess the impact of the factor of interest.

Cox proportional hazard models [21] were applied to assess the impact of HLA allele matching (mismatch vs match [hazard risk = 1.0] as a reference group) as well as KIR ligand matching (mismatch vs match in the GVH direction and mismatch vs match in the HVG direction) including the following confounders. The confounders considered were sex (donor–recipient pairs), patient age (older: linear), donor age (older: linear), type of disease (AML, CML, or ALL), risk of leukemia relapse (high vs standard),

Table 2. *Effects of HLA and KIR ligand matching for leukemia relapse*

	All Leukemia Cell Types			Acute Myeloblastic Leukemia			Acute Lymphoblastic Leukemia			Chronic Myeloid Leukemia		
	HR*	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
HLA-A	1.19	(0.89-1.59)	.251	0.92	(0.54-1.58)	.761	1.18	(0.76-1.86)	.462	1.63	(0.89-2.97)	.114
HLA-B	1.01	(0.65-1.59)	.953	1.36	(0.65-2.88)	.416	0.98	(0.48-1.98)	.952	0.62	(0.22-1.76)	.367
HLA-C	0.71	(0.53-0.96)	.025	0.8	(0.49-1.30)	.366	0.47	(0.28-0.78)	.003	1.2	(0.62-2.29)	.591
HLA-DRB1	1.05	(0.73-1.53)	.789	0.78	(0.40-1.52)	.466	0.91	(0.51-1.61)	.737	1.25	(0.55-2.85)	.59
HLA-DQB1	1.10	(0.77-1.58)	.579	1.55	(0.82-2.95)	.178	1.11	(0.63-1.95)	.71	0.86	(0.39-1.93)	.72
HLA-DPB1	0.68	(0.55-0.85)	.001	0.76	(0.52-1.09)	.137	0.92	(0.65-1.28)	.604	0.35	(0.21-0.58)	<.001
KIR2DL-G†	1.55	(0.92-2.63)	.103	1.05	(0.37-3.02)	.926	2.55	(1.18-5.52)	.017	1.23	(0.38-3.94)	.727
KIR2DL-R‡	0.73	(0.40-1.34)	.313	0.53	(0.15-1.78)	.305	1.30	(0.53-3.19)	.569	0.5	(0.14-1.80)	.292

HLA matching in GVH direction.

*Hazard ratio of mismatch with match as a reference adjusted for patient age, donor age, sex-matching disease, GVHD prophylaxis, total body irradiation, transplanted cell dose, risk status, and other matching status of HLA and KIR ligand.

†KIR2DL ligand mismatching in GVH direction.

‡KIR2DL ligand mismatching in HVG direction.

GVHD prophylaxis (tacrolimus-based vs cyclosporine-based and ATG vs cyclosporine-based), numbers of transplanted cells (linear), and preconditioning (non-TBI vs TBI). The numbers of nucleated cells before manipulation of bone marrow were replaced with the numbers of transplanted cells.

Multivariate analysis for clinical outcomes, including KIR ligand matching and HLA-C matching in all pairs (not restricted to HLA-C mismatch), made it possible to evaluate whether these factors are independent. The results of all pairs by multivariate analysis are presented in the Results section and in Tables 2, 3, and 4. HLA-C–mismatched pairs were selected for the analysis of cumulative incidence in KIR ligand matching.

RESULTS

Effects of HLA Locus Mismatch and KIR Ligand Mismatch on Leukemia Relapse

When all leukemia patients (AML, ALL, and CML) were analyzed together, HLA-C mismatch was found to be a factor reducing the relapse rate (HR = 0.71; P = .025) (Table 2). This GVL effect was remarkable in ALL patients (HR = 0.47; P = .003), especially in high risk (HR = 0.40; P = .004) but not in standard risk (HR = 0.85; P = .728). No such effect was observed in AML patients (HR = 0.80; P = .366) or CML patients (HR = 1.20; P = .591).

Cumulative incidence curves of relapse by leukemia cell type are shown in Figure 1. The relapse rate 5 years after transplantation was 16.7% (95% confidence interval [CI] = 11.6%-30.9%) for HLA-C mismatch and 29.8% (95% CI = 25.5%-34.3%) for HLA-C match in ALL patients (P = .012); 17.6% (95% CI = 12.2%-23.8%) and 25.9% (95% CI = 21.1%-30.9%), respectively, in AML patients (P = .342); and 11.7% (955 CI = 12.2%-23.8%) and 12.0% (95% CI = 9.0%-15.4%), respectively, in CML patients (P = .485).

HLA-DPB1 mismatch was shown to reduce the overall leukemia relapse rate (HR = 0.68; P = .001) (Table 2). This effect was significant in CML (HR =

Table 3. *Effects of HLA and KIR ligand matching for acute GVHD, chronic GVHD, and rejection in all leukemia cell types*

	Acute GVHD (Grade 2-4) (n = 1751)			Acute GVHD (Grade 3-4) (n = 1751)			Chronic GVHD (n = 1109)			Rejection (n = 1664)		
	HR*	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
HLA-A	1.22	(1.02-1.46)	.034	1.44	(1.11-1.86)	.006	1.41	(1.08-1.85)	.013	0.72	(0.24-2.14)	.555
HLA-B	1.43	(1.28-1.82)	.003	1.40	(1.00-1.95)	.05	1.00	(0.65-1.52)	.991	1.16	(0.32-4.16)	.82
HLA-C	1.29	(1.08-1.55)	.006	1.39	(1.06-1.83)	.017	1.38	(1.07-1.78)	.014	1.87	(0.72-4.86)	.201
HLA-DRB1	1.15	(0.90-1.47)	.254	1.09	(0.77-1.54)	.644	0.91	(0.63-1.31)	.607	0.49	(0.10-2.33)	.366
HLA-DQB1	1.02	(0.81-1.29)	.871	1.13	(0.81-1.59)	.465	1.20	(0.85-1.69)	.288	0.62	(0.07-5.16)	.536
HLA-DPB1	1.39	(1.19-1.63)	<.001	1.26	(1.00-1.60)	.053	0.86	(0.70-1.05)	.138	1.08	(0.59-2.41)	.843
KIR2DL-G†	1.70	(1.28-2.26)	<.001	2.35	(1.62-3.40)	<.001	1.13	(0.68-1.87)	.64	0.62	(0.07-5.16)	.655
KIR2DL-R‡	1.04	(0.77-1.42)	.78	1.33	(0.88-2.02)	.18	0.88	(0.55-1.42)	.603	4.39	(1.38-13.96)	.012

HLA matching in GVH direction for acute GVHD and chronic GVHD, and HLA matching in HVG direction for rejection.

*Hazard ratio of mismatch with match as a reference adjusted for patient age, donor age, sex-matching disease, GVHD prophylaxis, total body irradiation, transplanted cell dose, risk status, and other matching status of HLA and KIR ligand.

†KIR2DL ligand mismatching in GVH direction.

‡KIR2DL ligand mismatching in HVG direction.

Table 4. *Effects of HLA and KIR ligand matching for mortality*

	All Leukemia Cell Types			Acute Myeloblastic Leukemia			Acute Lymphoblastic Leukemia			Chronic Myeloid Leukemia		
	HR*	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
HLA-A	1.36	(1.16-1.59)	<.001	1	(0.75-1.34)	.978	1.46	(1.11-1.90)	.006	1.77	(1.35-2.33)	<.001
HLA-B	1.40	(1.13-1.73)	.002	1.43	(0.96-2.12)	.079	1.47	(1.03-2.09)	.036	1.18	(0.80-1.72)	.402
HLA-C	1.17	(0.99-1.37)	.067	1.18	(0.89-1.55)	.246	0.99	(0.74-1.31)	.928	1.42	(1.04-1.93)	.025
HLA-DRB1	0.92	(0.74-1.15)	.463	0.74	(0.50-1.10)	.136	1.04	(0.72-1.49)	.849	0.99	(0.65-1.50)	.951
HLA-DQB1	1.28	(1.04-1.58)	.018	1.29	(0.89-1.87)	.184	1.33	(0.93-1.90)	.108	1.18	(0.79-1.75)	.422
HLA-DPB1	1.06	(0.91-1.23)	.474	0.96	(0.75-1.24)	.772	1.33	(1.02-1.75)	.038	0.97	(0.74-1.27)	.827
KIR2DL-G†	1.80	(1.39-2.34)	<.001	1.93	(1.22-3.05)	.005	1.57	(0.96-2.56)	.069	2.23	(1.42-3.50)	<.001
KIR2DL-R‡	1.07	(0.81-1.41)	.612	1.08	(0.66-1.75)	.769	0.98	(0.59-1.61)	.934	1.07	(0.66-1.72)	.787

*Hazard ratio of mismatch with match as a reference adjusted for patient age, donor age, sex-matching disease, GVHD prophylaxis, total body irradiation, transplanted cell dose, risk status, and other matching status of HLA and KIR ligand.
†KIR2DL ligand mismatching in GVH direction.
‡KIR2DL ligand matching in HVG direction.

0.35; $P < .001$), and both high-risk and standard-risk CML had a significantly lower relapse rate of HLA-DPB1 mismatch (HR = 0.35; $P < .001$ and HR = 0.39; $P = .012$, respectively). No significant effect was observed in AML (HR = 0.76; $P = .137$) or ALL (HR = 0.92; $P = .604$).

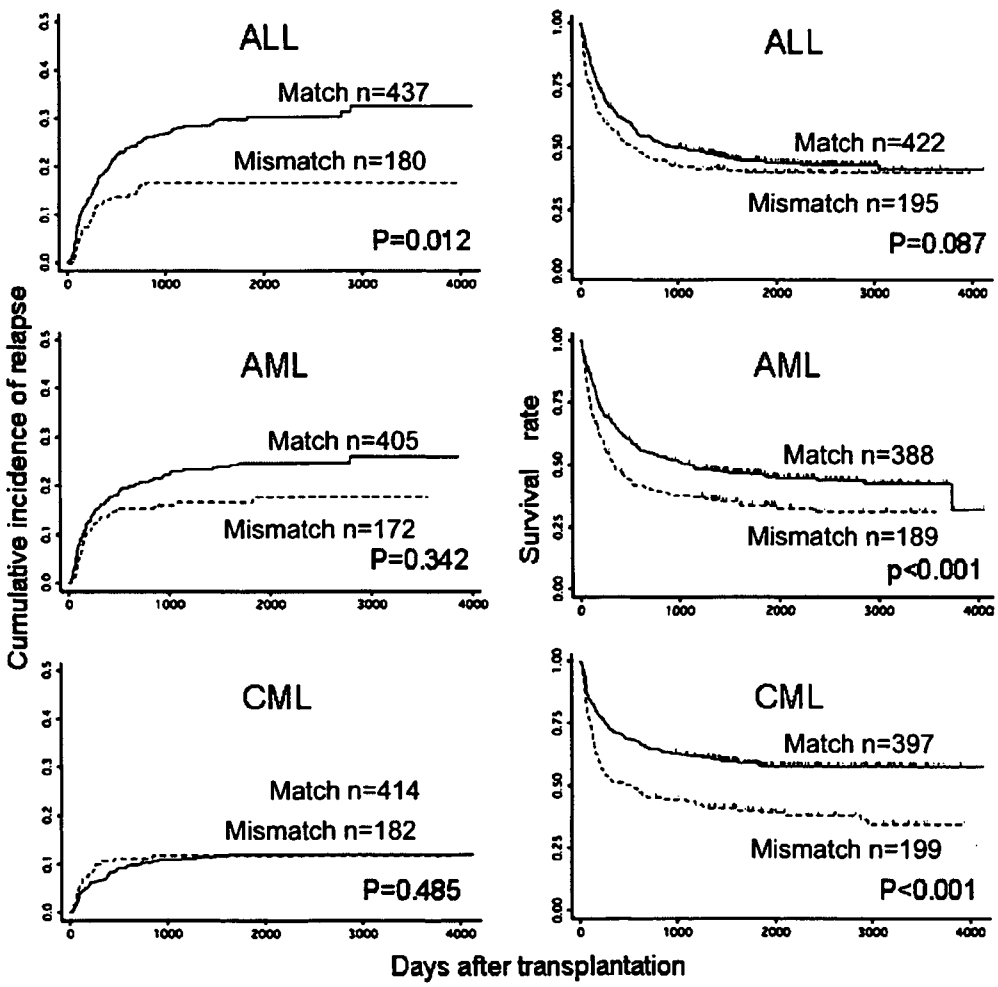

Figure 1. Cumulative incidence of relapse and survival by matching of HLA-C in patients with ALL, AML, and CML. All patients were analyzed. The direction of mismatching of HLA-C for relapse is GVH for relapse, and the direction for survival is GVH and/or HVG. The solid line represents match; the dotted line, mismatch.

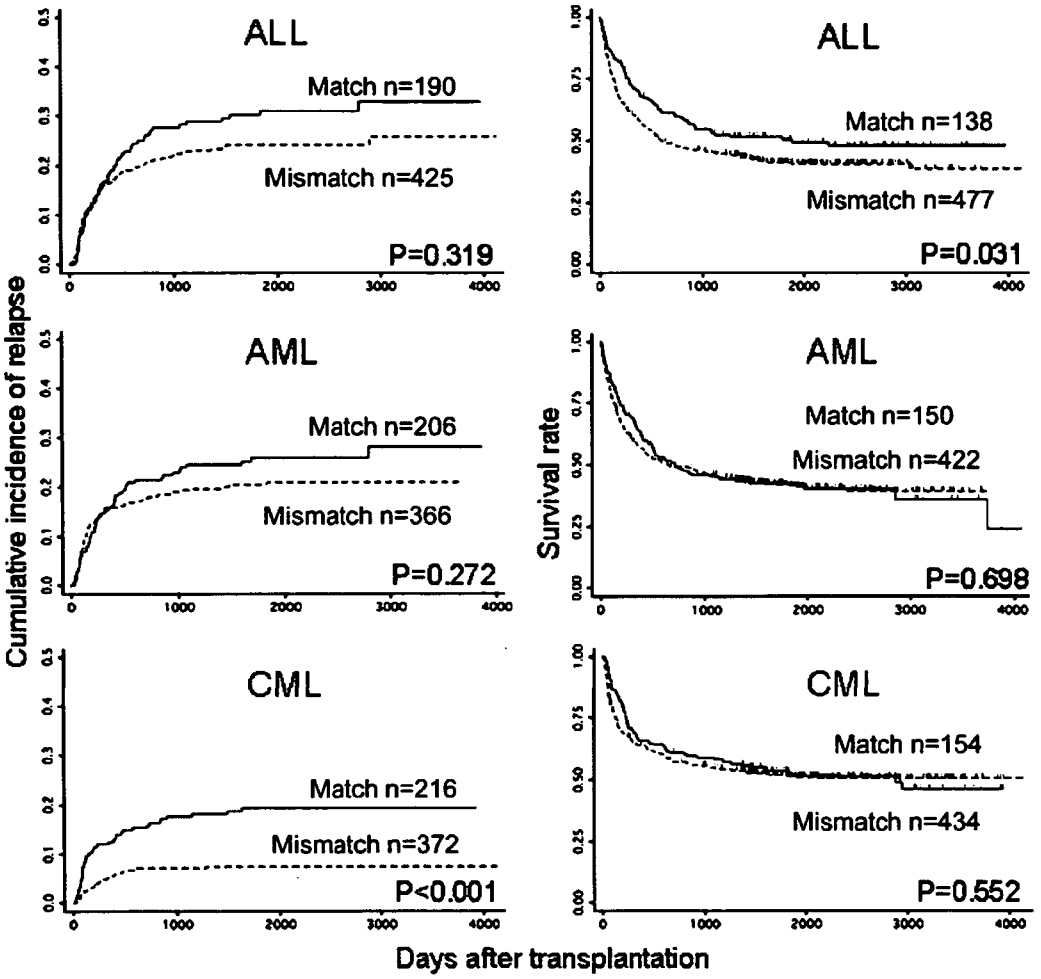

Figure 2. Cumulative incidence of relapse and survival by matching of HLA-DPB1 in patients with ALL, AML, and CML. All patients were analyzed. The direction of mismatching of HLA-DPB1 for relapse is GVH for relapse, and the direction for survival is GVH and/or HVG. Solid line, match; dotted line, mismatch.

As shown in Figure 2, the relapse rate 5 years after transplantation was 7.1% (95% CI = 5.0%-10.4%) for HLA-DPB1 mismatch and 19.3% (95% CI = 14.3%-24.9%) for HLA-DPB1 match in CML patients ($P < .001$); 20.4% (95% CI = 16.4%-24.8%) and 25.9% (95% CI = 19.9%-32.2%), respectively, in AML patients ($P = .272$); and 24.0% (95% CI = 19.9%-28.3%) and 30.2% (95% CI = 23.7%-37.0%), respectively, in ALL patients ($P = .319$).

Mismatch of HLA-A, -B, -DRB1, and -DQB1 was not a significant risk factor for leukemia relapse by multivariate analysis (Table 2).

Patients with KIR-L-MM-G had a higher relapse rate than those with KIR2DL ligand match in ALL (HR = 2.55; $P = .017$) (Table 2). This adverse effect on leukemia relapse was remarkable in high-risk ALL (HR = 3.03; $P = .013$), but not in standard-risk ALL (HR = 1.11; $P = .921$). In AML and CML, KIR-L-MM-G had no effect on leukemia relapse (HR = 1.05; $P = .926$ and HR = 1.23; $P = .727$, respectively).

Because KIR-L-MM occurs in HLA-C mismatch pairs, the cumulative incidence of leukemia relapse was analyzed in HLA-C mismatch patients in either direction by leukemia cell type (Figure 3). The relapse rate 5 years after transplantation was 31.0% (95% CI = 5.6%-47.9%) for KIR-L-MM-G and 16.3% (95% CI = 11.0%-22.4%) for match in ALL patients ($P = .026$); 11.1% (95% CI = 3.5%-23.6%) and 11.8% (95% CI = 7.4%-17.3%), respectively, in CML patients ($P = .634$); and 12.9% (95% CI = 4.1%-27.0%) and 16.3% (95% CI = 11.0%-22.6%), respectively, in AML patients ($P = .757$).

Significant clinical risk factors for leukemia relapse by multivariate analysis included status at transplantation (standard vs high, HR = 3.00; $P < .001$) and disease (HR = 0.75; $P < .001$) in all leukemia patients.

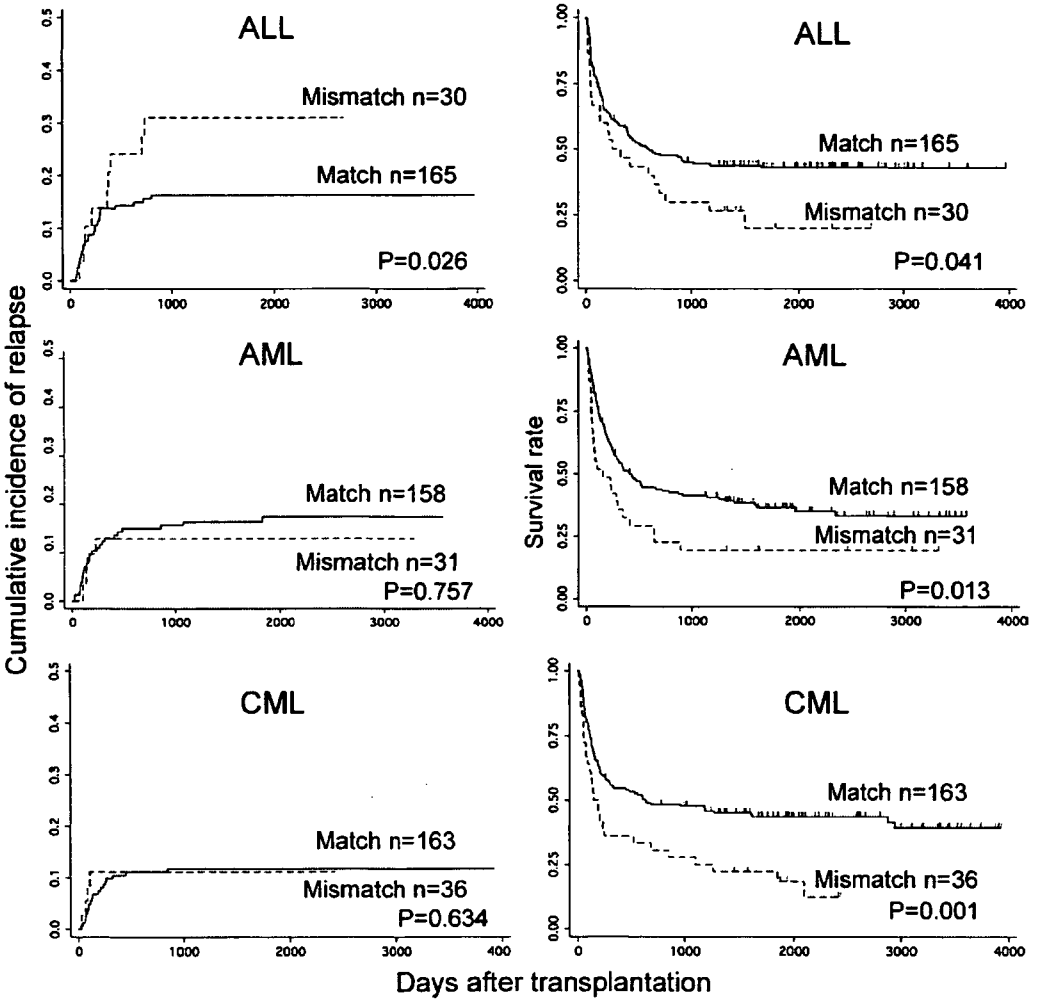

Figure 3. Cumulative incidence of relapse and survival by matching of KIR2DL ligand in the GVH direction in HLA-C–mismatched patients with ALL, AML, and CML. HLA-C–mismatched patients were selected for this analysis. The directions of HLA-C mismatching were GVH and/or HVG. The solid line represents KIR2DL ligand match in the GVH direction; the dotted line, KIR2DL mismatch in the GVH direction.

Effects of HLA Locus Mismatch and KIR Ligand Mismatch on Rejection

Rejection rates in patients who engrafted marrow and survived more than 21 days were analyzed. KIR-L-MM-R was found to be a significantly higher risk factor for rejection compared with match (HR = 4.39; P = .012), and no HLA mismatch was considered significant by multivariate analysis (Table 3). Older donor age was a significant clinical risk factor for rejection (HR = 1.08; P = .002); other clinical factors were not significant.

The cumulative incidence of graft rejection was 5.7% (95% CI = 2.3%-11.3%) in KIR-L-MM-R (n = 106) and 1.8% (95% CI = 0.8%-3.3%) in match (n = 447) (P = .019) 1 year after transplantation in HLA-C–mismatched patients in either direction. Engraftment rate was not influenced by HLA and KIR ligand matching (data not shown).

Effects of HLA Locus Mismatch and KIR Ligand Mismatch on Acute GVHD

HLA allele mismatch of each HLA-A, -B, and -C locus was found to be an independent risk factor for grade 3-4 aGVHD and grade 2-4 aGVHD, and the mismatch of each HLA-DRB1 and -DQB1 locus was not a significant risk factor. HLA-DPB1 mismatch was a significant risk factor for grade 2-4 aGVHD and a marginal risk factor for grade 3-4 aGVHD (Table 3). When analyzed by leukemia cell type, AML showed no significant HLA mismatch locus for aGVHD (data not shown).

KIR-L-MM-G was associated with a significantly higher risk of grade 2-4 aGVHD (HR = 1.70; $P < .001$) and grade 3-4 aGVHD (HR = 2.35; $P < .001$) compared with KIR ligand match (Table 3). By leukemia cell type, the HR of KIR-L-MM-G in grade 3-4 aGVHD was 2.76 for AML ($P = .005$), 1.75 for ALL ($P = .111$), and 2.79 for CML ($P < .001$).

In HLA-C mismatch patients, the incidence of 40.3% in KIR-L-MM-G (95% CI = 29.3%-50.9%) was significantly higher than the 25.8% in match (95% CI = 21.9%-30.0%) ($P = .011$) for grade 3-4 aGVHD.

Significant clinical risk factors for grade 3-4 GVHD by multivariate analysis were GVHD prophylaxis (tacrolimus vs cyclosporine, HR = 0.72; $P = .016$), patient age (HR = 0.99; $P = .019$), donor age (HR = 1.02; $P = .001$), and disease (HR = 1.28; $P = .001$) in all leukemia patients.

Effects of HLA Locus Mismatch and KIR Ligand Mismatch on Chronic GVHD

The occurrence of cGVHD was analyzed in patients who survived more than 100 days after transplantation. HLA-A mismatch and HLA-C mismatch were found to be significant factors (HR = 1.41; $P = .013$ and HR = 1.38; $P = .014$, respectively). KIR-L-MM-G was not significant (HR = 1.13; $P = .640$) (Table 3).

In HLA-C mismatch patients, the cumulative incidence of cGVHD 3 years after transplantation was 43.2% in KIR-L-MM-G (95% CI = 27.2%-58.3%) and 40.4% in KIR2DL ligand match (95% CI = 35.4%-46.1%) ($P = .727$). Significant clinical risk factors for cGVHD by multivariate analysis were patient age (HR = 1.01; $P = .0004$), disease (HR = 1.23; $P = .003$), and TBI (HR = 1.54; $P = .004$).

Effects of HLA Allele Mismatch and KIR Ligand Mismatch on Survival

In all leukemia patients, HLA allele mismatch of each HLA-A, -B, and -DQB1 locus was found to be an independent risk factor for mortality after transplantation, and the mismatch of HLA-C was of marginal risk. HLA mismatch in each HLA-DRB1 and -DPB1 locus was not a significant factor. By leukemia cell type, mismatch of HLA-A, -B, and -DPB1 was a significant risk factor in ALL, and mismatch of HLA-A and -C was a significant risk factor in CML (Table 4).

Survival 5 years after transplantation was 39.8% in HLA-C mismatch (95% CI = 32.8%-46.7%) and 44.5% in HLA-C match (95% CI = 39.6%-49.3%) in ALL ($P = .088$); 33.7% (95% CI = 26.9%-40.6%) and 46.3% (95% CI = 41.2%-51.2%), respectively, in AML ($P < .001$); and 39.7% (95% CI = 32.8%-46.5%) and 58.3% (95% CI = 53.2%-63.1%), respectively, in CML ($P < .001$) (Figure 1).

Survival 5 years after transplantation was 40.9% in HLA-DPB1 mismatch (95% CI = 36.3%-45.4%) and 50.3% in HLA-DPB1 match (95% CI = 41.5%-58.4%) in ALL ($P = .031$); 41.8% (95% CI = 37.0%-46.6%) and 42.6% (95% CI = 34.5%-50.4%), respectively, in AML ($P = .698$); and 51.4% (95% CI = 46.5%-56.1%) and 53.4% (95% CI = 45.1%-61.0%), respectively, in CML ($P = .522$) (Figure 2).

KIR-L-MM-G was also found to be a significant risk factor for mortality (HR = 1.80; $P < .001$). Particularly in AML and CML patients, KIR-L-MM-G had a significantly higher adverse effect than match (HR = 1.93; $P = .005$ and HR = 2.23; $P < .001$, respectively); its effect was moderate in ALL patients (HR = 1.57; $P = .069$) (Table 4).

In HLA-C mismatch patients in either direction, the survival rate 5 years after transplantation was 20.0% for KIR-L-MM-G (95% CI = 6.9%-38.0%) and 43.0% in match (95% CI = 35.3%-50.5%) in ALL ($P = .041$); 19.4% (95% CI = 7.9%-34.6%) and 36.5% (95% CI = 28.8%-44.2%), respectively, in AML ($P = .013$); and 22.2 (95% CI = 10.5%-36.7%) and 43.6% (95% CI = 35.8%-51.1%), respectively, in CML ($P = .001$) (Figure 3).

Significant clinical factors for mortality by multivariate analysis were patient age (HR = 1.02; $P < .001$), donor age (HR = 1.01; $P = .037$), disease (HR = 0.88; $P = .006$), and the status at transplantation (high vs standard, HR = 2.14; $P < .001$).

DISCUSSION

In the present study, we attempted to elucidate how disparities of HLA and KIR affect leukemia relapse and the other transplantation-related immunologic events and to explore how these findings can be applied to induce a GVL effect and improve patient survival in the unrelated setting. Simultaneous analysis of HLA and KIR ligand matching by multivariate analysis made it possible to clarify the role of these antigens in UR-HSCT.

To the best of our knowledge, this is the first report to elucidate the HLA locus responsible for the GVL effect by leukemia cell type in T-cell–replete UR-HSCT. The sequentially registered 577 AML, 617 ALL, and 596 CML patients sufficed to analyze the effects of HLA and KIR ligand matching in the 3 major leukemia cell types.

HLA-C mismatch reduced the relapse rate overall, as reported previously [4]. The GVL effect of HLA-C mismatch depended on the leukemia cell type. ALL patients with HLA-C mismatch showed a significantly lower leukemia relapse risk than those with match, whereas AML and CML patients did not. Furthermore, CML patients with HLA-DPB1 mismatch

showed a significantly lower leukemia relapse rate than those with match, whereas AML and ALL patients did not. Although the reason why the HLA locus responsible for the GVL effect differs with leukemia cell type remains unknown, the different expression of HLA antigens, such as HLA-C, HLA-DPB1, or co-stimulatory molecules on leukemia cells, might modify the immune response of effector cells to leukemia cells. The finding of HLA-DPB1 is in line with a previous report in CML and ALL patients treated with T cell–depleted UR-HSCT [12].

In contrast, an impact of HLA-A and -B allele mismatch on leukemia relapse was not observed. Because HLA-A and/or -B allele mismatch induces severe aGVHD, no GVL effect of HLA-A and /or -B allele mismatch might imply that the target antigenic peptide recognized by effector T cells responsible for aGVHD is not expressed on leukemia cells.

Unexpectedly, KIR-L-MM-G increased the leukemia relapse rate overall. A significantly increased relapse rate in the mismatched group was observed in ALL, but not in AML and CML. Simultaneous multivariate analysis of HLA-C mismatch and KIR-L-MM-G revealed that contrary reactions of these mismatches occurred independently. Although the mechanism involved in this detrimental effect of KIR-L-MM-G on leukemia relapse is not known, the activation of KIR-positive NK cells or T cells might cause immune dysfunction, which abrogates the GVL effect.

The GVL effect of donor-derived KIR-positive NK cells transplanted purified $CD34^+$ stem cells with HLA haploidentical donor was reported in AML patients, but not in ALL patients [22]. In T-cell–replete UR-HSCT, published reports show contradictory effects of KIR ligand mismatch on leukemia relapse. A GVL effect in myeloid malignancies [23-25], a higher leukemia relapse rate [26], and no significant effect [27-29] all have been reported. The use of ATG for GVHD prophylaxis might be a key to understanding these diverse results. Our analysis of T-cell–replete UR-BMT with no use of ATG provided reliable evidence for the adverse effect of KIR-L-MM-G on relapse of ALL relapse. No effect on relapse of AML or CML was reported in a recent large-scale study of myeloid malignancy from the Center for International Blood and Marrow Transplant Research, the European Blood and Marrow Transplant Registry, and the Dutch Registry [30]. Whether KIR ligand match affects leukemia relapse adversely or beneficially is a critical issue for clinical transplantation and immunotherapy using NK cells, and further large-scale comparative studies considering GVHD prophylaxis are warranted.

A higher rejection rate (HR = 4.39; $P = .012$) was found for KIR-L-MM-R; that is, in this mismatch combination, patient KIR2DL-positive effector cells lacking donor KIR ligand are reconstituted and activated after transplantation, which induces the rejection of engrafted donor-derived hematopoietic stem cells. "Hybrid resistance" has been extensively analyzed in mice to induce graft rejection by NK cells [31]. The same mechanism of rejection induced by NK cells might be considered in humans, although 88% of KIR ligand mismatch pairs and 86% of match pairs were given cyclophosphamide as a preconditioning. The effects of HLA class I mismatch for graft rejection were reported [5,32,33]; our data suggest that the effect of HLA-C mismatch were mainly because of KIR2DL ligand mismatch in the HVG direction, and may not result from the HLA-C allele mismatch itself. Our findings are in agreement with a report showing the effect of rejection but not engraftment by KIR2DL ligand mismatch in UR-HSCT [29].

Since the first JMDP report [4], HLA-class I mismatch has been known to significantly increase aGVHD, whereas HLA-DRB1 mismatch has only a marginal effect on aGVHD. The present study has confirmed those earlier findings. We could add the new data on HLA-DPB1 matching showing that HLA-DPB1 mismatch induces moderate aGVHD. Our finding of the effect of HLA-DPB1 on aGVHD concurs with other reports [9-11], although there we found no difference in aGVHD between 2 allele mismatches and 1 allele mismatch of HLA-DPB1.

The international collaborative study is expected to reconcile discrepancies of allele matching in ethnically diverse transplantation populations. Furthermore, the identification of nonpermissive HLA allele mismatch and amino acid substitution responsible for aGVHD, leukemia relapse, and survival might explain these discrepancies in diverse ethnic populations.

Interestingly, KIR-L-MM-G had a higher HR of severe aGVHD than did match. Because these values were adjusted by HLA allele matching and clinical factors, this finding demonstrates that KIR-L-MM-G is a factor independent of HLA allele matching. In fact, among HLA-C mismatch patients, KIR-L-MM-G was associated with a higher rate of grade 3-4 aGVHD than match. In KIR-L-MM-G, the donor-derived KIR2DL2/3- or KIR2DL1-positive effector cells are suspected to react with patient cells that lack the corresponding KIR2DL epitope of HLA-C. These effector cells induce aGVHD through several possible mechanisms. First, NK cells derived from donor graft might directly attack the patient target cells. This is unlikely, however, because in vivo infusion of alloreactive NK cells were found to not cause aGVHD [34], and NK cells were seen to play mainly a protective role for GVHD in a murine experimental model [35]. Alternatively, activated NK cells might

affect donor-derived T cells that induce aGVHD. Third, KIR2DL-positive T cells might induce aGVHD directly. The presence of KIR2DL-positive T cells was reconstituted after UR-HSCT [36].

Conflicting findings have been reported in terms of the effect of KIR-L-MM-G on aGVHD in T-cell–replete UR-HSCT. Some studies have found a trend toward less aGVHD [23], whereas others have reported an increased risk of aGVHD [27,29]. The variety of GVHD prophylaxis, HLA matching, and other clinical factors, and limited patient numbers in each study makes it difficult to determine the role of KIR ligand matching in clinical outcomes. The use of ATG and/or the T-cell depletion method for GVHD prophylaxis will be a key strategy in resolving the discrepancy regarding aGVHD in UR-HSCT [35,37] and in HLA haplotype–identical related HSCT with T-cell depletion [38]. That is, T cell and NK-cell reconstitution after transplantation might affect immunologic events induced by the interaction of KIR and HLA-C epitopes. In addition, genotyping of KIR genes, especially for activating KIR such as KIR2DS, is required to understand the mechanism of KIR involved in aGVHD and the GVL effect [39]. The East Asian population, including Japanese, is known to have several characteristic HLA types. Similarly, the frequencies of both the KIR ligand epitope and the KIR genotype are distinctive in the Japanese population. For example, a higher frequency of C1 epitope and dominance of the KIR "A" haplotype were reported [40]. Those features might contribute considerably to our results. The combination of KIR2DL1 and C2 epitope has been reported to show higher affinity and a stronger inhibitory signal compared with the combination of KIR2DL2/3 and C1 epitope [14].

HLA-A and HLA-C mismatch have been identified as significant independent factors inducing cGVHD, underscoring our previous finding of the importance of HLA class I matching. No influence of KIR-L-MM-G on cGVHD (in contrast to aGVHD) indicates that the KIR-related immunologic reaction has no relation to cGVHD.

There is another model regarding the KIR ligand effect in HSCT, the so-called "missing KIR ligand theory." Hsu et al reported this effect on survival and relapse of AML and myelodysplastic syndrome in T-cell–depleted HLA-matched related HSCT [41] and on relapse in AML, ALL, and CML in UR-HSCT in non-JMDP populations [42]. Lack of either KIR2DL ligand in a patient should activate the corresponding donor NK cells and induce the GVL effect.

In the analysis of KIR matching including HLA mismatch pairs, the mismatch pairs in the "missing KIR ligand theory" with either C1C1 or C2C2 patient epitope were divided into match and mismatch in the "KIR ligand matching theory" by donor epitope. When the donor has either C1C1 or C2C2, the KIR ligand matching theory indicates match, and when the donor has C1C2, the theory indicates mismatch. In this combination, donors with C1C2 (n = 92) had a significantly higher rate of severe aGVHD (44.4%) than donors with either C1C1 or C2C2 (19.2%) (n = 1413; $P < .001$). Therefore, we considered the "ligand matching model" to be applied in this JMDP study.

Finally, because survival after transplantation is influenced not only by leukemia relapse, but also by transplantation-related mortality resulting from aGVHD, cGVHD, fatal infections, or graft failure, the effect of HLA matching and KIR ligand matching should be discussed in light of these events.

The present study has more precisely elucidated the impact of HLA matching on leukemia patient survival. The mismatch of HLA-A and -B alleles resulted in significantly higher mortality. HLA-C and HLA-DQB1 mismatch emerged as a risk factor for poorer survival for the first time in the JMDP study. Increased survival in ALL with HLA-C mismatch cannot be linked to the compensation from a lower leukemia relapse rate. HLA-DPB1 mismatch did not significantly affect overall mortality despite the increase in moderately aGVHD. These observations of HLA-C and -DQB1 mismatch in the JMDP are in line with those of other recent reports. The NMDP reported an adverse effect of HLA-C mismatch [8], and another study reported that not only HLA-C mismatch in early-stage CML, but also HLA-DQB1 mismatched CML patients with multiple mismatch posed increased risk for mortality [43].

It should be noted that KIR-L-MM-G resulted in higher mortality in UR-HSCT with T-cell–replete marrow regardless of leukemia cell type. KIR-L-MM-G might induce an immunodeficient state that would result in a higher risk for opportunistic infections [44,45]. Thus, infectious complications by cytomegalovirus and the like should be explored in relation to KIR.

We estimate that about 30% of patients in the Japanese population have HLA-C mismatch donors, of whom 15.0% are KIR-L-MM in the GVH direction, 20.8% are KIR-L-MM in the HVG direction, and 35.6% are KIR-L-MM in either direction, when HLA-A, -B, and -DRB1 genotyping is used as the donor confirmatory typing. Because both KIR2DL ligand matching and/or HLA matching itself affect aGVHD, cGVHD, rejection, ALL relapse, and survival, as described earlier, HLA-C typing is essential in selecting a suitable donor to reduce the risk of aGVHD and improve survival in practice.

In conclusion, our analysis has produced important findings for transplantation immunology and the selection of donors in UR-HSCT. First, HLA-C and HLA-DPB1 mismatches are expected to induce a ben-

eficial GVL effect, which should be considered in terms of the leukemia cell type of individual patients. Second, KIR-L-MM should be avoided, because it induces only adverse effects on transplantation outcome and provides no benefits for patients undergoing T-cell–replete UR-HSCT.

ACKNOWLEDGMENTS

The authors thank the staff members of the transplant center, donor centers, and JMDP office. They also thank Ms. Ryouko Yamauchi for data management. This work was supported in part by a Health and Labor Science Research Grant from the Ministry of Health, Labor and Welfare of Japan (Research on Human Genome, Tissue Engineering); a grant from Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation; Grant-in-Aid B (15390309) from the Japan Society for the Promotion of Science, and a grant (30) from Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan. The institutions participating and registering patients in this study include Hokkaido University Hospital, Sapporo University Hospital, Sapporo Hokuyu Hospital, Japanese Red Cross Asahikawa Hospital, Asahikawa Medical College Hospital, Hirosaki University Hospital, Iwate Medical University Hospital, Tohoku University Hospital, Yamagata University Hospital, Akita University Hospital, Fukushima Medical College, Toranomon Hospital, National Cancer Center Central Hospital, National Center for Child Health and Development, Institute of Medical Science at the University of Tokyo, Toho University Hospital, Omori Hospital, Tokyo Metropolitan Komagome Hospital, Nihon University Hospital, Itabashi Hospital, Jikei University Hospital, Keio University Hospital, Tokyo Medical College Hospital, Tokyo Medical and Dental University Hospital, Tokyo University Hospital, Yokohama City University Hospital, Kanagawa Children's Medical Center, Kanagawa Cancer Center, Tokai University Hospital, St. Marianna University Hospital, Chiba University Hospital, Chiba Children's Hospital, Kameda General Hospital, Saitama Children's Medical Center, Saitama Cancer Center Hospital, Saitama Medical School Hospital, Ibaraki Children's Hospital, Jichi Medical School Hospital, Tsukuba University Hospital, Dokkyo University Hospital, Saiseikai Maebashi Hospital, Gunma University Hospital, Niigata University Hospital, Niigata Cancer Center Hospital, Shinshu University Hospital, Hamamatsu University Hospital, Hamamatsu Medical Center, Shizuoka General Hospital, Shizuoka Children's Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, Nagoya University Hospital, Nagoya Ekisaikai Hospital, Nagoya Medical Center, Aichi Cancer Center Hospital, Aichi Medical University Hospital, Nagoya City University Hospital, Showa Hospital, Anjo Kousei Hospital, Fujita Health University Hospital, Mie University Hospital, Yamada Red Cross Hospital, Kanazawa University Hospital, Kanazawa Medical University Hospital, Toyama Prefectural Central Hospital, Fukui Medical School Hospital, Shiga University of Medical Science, Center for Adult Disease in Osaka, Kinki University Hospital, Osaka University Hospital, Osaka City University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, Matsushita Memorial Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Kobe City General Hospital, Kobe University Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Kyoto City Hospital, Kansai Medical University Hospital, Tenri Hospital, Nara Medical University Hospital, Tottori University Hospital, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Yamaguchi University Hospital, Ehime Prefectural Central Hospital, Okayama Medical Center, Kurashiki Central Hospital, Kyushu University Hospital, Harasanshin General Hospital, Hamanomachi General Hospital, National Kyushu Cancer Center, St. Mary's Hospital, Kokura Memorial Hospital, Nagasaki University Hospital, Kumamoto Medical Center, Oita Medical University Hospital, Imamura Hospital, and Kagoshima University Hospital.

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Immunogenetics (2007) 59:99–108
DOI 10.1007/s00251-006-0186-2

ORIGINAL PAPER

Mapping of susceptibility and protective loci for acute GVHD in unrelated HLA-matched bone marrow transplantation donors and recipients using 155 microsatellite markers on chromosome 22

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Received: 15 August 2006 / Accepted: 21 November 2006 / Published online: 3 January 2007

Abstract Despite matching donors and recipients for the human leukocyte antigens (HLAs) expressed by the major histocompatibility genomic region of the short arm of chromosome 6, several recipients still develop acute graft-versus-host disease (aGVHD) after bone marrow transplantation (BMT). This is possibly due to non-HLA gene polymorphisms, such as minor histocompatibility antigens (mHas) and genes coding for cytokines. However, a detailed genetic background for aGVHD has not yet been established. To find novel susceptibility and/or protective loci for aGVHD, a whole genome-wide association study of donors and recipients needs to be performed. As the first step to such a study, we retrospectively analyzed polymorphisms of 155 microsatellite markers spread across the long arm of chromosome 22 in 70 pairs of HLA-matched unrelated BMT donors and recipients. We performed individual typing and then compared the markers' allele frequencies (1) between all the aGVHD (grades III and IV GVHD) and GVHD-free (grade 0 GVHD) groups in donors and recipients and (2) between the aGVHD and aGVHD-free groups in donor/recipient pairs that were matched and mismatched for the microsatellite marker's allele. Screening of the microsatellite markers revealed five loci with a significant difference between the aGVHD and GVHD-free groups and revealed eight loci on chromosome 22, where the microsatellite allele mismatched markers were associated with aGVHD. This screening analysis suggests that several aGVHD-associated susceptible and protective loci exist on chromosome 22, which may encompass novel gene regions that need to be elucidated for their role in aGVHD.

Keywords Microsatellite · Bone marrow transplantation · Acute GVHD · Chromosome 22 · Non-HLA

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Introduction

The occurrence of acute graft-versus-host disease (aGVHD) is still a major cause of mortality in the bone marrow transplantation (BMT) recipients who are not related familially to donors. Despite successfully matching the human leukocyte antigen (HLA) alleles of donors and recipients for hematopoietic stem cell transplantation, a significant proportion of transplantation recipients develop aGVHD because of genetic differences attributed to minor histocompatibility antigens (mHa) (Chao 2004; Falkenburg et al. 2003), non-HLA genes coding for cytokines, and other molecules involved in the pathogenesis of aGVHD (Charron 2003; Kallianpur 2005; Dickinson and Charron 2005; Mullighan et al. 2004).

Genetic association studies of aGVHD can be performed at least in two ways: the candidate gene approach and genome-wide approach. The former approach is hypothesis-driven and dependent on the systematic knowledge of the aGVHD biological process. By using the candidate gene approach, single nucleotide polymorphisms (SNPs) were found within cytokine or cytokine receptor genes, which affect the aGVHD (Charron 2003; Kallianpur 2005; Dickinson and Charron 2005; Mullighan et al. 2004). However, aGVHD is a complex pathophysiological disease, and undoubtedly, a number of unknown genes contribute to or affect the GVHD mechanism. In this regard, the candidate gene approach would fail to find novel genes that are not already reported or thought to be immunoregulatory genes involved with aGVHD. In comparison, the genetic association studies using the genome-wide approach and genetic markers to test all possible variants systemically across the whole genome would be a more experimentally ideal approach to find novel genes involved with aGVHD. In addition, genomic matching by using SNP and/or microsatellite markers for finding compatibility of minor antigens in BMT may improve survival and other clinical outcomes.

Microsatellites and SNPs are two types of genetic markers that can be applied to genome-wide disease association studies, with each type of marker presenting certain advantages as well as inconveniences. Microsatellites are direct tandem-repeated sequences of DNA with a repeat size ranging from 2 to 6 bp. The number of repeats within a microsatellite sequence is usually less than 100. Because the microsatellite polymorphism is based on the differences in number of repeats, microsatellites are highly polymorphic with a high degree of heterozygosity. Polymorphic microsatellites are fewer in number than SNPs, but like SNPs, they are widely distributed across the human genome enabling efficient and accurate calculations of linkage disequilibrium (LD) between pairs of microsatellite loci separated by less than 100 kb of genomic sequence. Indeed, we have already established and described a set of 27,039 microsatellite markers for the systematic analysis of the whole human genome and, together with SNP analysis, revealed at least seven potential susceptibility gene loci of rheumatoid arthritis (Tamiya et al. 2005). Therefore, the main advantage of using microsatellites as the primary or "first pass" genotyping method is that they allow for a genome association analysis to become an immediate and efficient reality.

To date, there are only a few association studies using microsatellite analysis to determine the potential clinical outcomes in hematopoietic stem cell transplantation, and these studies are limited mainly to the cytokine genes and the HLA region (Karabon et al. 2005; Li et al. 2004; Cullup et al. 2003; Nordlander et al. 2002; Witt et al. 1999). As a set of 27,039 microsatellite markers for the systematic analysis of the whole human genome has been established, we decided to use them in a genome-wide search of allele frequency differences to find and map novel susceptibility and/or protective loci for aGVHD. Although our ultimate goal is a complete genome-wide study, we have started our search for aGVHD susceptibility/protective loci within chromosome 22 (chr 22) for simplicity and economic convenience. A number of studies (Abecasis et al. 2001; Keicho et al. 2000; Oka et al. 1999; Ota et al. 1999; Li et al. 2004) suggest that association analysis using microsatellite markers as a first step of the genome-wide approach is a useful way to find candidate genes and specifically the mHa genes on chr 22 of BMT donors and recipients.

Human chr 22 is the second smallest of the autosomes comprising 1.6–1.8% of the genomic DNA (Dunham et al. 1999). There is no evidence to indicate the presence of any protein coding genes on the short arm of chr 22 (22p). In contrast, the long arm of the chr 22 (22q) is rich in genes compared with other chromosomes. In addition, alteration of gene dosage on the part of 22q is responsible for the etiology of 29 Mendelian disorders and a number of congenital abnormality disorders including cat eye syndrome and DiGeorge syndrome (McDermid and Morrow 2002). Linkage studies have shown an association of chr 22 loci to several disorders, such as schizophrenia, epilepsy, multiple sclerosis, and myopia (DeLisi et al. 2002; Berkovic et al. 2004; Liguori et al. 2004; Stambolian et al. 2004).

Interestingly, two recent reports have highlighted that there are many signal transducers and activators of transcription (STAT) and NF-kappaB-binding sites distributed across chr 22 (Martone et al. 2003; Hartman et al. 2005). STAT and NF-kappaB family members play an essential role in regulating the induction of genes involved in physiological processes, such as apoptosis, immunity, and inflammation, and they may also affect immunoregulatory genes relevant to the recognition and rejection of

foreign tissue. In addition, Gubarev et al. (1996) reported the localization of a gene encoding mHa to chr 22. On the basis of these reports and in an attempt to improve efficiency by screening chromosomal regions of high gene density, chr 22 is a very attractive target for genome-wide association research of GVHD and other immune-related diseases.

As the first step to our genome-wide study, we retrospectively genotyped 155 microsatellite markers on chr 22 in 70 HLA-matched unrelated BMT recipient and donor pairs and associated at least eight significant allele frequency differences with aGVHD. In accordance with our previous study using microsatellite markers to identify mHa (Li et al. 2004), we performed individual DNA typing to investigate the association between statistically significant donor/recipient microsatellite marker mismatches.

Materials and methods

Recipient and donor pairs

A total of 70 unrelated donor/recipient pairs after BMT who were treated through the Japan Marrow Donor Program and completely allele-matched for the HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 genes at the high resolution level were enrolled for this study after BMT (Sasazuki et al. 1998; Morishima et al. 2002). All 70 recipients underwent BMT from 1995 to 2000 for hematopoietic malignancy. None of the recipients received T-cell-depleted grafts. Patient, transplantation, and treatment information data are summarized in Table 1. All the donors and recipients provided informed consent for study, which was approved by the relevant institutional ethical committee.

Diagnosis and evaluation of the acute GVHD study group

Acute GVHD was diagnosed clinically and classified into four groups according to standard criteria (grades 0, I, II, III, and IV; Glucksberg et al. 1974; Thomas et al. 1975). The 30 recipients who experienced grades III and IV were designated in this study as the aGVHD group. The 40 recipients who had not developed aGVHD (grade 0) were designated as the aGVHD-free group. The recipients with GVHD grades I or II were excluded from this analysis to differentiate more efficiently between the aGVHD and aGVHD-free groups.

Microsatellite markers set

The association study was performed using 155 microsatellite markers spanning the long arm of chromosome 22.

Table 1 Numbers and ratios for the major clinical characteristics

Clinical characteristics	Number or Ratio
Recipient's age (median year, range)	27.5, 1–50
Donor's age (median year, range)	33.9, 21–52
Recipient's sex (M/F)	38:32
Sex combination (recipient/donor)	M/M 29
	M/F 19
	F/F 13
	F/M 9
Diagnosis	
Acute myeloid leukemia	28
Acute lymphoblastic leukemia	21
Chronic myeloid leukemia	21
Conditioning regimen	
CY+TBI	26
CY+CA+TBI	17
BU+CY+CA	2
BU+CY	7
CY+BU+TBI	3
CY+VP+TBI	4
BU+CY+TLI	1
LP+TBI	2
BU+VP+LP	1
CA+VP+TBI	1
CA+TBI	2
VP+TBI	1
BU+LP+TBI	1
CA+TBI	2
aGVHD frequency	
Grade 0	40
Grade III	20
Grade IV	10
GVHD prophylaxis	
CsA+MTX	64
FK+MTX	2
FK+PDR	1
CsA	1
CsA+MTX+PDR	1
CsA+MTX+FK	1

M Male, *F* female, *CY* cyclophosphamide, *TBI* total body irradiation, *CA* cytosine arabinoside, *BU* busulfan, *VP* etoposide, *TLI* total lymph node irradiation, *LP* melpharan, *CsA* cyclosporine A, *MTX* methotorexate, *FK* tacrolimus hydrate, *PDR* predonisolone

These markers were selected from Japan Biological Information Research Center (JBIRC) database (http://jbirc.jbic.or.jp/gdbs/). The markers covered the human genome from 15647099b (D22S0283i) to 49510061b (D22S0211i) on 22q with an average spacing of 200 kb.

Microsatellite genotyping

Genomic DNA was isolated from the peripheral blood lymphocytes of patients and donors. The PCR procedure was performed in 10 μl reactions using fluorescent-dye conjugated PCR primers that were unilaterally labeled at

the 5'-end with the fluorescent reagent, 6-FAM (Applied Biosystems Japan, Tokyo, Japan). The PCR reaction mixture contained 10 ng of genomic DNA, 1 μl of deoxyribonucleotide triphosphate (5 mM each), 1 μl of 10× buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), and 20 pmol of forward and reverse primers as well as 0.5 unit of Ampli Taq Gold DNA polymerase (Applied Biosystems Japan). After initial denaturation for 5 min at 96°C, amplification was carried out in an automated thermal cycler (Applied Biosystems Japan Co.) for 40 cycles of 1 min at 96°C, 45 s at 57°C, and 45 s at 72°C with a final extension of 7 min at 72°C. Each PCR product was diluted 1:40 with water. The samples containing 1 ul of the diluted PCR product, 10 μl Hi–Di formamide (Applied Biosystems Japan Co.) and 0.1 μl GeneScan-500LIZ size standard (Applied Biosystems Japan) were denatured for 3 min at 95°C, separated on capillary gels using an ABI PRISM 3730 automated sequencer, and the electrophoretic runs were analyzed with GeneMapper software (Applied Biosystems Japan).

Statistical analysis

Microsatellite allele frequency was calculated by direct counting. The strength of association was expressed by odds ratio (O.R), which was calculated from 2×2 contingency tables. Statistical significance was examined by the Fisher's double-sided exact test and the $m \times n$ contingency table. Univariate analysis was performed to determine the association between microsatellite mismatch and aGVHD incidence. The P value, except when comparing a mismatch, was corrected by multiplying the number of microsatellite alleles (corrected Pc value). The Pc value less than 0.05 was accepted as statistically significant, and the Pc value between 0.05 and 0.1 was indicative of a trend.

Definition of a microsatellite mismatch

Donor/recipient pairs were classified as matched or mismatched at each microsatellite marker locus. Pairs were defined as mismatched only when one or more recipient alleles are not shared by the corresponding donor (direction aGVHD).

Results

The overall genotyping results for paired transplantation donors and recipients

A total of 155 microsatellite markers spanning the long arm of chromosome 22 were used to genotype 70 pairs of transplantation patients and donors. Of the 70 transplantation recipients, 30 (42.8%) developed aGVHD with GVHD grade III in 20 patients and GVHD grade IV in 10 patients. The genotyping results obtained for the 70 transplantation recipients and the 70 transplantation donors were then analyzed and compared between the aGVHD-free group (grade 0 aGVHD) and the severe aGVHD group (group III to IV aGVHD).

Of the 155 markers, there were three markers with significant allele frequency differences between all donors and all recipients (D22S0052i-385; D22S0099i-412; D22S0115i-225; data not shown). As these three markers have different allele distribution between all donors and all recipients, they were considered to be inappropriate markers for the comparison between the aGVHD-free and the aGVHD group and were therefore excluded from further analysis. The remaining 152 markers were retained for further analysis in this study because they showed no significantly different allele distribution between all donors and recipients (data not shown).

Recipient age and GVHD prophylaxis

The recipient age was not significantly higher in the patients with aGVHD than the aGVHD-free group (P= 0.27 Student's t test). In regard to GVHD prophylaxis (Table 1), there was no significant association (p>0.07) of aGVHD factor risk between the patients in the total body irradiation (TBI) group and those in the non-TBI group.

Comparison of allele frequency differences between the aGVHD-free and the aGVHD group for the microsatellite polymorphisms in donors and recipients

The frequency differences for the microsatellite alleles between the aGVHD-free group and the aGVHD group were compared separately for the donors and recipients. The significant association (P<0.05) of markers with the occurrence of aGVHD was found for five markers (Table 2), with a significant difference (P and Pc<0.05) for two donor markers (D22S283 and D22S0141i) and for three recipient markers (D22S0021i, D22S0199i, D22S0222i). The comparison of individual allele frequencies of the microsatellite markers in the grade 0 (aGVHD-free) and grade III+IV (aGVHD) groups revealed the presence of possible risk (R) alleles (O.R>1) and protective (P) alleles (OR<1; Table 2). In the donors, the allele D22S0141i-431 was increased significantly (Pc=0.049) and the allele D22S283-132 was decreased significantly (Pc=0.008) in aGVHD when compared to the aGVHD-free group. Both of the marker loci were in position 22q12.3. In the recipients, the frequency of the allele D22S0021i-348 was significantly increased (Pc= 0.035) and three alleles (D22S0021i-357, D22S0199i-444,

Table 2 Statistically significant alleles associated with aGVHD grade in patients and donors, respectively

Marker	Position	No. of alleles	Significant allele	aGVHD grade (N=40)	aGVHD grade III+IV (N=30)	Odds ratio (95% confidence interval)	Protective (P) or at risk (R)	P value	Pc
Donor									
D22S283	22q12.3	12	132	35 (87.5%)	15 (50.0%)	0.14 (0.04–46)	P	0.0007	0.008
D22S0141i	22q12.3	7	431	4 (10.0%)	14 (46.7%)	7.87 (2.24–27.7)	R	0.007	0.049
Recipient									
D22S0021i	22q13.2	5	348	21 (52.5%)	25 (83.2%)	4.52 (1.44–14.2)	R	0.007	0.035
			357	38 (90.0%)	18 (67.7%)	0.16 (0.06–0.48)	P	0.004	0.020
D22S0199i	22q13.2	4	444	22 (55.0%)	7 (23.3%)	0.25 (0.09–0.72)	P	0.007	0.028
D22S0222i	22q13.3	7	258	17 (42.5%)	4 (13.3%)	0.21 (0.06–0.71)	P	0.007	0.049

and D22S0222i-258) were significantly decreased in aGVHD when compared to the aGVHD-free group.

In regard to the D22S0021i locus, we found both a risk allele (D22S0021i-348) and a protective allele (D22S0021i-357). On the basis of a genotype analysis, there was a significant association (P=0.001) between the D22S0021i genotype and aGVHD occurrence (Table 3).

Comparison of differences between the aGVHD-free and the aGVHD groups for microsatellite alleles that were matched or mismatched in donors and recipients

As a further comparison between the aGVHD-free and the aGVHD groups, we determined the significant differences between the number of alleles of the aGVHD-free and the aGVHD groups that were matched and mismatched for the donor and recipient pairs. We estimated that there were eight significant marker mismatches for an association with the occurrence of aGVHD (Table 4). Of these eight markers, three (D22S0267i, D22S0220i, and D22S683) were more often mismatched in the severe GVHD group (O.R>1), and therefore, these markers appear to be protective against the occurrence of severe aGVHD. As D22S0220i and D22S683 are located in a relatively close position to each other on 22q12.3 where they are 780 kb apart, we selected an additional five markers between D22S0220i and D22S683. As a result, three markers (Z67524, P=0.09, O.R=0.35; D22S0132i, P=0.07, O.R=2.54; D22S0075i, P=0.07, OR=0.03 in order from the centromere to telomere) showed a tendency of association with aGVHD (Fig. 1).

Table 3 Univariate analysis of D22S0021i genotype

Allele genotype	aGVHD grade 0 (N=40)	aGVHD grade III+IV (N=30)	P value
348/348	2	10	
348/357	19	15	
357/357	17	3	
			0.001

On the other hand, five markers (D22S0152i, P=0.0005; D22S0145i, P=0.017; Z66750, P=0.014; D22S0085i, P=0.035; D22S0197i, P=0.005) were more often mismatched in the aGVHD-free group (O.R<1), suggesting that they are significant susceptibility markers for aGVHD. Of these markers, D22S0152i and D22S0145i were located in a relatively close position to each other on 22q11.23 where they were 960 kb apart. We, therefore, genotyped an additional six markers (D22S0068i, D22S0186i, D22S0163i, D22S0169i D22S0184i, and D22S1174) but found that none of them were significantly associated with aGVHD (data not shown).

Candidate genes within the aGVHD susceptibility regions

Table 5 lists the candidate susceptibility genes that are located within or near to the genomic susceptibility region which was identified by microsatellite genotyping. These genes are in the close vicinity of the significant microsatellite markers that were found within intron 3 of CACNG2, intron 3 of PEX26, intron 4 of KIAA0376, intron 7 of LARGE, and intron 8 of TOM1. Other genes, such as MYH9, EP300, TCF20, ARSA, FLJ31568, EMID1, APOL3, and FLJ44385, are located within 10 kb to 172 kb of the significantly associated microsatellite markers.

Genomic map of the association of microsatellite polymorphisms on 22q12.3 with the occurrence of aGVHD

The P values for comparing the matching of microsatellite marker alleles between those of the aGVHD-free group and the aGVHD group were determined and plotted as a P value plot against the physical location of the microsatellite markers and the known genes on 22q12.3. Figure 1 shows a P value plot and the gene map of one of the aGVHD susceptibility regions determined by the association analysis using the microsatellite markers from D22S0220i to D22S683 and beyond the border of 22q12.3 and 22q13.1. The figure shows that the genes TOM1, HMOX1, and

Table 4 Correlation between matched mismatch donor–recipient pairs and aGVHD grade for each of the significant microsatellite markers on chromosome 22

Marker	Position	aGVHD grade 0		aGVHD grade III+IV		Odds ratio (95% CI)	Protective (P) or at risk (R)	*P* value
		Matched	Mismatched	Matched	Mismatched			
D22S0267i	22q11.21	38	2	23	7	5.78 (1.10–30.24)	P	0.028
D22S0152i	22q11.23	25	15	29	1	0.05 (0.01–0.41)	R	0.0005
D22S0145i	22q11.23	8	32	14	16	0.29 (0.10–0.82)	R	0.017
Z66750	22q12.1	28	12	28	2	0.17 (0.03–0.81)	R	0.014
D22S0085i	22q12.3	6	34	11	19	0.30 (0.10–0.96)	R	0.035
D22S0220i	22q12.3	17	23	4	26	4.80 (1.41–16.35)	P	0.008
D22S683	22q12.3	6	34	0	30	11.86 (0.64–219.35)	P	0.027
D22S0197i	22q13.33	16	24	22	8	0.24 (0.087–0.67)	R	0.005

MCM5 are in the region of the most significant *P* values and in close vicinity to the protective microsatellite marker D22S0220i.

Discussion

Of the 155 markers analyzed for differences between the aGVHD-free group and the aGVHD group and separately for the recipients and donors, only five markers on chr 22 (Table 2) were found to be significantly associated with aGVHD (Pc<0.05). Interestingly, of these five positive markers, the donor positive marker D22S283 was previously reported to be associated with schizophrenia (DeLisi et al. 2002), Sorsby's fundus dystrophy (Assink et al. 2000), and CDAGS (Mendoza-Londono et al. 2005). Although the susceptibility genes on chr 22 for those diseases are still unknown, the positive microsatellite marker D22S283 is located within the SC2D4 schizophrenia susceptibility locus (NCBI GeneID 6379) and could be associated with neuropsychological impairment that may evolve with aGVHD (Sostak et al. 2003).

The other four positive markers, D22S0141i, D22S0021i, D22S0199i, and D22S0222i, which were associated with aGVHD (Table 2) had not been previously associated with any human disease. However, these markers are located in a region of human chr 22 that was previously associated with the presence of strong mucosal and T-cell immune response against HIV-1 (Kanari et al. 2005) and, therefore, that could also affect the aGVHD. The p300 gene, which is a transcriptional factor located 10 kb from D22S0021i, is believed to participate in the activities of hundreds of

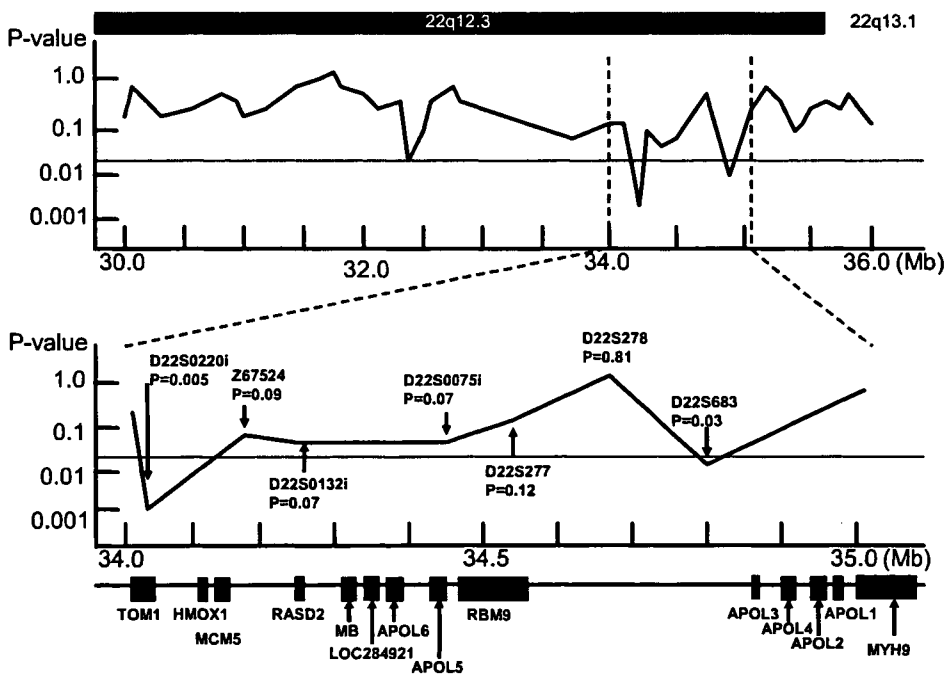

Fig. 1 aGVHD susceptibility gene mapping by association analysis using microsatellite markers on 22q12.3. *P* value (*y-axis*) was plotted against physical location of the microsatellite markers on 22q12.3 (*x-axis*), their distance (in Mb) in order from the centromere to the telomere. All markers were plotted according to their genetic map position taken from JBIRC database (http://jbirc.jbic.or.jp/gdbs/). The gene map at the *bottom* of the figure shows the representative genes that are indicated by *black boxes* on the locus near the two positive markers D22S0220i and D22S683 on 22q12.3. The *dotted horizontal line* shows the threshold for 5% significance