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ORIGINAL ARTICLE

A single-nucleotide polymorphism of the Fcγ receptor type IIIA gene in the recipient predicts transplant outcomes after HLA fully matched unrelated BMT for myeloid malignancies

A Takami[1,13], JL Espinoza[1,13], M Onizuka[2], K Ishiyama[1], T Kawase[3], Y Kanda[4], H Sao[5], H Akiyama[6], K Miyamura[7], S Okamoto[8], M Inoue[9], S Ohtake[1], T Fukuda[10], Y Morishima[11], Y Kodera[12] and S Nakao[1], for the Japan Marrow Donor Program

[1]*Department of Hematology and Oncology, Kanazawa University Hospital, Kanazawa, Japan;* [2]*Department of Hematology and Oncology, Tokai University School of Medicine, Isehara, Japan;* [3]*Division of Epidemiology, Aichi Cancer Center Research Institute, Nagoya, Japan;* [4]*Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama, Japan;* [5]*Department of Hematology, Meitetsu Hospital, Nagoya, Japan;* [6]*Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan;* [7]*Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan;* [8]*Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo, Japan;* [9]*Department of Hematology and Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan;* [10]*Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo, Japan;* [11]*Department of Hematology and Cell Therapy, Aichi Cancer Center, Nagoya, Japan and* [12]*Department of Promotion for Blood and Marrow Transplantation, Aichi Medical University, Nagoya, Japan*

Fcγ receptor type IIIA (FCGR3A) has a functional single-nucleotide polymorphism (rs396991), at which a G-to T-point mutation results in an amino acid substitution at position 158 (valine to phenylalanine; V158F). This study examined the effect of the FCGR3A polymorphism in donors and recipients on the clinical outcomes in unrelated HLA fully matched myeloablative BMT. The FCGR3A-V158F genotype was retrospectively analyzed in a total of 99 recipients with myeloid malignancies, and their unrelated donors. The presence of the 158V genotype in recipients showed a statistically better OS (adjusted hazard ratio (HR) 0.49; 95% confidence interval (CI) 0.26–0.93; $P=0.03$) and TRM (HR 0.30; 95% CI 0.14–0.67; $P=0.003$) without significant influence on the relapse rate. The recipient 158V genotype was also associated with a significantly reduced risk of chronic GVHD (HR 0.45; 95% CI 0.20–0.99; $P=0.049$) and a trend toward a reduced risk of grade II–IV acute GVHD (HR 0.55; 95% CI 0.27–1.10; $P=0.09$), leading to a significantly reduced GVHD-related mortality (HR 0.22; 95% CI 0.06–0.77; $P=0.02$). The donor FCGR3A polymorphism did not have any effect on the transplant outcomes. These results suggest an association between the recipient FCGR3A genotype and the clinical outcomes after BMT.

Correspondence: Dr A Takami, Department of Hematology and Oncology, Kanazawa University Hospital, 13-1 Takaramachi, Kanazawa, 920-8641, Japan.
E-mail: takami@med3.m.kanazawa-u.ac.jp
[13]These authors contributed equally to this work.
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Introduction

Hematopoietic SCT is a potentially curative therapy in a range of malignant and nonmalignant diseases. However, its utility is limited because of transplant-related life-threatening complications, including GVHD, infections and disease relapse.[1] Although HLA matching represents the major genetic determinant in clinical outcome after allo-SCT, recent evidence suggests that non-HLA immune-associated genes are also implicated.[2] Previous investigations have revealed that several single-nucleotide polymorphisms (SNPs) that effect individual immune response to infections and inflammatory reactions are associated with transplant outcomes.[3–9]

Fcγ receptor type IIIA (FCGR3A), a low-affinity receptor capable of interaction with complexed or monomeric IgG, is expressed on neutrophils, eosinophils, natural killer cells, macrophages, monocytes, DC, γδ-positive T cells and keratinocytes.[10–13] FCGR3A mediates Ab-dependent cell-mediated cytotoxicity, phagocytosis, cytokine production and regulation of Ig production. FCGR3A has a functional SNP (rs396991), at which a G-to-T point mutation results in an amino acid substitution at position 158 (valine to phenylalanine; V158F) in the second Ig-like domain.[14] The cells bearing the FCGR3A-158V genotype show a higher affinity for IgG1 and IgG3 than those without 158V, and are capable of binding IgG4,[15] and thus

can exert Ab-dependent cell-mediated cytotoxicity more efficiently.[16] The 158V genotype is associated with susceptibility to rheumatoid arthritis[17] and immune-mediated thrombocytopenic purpura,[18] better clinical response to rituximab in B-cell lymphomas[19,20] and a lower risk of recurrent periodontitis.[21] In contrast, systemic lupus erythematosus and better clinical outcome of cetuximab against metastatic colorectal cancer correlates with the 158F genotype.[22,23] This study analyzed the effect of donor and recipient SNP (rs396991) in the FCGR3A gene on the clinical outcomes in patients after allogeneic myeloablative BMT using an HLA allele-matched unrelated donor. The data show that the presence of the FCGR3A-158V genotype in the recipient was associated with significantly better transplant outcomes on the OS, TRM and GVHD.

Patients and methods

Patients

FCGR3A genotyping was performed on a total of 99 recipients with myeloid malignancies and their unrelated donors who underwent transplantation after myeloablative conditioning through the JMDP (Japan Marrow Donor Program) with T-cell-replete marrow from an HLA-A, -B, -C and -DRB1 allele-matched donor between November 1995 and March 2000. HLA genotypes of HLA-A, -B, -C and -DRB1 allele of patient and donor were determined by the Luminex microbead method described previously (Luminex 100 System; Luminex, Austin, TX, USA).[24,25] No patient had a history of any previous transplantation. The final clinical survey of these patients was completed by 1 November 2007. Diagnoses were AML in 47 (47%), CML in 42 (42%) and myelodysplastic syndrome in 10 patients (10%; Table 1). The recipients were defined as having standard risk disease if they had AML in first CR, CML in any chronic phase or myelodysplastic syndrome. All others were designated as high-risk disease. CYA- or tacrolimus-based regimens were used in all patients for GVHD prophylaxis, and anti-T-cell therapy, such as antithymocyte globulin and *ex vivo* T-cell depletion, was not. All patients and donors gave their written informed consent to participate in molecular studies of this nature according to the declaration of Helsinki at the time of transplantation. The project was approved by the institutional review board of Kanazawa University Graduate School of Medicine and JMDP.

FCGR3A rs3969913 genotyping

Genotyping of FCGR3A was performed using the TaqMan-Allelic discrimination method[26] with a 7900-HT Real Time PCR system (Applied Biosystems, Foster City, CA, USA), and results were analyzed using the Allelic Discrimination software program (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, the TaqMan universal master mix and the specific probe rs396991 designed for SNP of FCGR3A (product No C_25815666_10; Applied Biosystems).

Table 1 Donor and recipient characteristics

Variable	*Myeloid malignancies (n = 99)*				
	Recipient FCGR3A genotype				P-*value*
	158V positive n = *46, 46%*		*158V negative* n = *53, 54%*		
	No.	*Ratio*	*No.*	*Ratio*	
Age, years					
Recipient					0.89
Median	31		36		
Range	8–49		1–50		
Donor					0.45
Median	33		31		
Range	21–50		22–48		
Donor FCGR3A genotype					0.15
With 158V (V/F or V/V)	24	86%	20	83%	
Without 158V (F/F)	22	79%	33	138%	
Sex, male					0.68
Recipient	30	65%	35	66%	
Donor	29	63%	39	74%	
Recipient/donor sex					0.69
Sex matched	25	54%	31	58%	
Male/female	11	24%	9	17%	
Female/male	10	22%	13	25%	
Disease					0.48
AML	23	50%	24	45%	
MDS	6	13%	4	8%	
CML	17	37%	25	47%	
Disease risk					0.26
Low	30	65%	40	75%	
High	16	35%	13	25%	
ABO matching					0.69
Match	26	57%	32	60%	
Major mismatch	10	22%	13	25%	
Minor mismatch	10	22%	8	15%	
Conditioning regimen					0.25
With TBI	39	85%	40	75%	
Without TBI	7	15%	13	25%	
Pre-transplant CMV serostatus					0.28
CMV-negative recipient	9	20%	13	25%	
Missing	7	15%	3	6%	
GVHD prophylaxis					0.20
With CYA	40	87%	50	94%	
With tacrolimus	6	13%	3	6%	
TNC, $\times 10^8$/kg					0.45
Median	5.4		5.6		
Range	2.3–16.2		2.4–52.6		
Engraftment	1	2%	2	4%	0.64

Abbreviations: FCGR3A = Fcγ receptor type IIIA; MDS = myelodysplastic syndrome; TNC = total nucleated cell count harvested.

Data management and statistic analysis

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. The pre-transplant CMV serostatus was routinely tested only for

Table 2 Univariate analysis of the association between the FCGR3A genotype and the clinical outcomes after transplantation

	Donor FCGR3A genotype			*Recipient FCGR3A genotype*		
	With 158V	*Without 158V*	P-*value*	*With 158V*	*Without 158V*	P-*value*
No.	44	55		46	53	
5-year OS	50%	60%	0.52	65%	49%	0.15
5-year TRM	36%	27%	0.28	22%	40%	0.07
5-year relapse	16%	16%	0.92	17%	15%	0.79
II–IV aGVHD	39%	40%	0.81	33%	45%	0.19
cGVHD	34%	36%	0.81	26%	43%	0.07
GVHD-related mortality	15%	18%	0.63	7%	25%	0.01
Infection-related mortality	27%	20%	0.46	20%	26%	0.33

Abbreviations: aGVHD = acute GVHD; cGVHD = chronic GVHD; FCGR3A = Fcγ receptor type IIIA.

patients but not for their donors. Engraftment was confirmed by an ANC of $>0.5 \times 10^9$/L for at least 3 consecutive days. Acute and chronic GVHD were diagnosed and graded using established criteria.[27,28] The OS was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. TRM was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. The data on the causative microbes of infections, postmortem changes in the cause of death and staging of acute GVHD, as well as the data on supportive care including infection prophylaxis and therapy of GVHD, which were given on institution basis, were not available in this cohort. The analysis was performed using the Excel 2007 (Microsoft Corp., Redmond, WA, USA), OriginPro version 8.0J (Lightstone Inc., Tokyo, Japan) and R (The R Foundation for Statistical Computing, Perugia, Italy) software programs.[29] The probability of OS was calculated using the Kaplan–Meier method and compared using the log-rank test. The probabilities of TRM, disease relapse, acute GVHD, chronic GVHD and each cause of death were compared using the Grey test[30] and analyzed using the cumulative incidence analysis,[29] considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD and death without each cause as respective competing risks. The variables included the recipient age at the time of transplantation, sex, CMV serostatus before transplantation, disease characteristic (disease type and disease risk at transplantation), donor characteristics (age, sex, sex compatibility and ABO compatibility), transplant characteristics (TBI-containing regimen, tacrolimus vs CYA and total nucleated cell count harvested per recipient weight). The median values were used as the cutoff point for continuous variables. The χ^2 test and Mann–Whitney test were used to compare two groups. The Hardy–Weinberg equilibrium for the FCGR3A gene polymorphism was tested using the Haploview program.[5] Multivariate Cox models were used to evaluate the hazard ratio (HR) associated with the FCGR3A polymorphism. Covariates found to be significant in univariate analyses ($P \leqslant 0.10$) were included in the models. The *P*-values were two sided and outcomes were considered to be significant with $P \leqslant 0.05$ in both the univariate and multivariate analyses.

Results

Frequencies of the FCGR3A genotyping

The FCGR3A gene polymorphism (rs396991) was analyzed in 99 unrelated BM donor-myeloablative transplant recipient pairs (Table 1). The genotype frequencies of 158V/V, 158V/F and 158F/F were 4, 42 and 54% in donors, and 3, 41 and 56% in recipients. These were similar to a previous report[14,31] in Japanese populations and were in accord with the Hardy–Weinberg equilibrium ($P = 0.91$).

Transplant outcome according to the FCGR3A genotype

The median follow-up duration in the cohort was 109 months among the survivors (range 43–134 months), and 16 recipients (16%) had relapsed or progressed and 47 (47%) had died. Three patients (3%) died before undergoing engraftment.

The transplant outcomes according to the FCGR3A genotype are summarized in Table 2. The recipient 158V genotype was associated with a significantly reduced incidence of GVHD-related mortality (7 vs 25%, $P = 0.01$; Figure 1b), and a trend toward a reduced incidence of chronic GVHD (33 vs 45%, $P = 0.07$) and reduced 5-year TRM (22 vs 40%, $P = 0.07$; Figure 1a). The donor genotype had no significant effects on the transplant outcomes.

Multivariate analysis

All factors that were found to be significant in univariate analyses were included in the model. The presence of the 158V genotype in recipients were statistically significant in the multivariate analyses for better OS (HR 0.49; 95% confidence interval (CI) 0.26–0.93; $P = 0.03$; Table 3) and TRM (HR 0.30; 95% CI 0.14–0.67; $P = 0.003$). In addition, the recipient 158V genotype was associated with a significantly reduced incidence of chronic GVHD (HR 0.45; 95% CI 0.20–0.99; $P = 0.049$) and a trend toward a lower incidence of grade II–IV acute GVHD (HR 0.55; 95% CI 0.27–1.10; $P = 0.09$), resulting in a significantly reduced GVHD-related mortality (HR 0.22; 95% CI 0.06–0.77; $P = 0.02$). A correlation between the recipient 158V genotype and low infection-related death was also observed (HR 0.42; 95% CI 0.17–1.01; $P = 0.05$). The donor 158V genotype did not significantly influence the transplant outcomes.

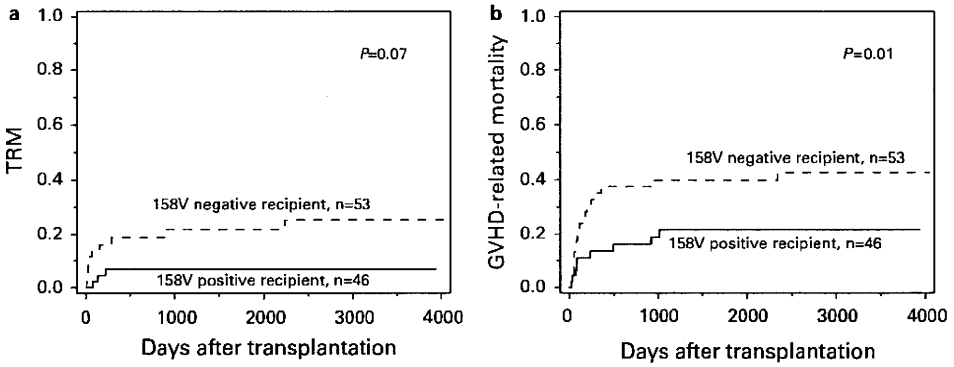

Figure 1 Estimated cumulative incidence curves of TRM (**a**) and GVHD-related mortality (**b**) according to the recipient FCGR3A genotype. Solid lines and dashed lines represent the 158V-positive recipient and the 158V-negative recipient, respectively.

Table 3 A multivariate analysis of the association of the FCGR3A genotype with the clinical outcomes after transplantation

Variable	*Donor 158V positivity*			*Recipient 158V positivity*			*Minor ABO incompatibility*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
OS	1.29	0.70–2.38	0.42	0.49	0.26–0.93	0.03	—	—	—
TRM	1.73	0.84–3.54	0.14	0.30	0.14–0.67	0.003	—	—	—
Relapse	0.82	0.27–2.55	0.74	0.67	0.22–2.02	0.47	—	—	—
II–IV aGVHD	1.24	0.63–2.46	0.53	0.55	0.27–1.10	0.09	—	—	—
cGVHD	0.55	0.25–1.21	0.14	0.45	0.20–0.99	0.049	0.26	0.03–1.88	0.18
GVHD-related mortality	1.65	0.61–4.45	0.32	0.22	0.06–0.77	0.02	—	—	—
Infection-related mortality	1.36	0.58–3.20	0.48	0.42	0.17–1.01	0.05	—	—	—
	High-risk disease			*Age (>27 years)*			*Donor age (>32 years)*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
OS	2.17	1.17–4.04	0.01	—	—	—	—	—	—
TRM	—	—	—	—	—	—	2.56	1.22–5.37	0.01
Relapse	6.44	1.92–21.59	0.003	0.43	0.13–1.43	0.17	—	—	—
II–IV aGVHD	—	—	—	0.38	0.19–0.74	0.005	2.84	1.42–5.69	0.003
cGVHD	—	—	—	—	—	—	—	—	—
GVHD-related mortality	0.43	0.10–1.90	0.27	—	—	—	—	—	—
Infection-related mortality	2.78	1.19–6.46	0.02	—	—	—	3.27	1.31–8.12	0.01

Abbreviations: aGVHD = acute GVHD; CI = confidence interval; cGVHD = chronic GVHD; FCGR3A = Fcγ receptor type IIIA; HR = hazard ratio.

Discussion

This study showed a considerable effect of the recipient FCGR3A-158V genotype on GVHD development and GVHD-related mortality, thus positively contributing to a significantly better TRM and OS for patients with myeloid malignancies receiving HLA-matched myeloablative BMT. The presence of 158V in recipients did not influence disease relapse. Therefore, recipients with the 158V genotype may be capable of avoiding GVHD without compromising a GVL effect. The recipient 158V genotype also showed a trend toward reduced infection-related mortality, which might result from a reduced need for immunosuppressive therapy due to a low incidence of GVHD, although the data regarding treatment for GVHD were unavailable in this cohort. This is the first report to show that the FCGR3A-V158F polymorphism influences transplant outcomes.

Little is known about the involvement of FCGR3A in the pathogenesis of GVHD. Indirect evidence in animal and human studies showed that B cells have an important role in the immunopathophysiology of acute and chronic GVHD,[12,32–35] in which FCGR3A is involved through Ab-mediated immune responses, including Ab-dependent cell-mediated cytotoxicity and FCGR3A-mediated endocytosis. Previous reports showing the effectiveness of B-cell depletion in the treatment of acute[33] and chronic GVHD[35] prompted a working hypothesis that the presence of the FCGR3A-158V genotype, which potentially mediates Ab-dependent cell-mediated cytotoxicity more efficiently in comparison to its absence,[16] may be a risk factor contributing to the development of acute and chronic GVHD. However, the current results contradict this hypothesis. One explanation of this conflict may be observed in reports that B-cell-deficient mice experience more exacerbated acute GVHD than wild-type mice,[36] and that a high number of B-cell progenitors in the stem cell graft or in patients after allo-SCT is associated with a significantly lower rate of acute and chronic GVHD.[37,38] A study in HIV-infected men showed the presence of the FCGR3A-158V genotype to be associated with susceptibility to Kaposi's sarcoma and human herpesvirus-8

infection,[39] thus suggesting that the 158V genotype may possess an immunologically recessive nature in immunocompromised patients similar to transplant recipients. However, precisely how the recipient FCGR3A polymorphism influences the pathophysiology of GVHD is still unknown. The first assumption is that in the light of the pathogenesis of GVHD,[40,41] differential binding of IgG to FCGR3A on recipient DCs might alter downstream events, such as the release of cytokines or chemokines, which consecutively could influence pathway essential for the development of GVHD. The second assumption is that the expression of FCGR3A on keratinocytes[42] may have a role in neutralizing the autoantibodies contributing to GVHD.

An alternative explanation is that the association between the recipient FCGR3A polymorphism and the transplant outcomes could develop from polymorphisms in other genes in linkage disequilibrium with the FCGR3A gene. One possible candidate is the Fcγ receptor IIA (FCGR2A) H131R polymorphism shown to be in linkage disequilibrium with the FCGR3A polymorphism in the Caucasian populations.[31,43] However, the absence of a linkage disequilibrium between FCGR3A and FCGR2A genes in Japanese population[31] suggests that the FCGR3A polymorphism is more likely responsible for the transplant outcomes.

The current data are not consistent with a previous French study[44] that found no significant association between the FCGR3A-V158F SNP polymorphism and transplant outcomes. One of the reasons for this discrepancy may be that the population in the initial study included only HLA-identical sibling BMT recipients and was not stratified into myeloid and lymphoid malignancies to examine the effect of gene polymorphism on transplant outcomes. There is a possibility that the influence of the FCGR3A polymorphism is restricted exclusively to unrelated BMT and myeloid malignancies. Alternatively, these conflicting findings may result from ethnic differences in the study population. Verification of the present data in other cohorts has still to be made so that the implications of the findings can be fully accepted.

This study suggests that the genotyping of FCGR3A in transplant recipients before transplantation may provide a recipient bearing the 158F/F genotype an opportunity to avoid the risk of GVHD by favoring a BM or cord blood, and an HLA-matched graft, and planning more immunosuppressive regimens. Further studies are required to ascertain whether the findings of this study can be extended to other disease groups or other stem sources or HLA-mismatched transplantation.

Conflict of interest

The authors declare no conflict of interest.

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Impact of highly conserved HLA haplotype on acute graft-versus-host disease

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Impact of highly conserved HLA haplotype on acute graft-versus-host disease

*Satoko Morishima,[1] *Seishi Ogawa,[2] Aiko Matsubara,[2] Takakazu Kawase,[3] Yasuhito Nannya,[2] Koichi Kashiwase,[4] Masahiro Satake,[4] Hiroo Saji,[5] Hidetoshi Inoko,[6] Shunichi Kato,[7] Yoshihisa Kodera,[8] Takehiko Sasazuki,[9] and Yasuo Morishima,[1] for the Japan Marrow Donor Program

[1]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya; [2]21st Century COE Program, Graduate School of Medicine, University of Tokyo, Tokyo; [3]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya; [4]Tokyo Metropolitan Red Cross Blood Center, Tokyo; [5]HLA Laboratory, NPO, Kyoto; [6]Division of Molecular Science, Tokai University School of Medicine, Isehara; [7]Department of Cell Transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara; [8]Aichi Medical University, Aichi; and [9]International Medical Center of Japan, Tokyo, Japan

Although the effects of human leukocyte antigen (HLA) locus matching on clinical outcome in unrelated hematopoietic stem cell transplantations have been characterized, the biologic implications of HLA haplotypes have not been defined. We demonstrated the genetic fixity of Japanese conserved extended haplotypes by multi–single nucleotide polymorphism analysis in 1810 Japanese donor-recipient pairs matching with HLA-A, -B, -C, -DRB1, and -DQB1 alleles. Three major Japanese conserved extended haplotypes (named HP-P1, HP-P2, and HP-P3) were essentially completely conserved at least in the 3.3-Mb HLA region from HLA-A to -DPB1, and extended far beyond HLA-A. The risk of acute graft-versus-host disease (GVHD) of these HLA haplotypes was assessed with multivariate Cox regression in 712 patients transplanted from HLA fully (HLA-A, B, C, DRB1, DQB1, and DPB1) matched unrelated donors. HP-P2 itself reduced the risk of grade 2 to 4 acute GVHD (hazard ratio [HR] = 0.63; $P = .032$ compared with HP-P2-negative), whereas HP-P3 tended to increase the risk (HR = 1.38; $P = .07$). Among 381 patients with HP-P1, HP-P1/P3 (HR = 3.35; $P = .024$) significantly increased the risk of acute GVHD compared with homozygous HP-P1. This study is the first to demonstrate that a genetic difference derived from HLA haplotype itself is associated with acute GVHD in allogeneic hematopoietic stem cell transplantation. (*Blood.* 2010;115(23): 4664-4670)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)–matched unrelated (UR) donor has been established as a mode of curative therapy for hematologic malignancies and other hematologic or immunologic disorders, when an HLA-identical sibling donor is unavailable. Although the effect of donor and recipient HLA locus matching on the clinical outcome of UR-HSCT has been well elucidated,[1-4] the biologic implications of HLA haplotype itself have not been explored for HSCT.

HLA-identical sibling shares 2 identical major histocompatibility complex (MHC) haplotypes by descent, including non-HLA polymorphic genes, and it has been generally accepted that transplantation between these related pairs provides a superior outcome. On the other hand, there is no guarantee of matching for non-HLA genes between HLA-allele matched UR donor and recipient pairs, and mismatching of haplotype block in MHC has been suggested to lead to severe acute GVHD[5] and an inferior outcome[6,7] in UR-HSCT.

In human population, multiple DNA blocks in the MHC are strongly associated with each other, and these relatively long stretches of conserved DNA sequence in the MHC have been named conserved extended haplotypes (CEHs) or ancestral haplotypes.[8,9] CEHs are often population-specific and have been investigated as markers for disease susceptibility, particularly in autoimmune diseases.[10] However, the relation between CEHs and clinical outcome of UR-HSCT has not been yet reported.

Using the large-scale Japan Marrow Donor Program (JMDP) data, we evaluated the conservation of common HLA haplotypes among a Japanese population and elucidated its impact on acute graft-versus-host disease (GVHD) and other clinical outcomes in UR-HSCT.

Methods

Study population

A total of 5210 donor-recipient pairs who underwent transplantation through the JMDP between January 1993 and January 2006 were retrospectively genotyped for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 alleles as described elsewhere.[4] For the genome-wide association studies, 1810 pairs (3620 persons) who matched HLA-A, -B, -C, -DRB1, and -DQB1 alleles and were available for DNA sample were selected from these 5210 pairs. For the analysis of acute GVHD, 712 patients who had received T cell–replete bone marrow from an HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 allele-matched donor were selected from these 5210 pairs. The characteristics of these 712 patients are shown in Table 1. A final clinical survey of the patients was completed by June 2007. Informed consent was obtained from patients and donors in accordance with the Declaration of Helsinki, and approval of the study was obtained from the Institutional Review Board of Aichi Cancer Center and JMDP.

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*S.M. and S.O. contributed equally to this paper.

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Table 1. Clinical characteristics of patients according to HLA haplotype

Characteristic	Haplotype P1		Haplotype P2		Haplotype P3	
	Negative	Positive	Negative	Positive	Negative	Positive
No. of patients	381	331	601	111	608	104
Median patient age, y (range)	33 (0-70)	33 (1-68)	33 (0-70)	35 (1-65)	33 (1-68)	36 (0-70)
Sex (donor/ patient)						
Male/male	160	152	250	62	274	38
Male/female	71	63	117	17	112	22
Female/male	66	49	103	12	100	15
Female/female	84	67	131	20	122	29
Disease						
ALL	91	71	138	24	144	18
ANLL	90	106	167	29	166	30
CML	65	54	95	24	101	18
Hereditary disease	5	9	11	3	12	2
MDS	52	39	76	15	78	13
Malignant lymphoma	39	30	61	8	52	17
Multiple myeloma	4	4	7	1	7	1
Severe aplastic anemia	24	9	28	5	31	2
Other	11	9	18	2	17	3
Risk of leukemia relapse*						
Standard	137	112	211	38	214	35
High	109	119	189	39	197	31
Disease other than leukemia	135	100	201	34	197	38
GVHD prophylaxis						
Cyclosporine-based	199	203	345	57	339	63
Tacrolimus-based	182	128	256	54	269	41
ATG						
ATG	25	23	38	10	40	8
Non-ATG	356	308	563	101	568	96
Preconditioning						
TBI regimen	298	241	456	83	463	76
Non-TBI regimen	83	90	145	28	145	28

HLA indicates human leukocyte antigen; ALL, acute lymphoblastic leukemia; ANLL, acute nonlymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; ATG, anti–human thymocyte globulin; and TBI, total body irradiation.

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

SNP typing and HLA haplotype analysis

The single nucleotide polymorphism (SNP) array experiments were performed according to the standard protocol of Affymetrix GeneChip Mapping 500K Array (Affymetrix). After excluding those SNPs showing less than 95% call rate and deviation from Hardy-Weinberg equilibrium ($P < .001$), 10.8% of SNPs for the HLA region failed. And 4761 SNPs in the region spanning the MHC (20-46 Mb from the telomere in chromosome 6p) were analyzed to evaluate the conservation of common HLA haplotypes.

Persons who were homozygous for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 were isolated, and the 3 major HLA haplotypes were named HP-P1, HP-P2, and HP-P3. The homozygosity of consecutive SNPs of these HLA haplotypes was analyzed to assess the region of conservation. SNP alleles of the extended homozygous region in each HLA haplotype were analyzed to determine allele frequencies, and a consensus sequence of major alleles in each haplotype was established. Then, the SNP sequence of persons who carried at least one copy of HLA haplotype (shared the same HLA alleles as common HLA haplotype at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci) was compared with this consensus sequence. Missing alleles in a sample were not accounted for in this analysis.

Statistical analysis

Cumulative incidences of acute GVHD and relapse were assessed by the method described elsewhere to eliminate the effect of competing risk.[4] Overall survival was calculated using the Kaplan-Meier method. The competing event regarding acute GVHD was defined as death without acute GVHD. A log-rank test was applied to assess the impact by the factor of interest. Multivariable Cox regression analyses were conducted to evaluate the impact of the specific haplotype on acute GVHD, leukemia relapse, and mortality after transplantation. Confounders considered were sex (donor-recipient pair), patient age (linear), donor age (linear), transplantation year, type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (cyclosporine-based regimen vs tacrolimus-based regimen), anti–thymocyte globulin (anti–thymocyte globulin vs no anti–thymocyte globulin), and preconditioning (total body irradiation vs non–total body irradiation).

Results

Highly conserved common HLA haplotypes among Japanese

To evaluate for conservation of Japanese common HLA haplotypes, persons who were homozygous HLA haplotype (having homozygous alleles in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci) were selected from a total 3620 persons (1810 donor-recipient pairs) for whom genome-wide association study was performed. Among those, 72 persons were homozygous HLA-A*2402 -Cw*1202 -B*5201 -DRB1*1502 -DQB1*0601 -DPB1*0901 (named HP-P1), 10 persons were homozygous HLA-A*3303 -Cw*1403 -B*4403 -DRB1*1302 -DQB1*0604 -DPB1*0401 (named HP-P2), and 8 persons were homozygous HLA-A*2402 -Cw*0702 -B*0702 -DRB1*0101 -DQB1*0501 -DPB1*0402 (named HP-P3).

Homozygosity at consecutive SNP loci of persons with homozygous HLA haplotype was shown in Figure 1. The extended

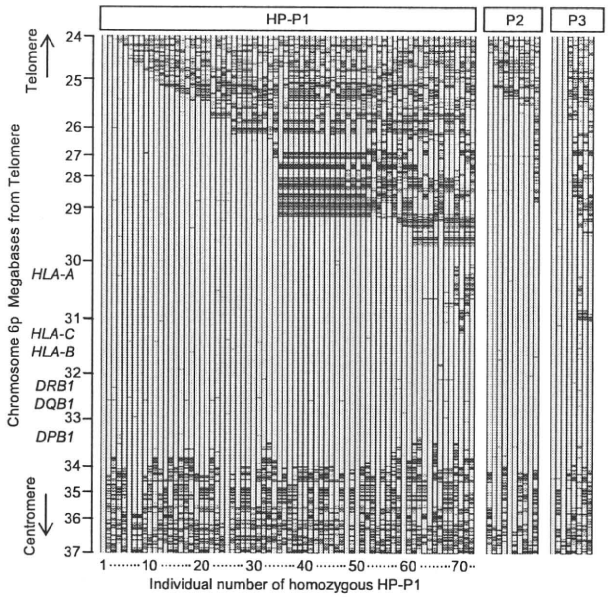

Figure 1. Representation of genotypes in persons with homozygous HLA haplotype. Data from chromosome 6p (24-37 Mb) of persons with homozygous HLA haplotype are shown. Each column indicates 1 person (72 persons with homozygous HP-P1, 10 persons with homozygous HP-P2, and 8 persons with homozygous HP-P3). Each of the 2389 evenly spaced rows represents 1 SNP locus. Blue row represents homozygous genotype; and red row, heterozygous genotype. Missing genotypes were not counted.

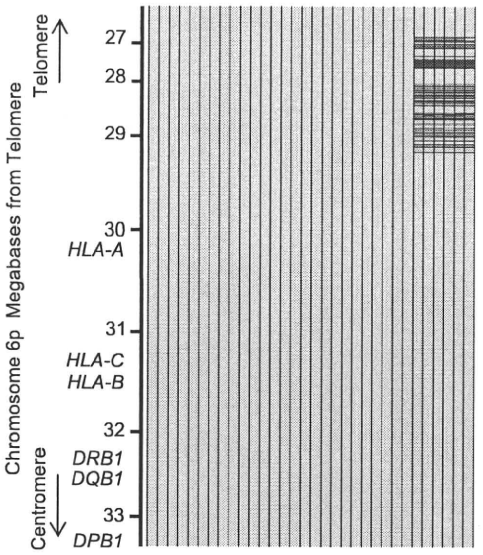

Figure 2. Subtypes of HLA haplotype P1. Data from chromosome 6p (from nucleotides 26252770-33187790) of 32 persons with consecutive homozygous SNPs throughout the 6.9-Mb region. The SNP sequence of persons was compared with consensus sequence across the 6.9-Mb region. Each column indicates 1 person. Each of 1395 evenly spaced rows represents 1 SNP locus. Gray row represents SNPs identical to the consensus alleles; and red row, SNPs different from the consensus alleles. Missing SNPs were not counted. A total of 26 of 32 persons had alleles identical to consensus alleles across 6.9 Mb (subtype A of HP-P1), whereas the remaining 6 persons had apparently different alleles in the telomeric region from nucleotide 29414635 (subtype B of HP-P1). These data indicated that the telomeric region of HP-P1 was clearly divided into 2 different haplotypes.

homozygous region of HP-P1 gradually broke up the region of HLA-A. The longest homozygous region in persons with HP-P1 was 18.7 Mb. Of 72 persons with homozygous HP-P1, 32 persons (nos. 1-32 of HP-P1 in Figure 1) had more than 99.0% of 1395 consecutive homozygous SNPs throughout the 6.9-Mb region from rs806971 to rs6937061 (nucleotides 26252770-33187790). Although haplotypes of all those 32 persons were identical centromeric rs9257745 (nucleotide 29414635), the telomeric region was clearly divided into 2 different haplotypes (Figure 2). A total of 26 of 32 persons had one of the homozygous haplotypes (named subtype A of HP-P1), and the remaining 6 persons (nos. 3, 9, 19, 23, 24, and 25 of HP-P1 in Figure 1) had another homozygous haplotype (subtype B of HP-P1). A total of 65 of 72 persons with homozygous HP-P1 (nos. 1-65 of HP-P1 in Figure 1) had more than 99.0% homozygous alleles for 888 consecutive SNPs throughout the 3.3-Mb region from rs1610630 to rs6937061 (nucleotides 29837265-33187790). Seven other persons (nos. 66-72 in Figure 1) had an apparently lower conserved region, with 1.0% to 10.0% heterozygous alleles within the 3.3-Mb region.

All 10 persons with homozygous HP-P2 had more than 99.0% homozygous alleles for consecutive SNPs throughout the 3.3-Mb region. Furthermore, 9 of 10 persons with homozygous HP-P2 showed homozygosity extending across the 7.7-Mb region from rs6912426 to rs6937061 (nucleotides 25517764-33187790), and those 9 persons had identical genotypes in almost all the 1540 consecutive SNPs throughout the 7.7-Mb region.

Among 8 persons with homozygous HP-P3, 5 persons had more than 99.0% homozygous alleles throughout the 3.3-Mb region, and the other 3 persons had 1.7% to 8.0% heterozygous alleles within that region. One person with homozygous HP-P3 showed an extraordinary long stretch of homozygosity across the 25.4-Mb region (nucleotides 20162518-45595922).

Consensus sequence of each haplotype was determined using analysis of persons who were homozygous for almost all the SNPs, ie, 6.9-Mb region in HP-P1 (subtypes A and B), 7.7-Mb region in HP-P2, and 3.3-Mb region HP-P3. The person with the longest homozygous telomeric region of HLA-A served to determine a further extended haplotype (Table 2). These persons had alleles identical to the consensus sequence of major alleles in each haplotype described above in this section.

We ascertained whether the consensus sequence of each HLA haplotype was present in the persons carrying at least one copy of HLA haplotype, that is, sharing the same HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 alleles of common HLA haplotypes (Figure 3; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Among 3620 persons analyzed for multi-SNP, 1000 of 1045 persons (95%) with HP-P1 had identical alleles for more than 99.5% of consecutive 888 SNPs as a consensus sequence across 3.3-Mb region from 181 kb telomeric HLA-A to 25 kb centromeric HLA-DPB1.

Table 2. Longest homozygous region of common HLA haplotype

	Start of homozygous region		End of homozygous region		
Type	**SNP**	**Position, kb**	**SNP**	**Position, kb**	**Region length, Mb**
HP-P1					
Subtype A	rs199026	23441.813	rs2395801	42231.646	18.8
Subtype B	rs573863	24080.365	rs1536501	33835.863	9.7
HP-P2	rs1175427	24387.055	rs1873254	34134.467	9.7
HP-P3	rs1688325	20162.518	rs6905847	45595.922	25.4

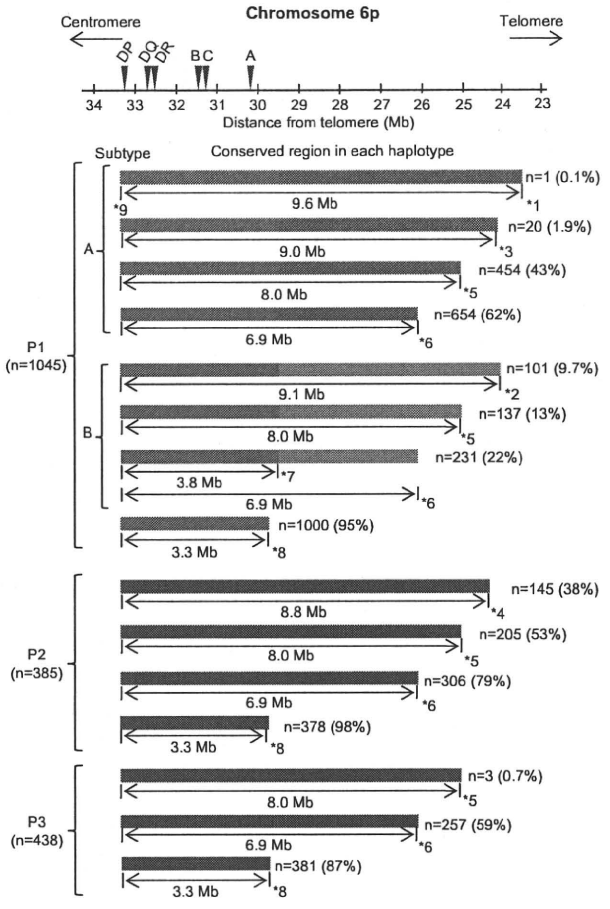

Figure 3. Conservation of common HLA haplotypes. The SNP sequence of persons who carried at least 1 copy of HLA haplotype (shared the same HLA alleles as common HLA haplotype) was compared with consensus sequence of common HLA haplotypes, and conserved regions in each HLA haplotype were illustrated schematically. The majority of persons who share the same HLA alleles in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 as common HLA haplotypes possess at least a 3.3-Mb conserved region from HLA-A to DPB1. HP-P1 splits into the A and B subtypes. The length of conservation in HP-P1, HP-P2, and HP-P3 is more extensive to the telomeric region of HLA-A. ID and position of SNPs are: *1 rs199026 (nucleotide 23551813), *2 rs573863 (nucleotide 24080365), *3 rs1397843 (nucleotide 24111433), *4 rs11754278 (nucleotide 24387055), *5 rs303031 (nucleotide 25144959), *6 rs806971 (nucleotide 26252770), *7 rs9257745 (nucleotide 29414635), *8 rs1610630 (nucleotide 29837265), and *9 rs6937061 (nucleotide 33187790).

Furthermore, 654 of 1045 (62%) persons with HP-P1 had identical alleles for more than 99.5% of consecutive 1395 SNPs as subtype A of HP-P1 across the 6.9-Mb region, and 231 persons (22%) had identical alleles as subtype B of HP-P1 across the 6.9-Mb region. Fewer persons showed the conserved region extending up to 9.0 Mb. Among 385 persons with HP-P2, 378 (98%) had identical alleles for 888 consecutive SNPs across the 3.3-Mb region. Furthermore, 305 (79%) had identical alleles for more than 99.5% of 1395 consecutive SNPs as a consensus sequence across the 6.9-Mb region, and 205 (53%) also did across the 8.0-Mb region (nucleotides 24111433-33187790). Among 438 persons with HP-P3, 381 (87%) had identical alleles across the 3.3-Mb region, and 257 (59%) had identical alleles for consecutive SNPs as a consensus sequence of HP-P3 across the 6.9-Mb region.

These results indicate that most of the persons with a common HLA haplotype had a conserved region at least 3.3 Mb from HLA-A to HLA-DPB1. Furthermore, a considerable number of unrelated persons with common HLA haplotype had a more extended conserved telomeric region of HLA-A.

Effect of HLA haplotype on acute GVHD

To elucidate the effect of specific HLA haplotype on acute GVHD, we analyzed 712 patients who underwent transplantation from HLA fully matched (12 of 12 HLA alleles) donor with T cell-replete marrow (Table 1). We excluded HLA-mismatched transplantation to avoid obscuring the relationship between HLA haplotype itself and acute GVHD by powerful allogeneic immune responses caused by HLA allele disparities. Among those patients, 331 (46.4%) had HP-P1, 111 (15.0%) had HP-P2, and 104 (14.6%) had HP-P3.

At first, grade 2 to 4 acute GVHD in patients with specific haplotype was compared with those without specific haplotype using multivariate analysis (Table 3). There was no significant difference in the hazard ratio (HR) in grades 2 to 4 acute GVHD between HP-P1-positive and -negative patients, and also no significant difference in the cumulative incidence of acute GVHD between HP-P1-positive and HP-P1-negative patients (31.5% vs 30.7%; Figure 4). Of note, HR of grades 2 to 4 acute GVHD in HP-P2-positive patients was 0.63 (95% confidence interval [CI], 0.41-0.96, $P = .032$) compared with HP-P2-negative patients, and the cumulative incidence of acute GVHD in patients with HP-P2 was significantly lower than HP-P2-negative patients (22.3% vs 33.7%, $P = .031$; Figure 4). On the other hand, a trend of increasing risk of acute GVHD was observed in HP-P3-positive patients (HR = 1.38; 95% CI, 0.97-1.95; $P = .07$). The cumulative incidence of acute GVHD in HP-P3-positive patients was 39.2% and HP-P3-negative patients 29.5% ($P = .064$; Figure 4).

A total of 331 patients with HP-P1 in HLA fully matched transplantation made it possible to elucidate the effect of another HLA haplotype on acute GVHD (supplemental Table 1 for patient characteristics). We did not determine unique haplotypes other than HP-P1, HP-P2, and HP-P3 in this study, so all the persons with HP-P1 and unknown haplotype were lumped together with HP-P1/other. The incidence of grade 2 to 4 acute GVHD in patients with HP-P1/P3 (49.9%) was significantly higher than those with homozygous HP-P1 (16.0%), and there was no significant difference between patients with homozygous HP-P1 and those with HP-P1/P2 (12.0%; Figure 5). Multivariate analyses of HR for acute GVHD showed the same results (Table 3). There was no significant difference in the risk of acute GVHD between patients with homozygous HP-P1 and those with HP-P1/P2 ($P = .64$). Compared with patients with homozygous HP-P1, patients with HP-P1/P3 (HR = 3.35; 95% CI, 1.18-9.55; $P = .024$) had a significantly higher risk of acute GVHD.

As for grade 3 to 4 acute GVHD, there were no significant differences between HP-positive and -negative patients. When 331 patients with HP-P1 in HLA fully match transplantation

Table 3. Hazard ratio of HLA haplotype on acute GVHD (grade 2-4)

HLA haplotype	Negative/positive	No.	Hazard ratio (95% CI)	P
P1	Negative	381	Referent 1.00	
P1	Positive	331	1.06 (0.81-1.39)	.665
P2	Negative	601	Referent 1.00	
P2	Positive	111	0.63 (0.41-0.96)	.032
P3	Negative	608	Referent 1.00	
P3	Positive	104	1.38 (0.97-1.95)	.07
P1/P1		36	Referent 1.00	
P1/P2		25	0.71 (0.17-2.93)	.64
P1/P3		19	3.35 (1.18-9.55)	.024
P1/other		251	2.49 (1.06-5.85)	.036

Multivariate analysis adjusted by clinical factors (see Table 2 and supplemental Table 1).

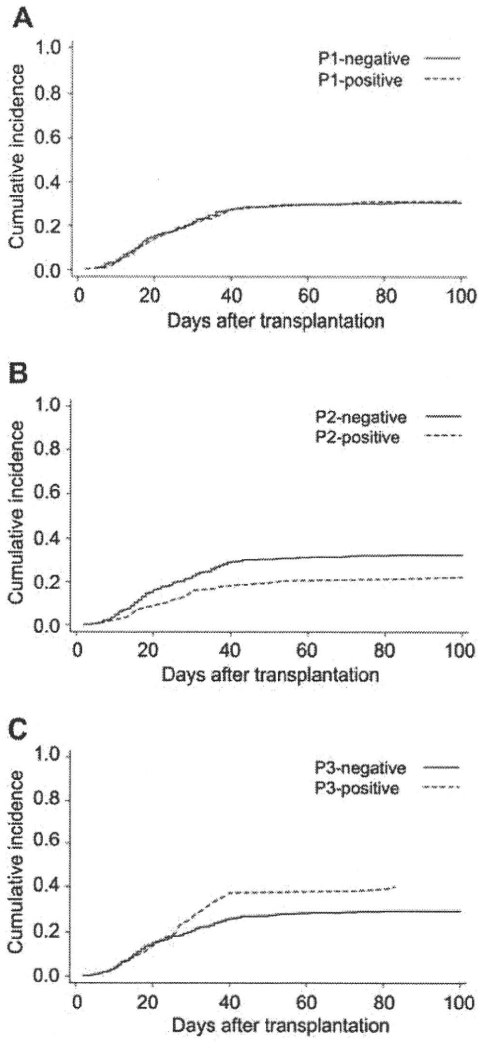

Figure 4. Cumulative incidence of grade 2 to 4 acute GVHD by common HLA haplotype. The endpoint was the time to diagnosis of grade 2 to 4 acute GVHD with censoring of date of death until 100 days after transplantation. *P* value was calculated with the log-rank test. (A) Patients with or without HP-P1. (B) Patients with or without HP-P2. (C) Patients with or without HP-P3.

were analyzed, grade 3 to 4 acute GVHD showed the same tendency with grade 2 to 4 acute GVHD. Incidence in patients with homozygous HP-P1 was 2.7%, HP-P1/P2 8.0%, and HP-P1/P3 23.6%.

The cumulative incidence of relapse showed a higher trend in HP-P2-positive patients compared with -negative patients (37.3% vs 29.6%, $P = .051$), and HR was 1.34 (95% CI, 0.94-1.91, $P = .108$). There were no significant differences in the relapse rate between HP-P1-positive and -negative patients, and also between HP-P3 -positive and -negative patients.

Overall survival showed no significant differences between HP-positive and -negative patients. When 331 patients with HP-P1 in HLA fully matched transplantation were analyzed, HR of mortality in HP-P1/P3 was 2.03 (95% CI, 0.92-4.49 $P = .08$) compared with patients with homozygous HP-P1, and HR of HP-P1/P2 1.69 (95% CI, 0.78-3.70, $P = .186$).

Discussion

First, we demonstrated that Japanese common HLA haplotypes were extraordinarily conserved. Preferential selection of HLA-A–, -B–, and -DR–matched donor through JMDP made it easy to identify a considerable number of persons with homozygous common HLA haplotype extending HLA-DPB1. HP-P1, HP-P2, and HP-P3 have been previously reported as common HLA haplotypes in the Japanese population.[11-13] The haplotype frequency of HP-P1 was 0.054 to 0.062, that of HP-P2 was 0.029 to 0.036, and that of HP-P3 was 0.016 to 0.040.

CEHs have been mainly identified by blocks of fragment in the MHC region, such as complement genes, alleles of HLA class I /II gene, and tumor necrosis factor-α gene. Recently, high-density SNP analysis in the HLA region made it possible to confirm the genetic fixity of HLA haplotype.[14-16]

We determined the consensus sequence of these HLA haplotypes using multi-SNP data of unrelated persons with homozygous HLA haplotype, and the majority of persons who share the same HLA alleles in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 as common HLA haplotypes possess at least a 3.3-Mb conserved region from HLA-A to -DPB1. Furthermore, we showed that those Japanese common HLA haplotypes extend far beyond the HLA-A. We also found, for the first time, that HP-P1 was divided into 2 subtypes based on the telomeric region from nucleotide 29414635 (subtypes A and B). The A1-B8-DR3 CEH, which is one of the most frequent haplotypes in northern European populations, showed that the region of conservation extended 6 Mb telomeric to HLA-A.[16,17] Caucasian common HLA haplotypes have often reportedly shown a lack of nonrandom association between HLA-DR, -DQ, and -DP,[18] whereas Japanese common HLA haplotypes have been subdivided into haplotypes with only a very limited number of HLA-DPB1.[11,12] The highly conserved HLA haplotype that extends to HLA-DPB1 might be attributable to ethnic isolation in the Japanese.

For the analysis of comparison between haplotype-positive and -negative patients (Figure 4; Table 3), patients with HP-P2 significantly reduced the risk of acute GVHD. On the other hand, patients with HP-P3 showed a tendency to increase the risk. Although the relapse rate showed a higher trend in patients with HP-P2 compared with HP-P2-negative patients, the grade 3 to 4 acute GVHD and overall survival did not differ between HP-P2-positive and -negative patients, nor between HP-P3-positive and -negative patients. We suspected the differences with weak power might be attributed to the effects of various other haplotypes combined with a particular haplotype. To confirm the differences in the effects on acute GVHD among a particular HLA haplotype, we analyzed the effect of another

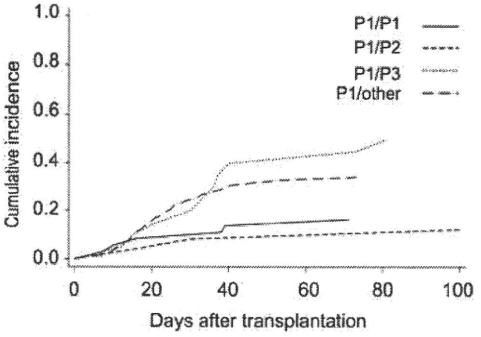

Figure 5. Cumulative incidence of grade 2 to 4 acute GVHD by common HLA haplotype in patients with HP-P1. The endpoint was the time to the diagnosis of grade 2 to 4 acute GVHD with censoring of date of death until 100 days after transplantation. *P* value was calculated with the log-rank test. A total of 331 patients with HP-P1 were analyzed for the effect of another HLA haplotype by HP-P1, HP-P2, HP-P3, and the other haplotypes, which were lumped together.

haplotype on acute GVHD among patients with HP-P1 (Figure 5). Patients with HP-P1/P3 showed a significantly higher risk of grade 2 to 4 acute GVHD compared with patients with homozygous HP-P1, a tendency to increase the incidence of grade 3 to 4, and also a tendency to decrease overall survival. Patients with homozygous HP-P1 and patients with HP-P1/P2 showed an extremely lower incidence of grade 2 to 4 acute GVHD (16.2% and 12.0%). Therefore, we could not detect any difference between HP-P1 and HP-P2.

Thus, we demonstrated, for the first time, that the HLA haplotype itself affected the occurrence of acute GVHD. These findings suggest that the genetic factor of specific haplotypes would contribute to reducing or increasing the risk of acute GVHD. Alternatively, we should consider the effect of donor and recipient mismatch SNPs because HP-P3 showed more variation than HP-P1 or HP-P2.

There are several possible explanations for the reduced or increased risk of acute GVHD in patients with the specific haplotype. In HSCT, GVHD has been known to result mainly from donor T cells recognizing minor histocompatibility antigens presented on HLA molecules of a recipient's organs.[19] Different HLA haplotypes possess a different combination of HLA alleles. Therefore, various HLA alleles in each haplotype might present different immunodominant peptides to T cells and evoke different alloreactivity in HLA-matched UR-HSCT. Presumably, the critical but as yet unidentified minor histocompatibility antigens linked to major histocompatibility antigen should be explored based on HLA haplotype, such as common gene deletion polymorphisms.[20] Distinct forms of GVHD were found in different MHC haplotypes in mice, and it has been argued that genes in the MHC locus can dominantly determine the forms of GVHD, probably through MHC-based selection of immunodominant antigens.[21]

In human retrospective analysis, several single-center studies have shown a reduced incidence of acute GVHD,[22] reduced relapse rate,[23] and improved overall survival[24] for HLA-DR15-positive patients transplanted from HLA-matched donors. HLA-DR15 has been known to be a marker of disease susceptibility and clinical response to immunosuppressive therapy in autoimmune-mediated bone marrow failure,[25,26] and it is speculated that immune responses specific to HLA-DR15 are induced. However, our analysis of the effect of HLA-DR15 using the same database showed no effect on acute GVHD (data not shown).

HLA haplotype serves as a model system for studies of disease association, especially in autoimmune disease or infection, and several candidate genes in the HLA region associated with specific haplotype have the potential to modulate immune or inflammatory responses.[10] The observed effect of HLA haplotypes on GVHD development could be explained by particular SNPs that are closely associated with those HLA haplotypes. Within the region of conserved HLA haplotypes, there exist several candidate genes whose SNPs may be related to immune responses. Genetic variants of tumor necrosis factor-α gene located in the HLA region might influence the risk of developing GVHD.[27,28] In addition, *TAP1/TAP2* and *LMP2/LMP7* genes encode subunit components of the proteasomes implicated in the processing of class I HLA-bound peptides,[29,30] and polymorphisms of these genes may affect antigen presentation on recipient tissues, leading to different susceptibility to GVHD. However, they probably do not explain the observed effects of haplotypes; correlations of each haplotype with known SNPs in these genes are generally weak, although D' among these alleles is high (> 0.98). Moreover, currently no haplotype-specific non-HLA polymorphisms have been identified in our series, although we could not exclude the possibility that there may exist some nonobserved SNPs that are closely associated with relevant HLA haplotypes.

We showed that Japanese common HLA haplotypes were conserved from HLA-DPB1 to extensively telomeric HLA-A, so it might be possible that the responsible gene is located in the telomeric region of the classic HLA. Interestingly, HP-P1 was divided into 2 subtypes based on the telomeric region from nucleotide 29414635 (subtypes A and B). Although we analyzed the effect of those subtypes on grade 2 to 4 acute GVHD among patients with HP-P1, we could not detect significant differences between patients with HP-P1 subtype A and subtype B. We also could not detect the differences between patients transplanted from a donor with HP-P1 subtype A and subtype B (data not shown).

In conclusion, in the present study, we demonstrated that highly conserved HLA haplotype might contribute to the occurrence of acute GVHD in HSCT. Although the clinical implications of our results should be considered cautiously, these results imply the proof of principle for an association between one or another HLA haplotype and GVHD. Our findings on the conservation of the MHC region may also provide background for exploring not only genetic factors associated with acute GVHD but also genetic disease susceptibility in our population. More extensive studies are warranted to identify specific genes associated with a particular haplotype contributing to acute GVHD.

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Authorship

Contribution: S.M., Y.M., S.O., H.S., M.S., H.I., and T.S. participated in the design of this study; S.O., K.K., A.M., and Y.N. performed histocompatibility analysis; Y.M., S.K., and Y.K. organized data collection for transplantation; T.K. performed statistical data analysis; S.M. and Y.M. performed analysis and wrote this paper; and all authors checked the final version of the paper.

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Correspondence: Seishi Ogawa, The 21st Century COE Program, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo, 113-0033 Japan; e-mail: sogawa-tky@umin.net; or Yasuo Morishima, Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan; e-mail: ymorisim@aichi-cc.jp.

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ORIGINAL ARTICLE

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

Y Inamoto[1,2], M Murata[1], A Katsumi[1], Y Kuwatsuka[2], A Tsujimura[2], Y Ishikawa[1], K Sugimoto[1], M Onizuka[2], S Terakura[1], T Nishida[2], T Kanie[2], H Taji[2], H Iida[2], R Suzuki[2], A Abe[1], H Kiyoi[3], T Matsushita[1], K Miyamura[2], Y Kodera[2] and T Naoe[1]

[1]*Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [2]*Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan and* [3]*Department of Infectious Diseases, Nagoya University Graduate School of Medicine, Nagoya, Japan*

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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Correspondence: Dr A Katsumi, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan.
E-mail: katsumi@med.nagoya-u.ac.jp
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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.

Allellic 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

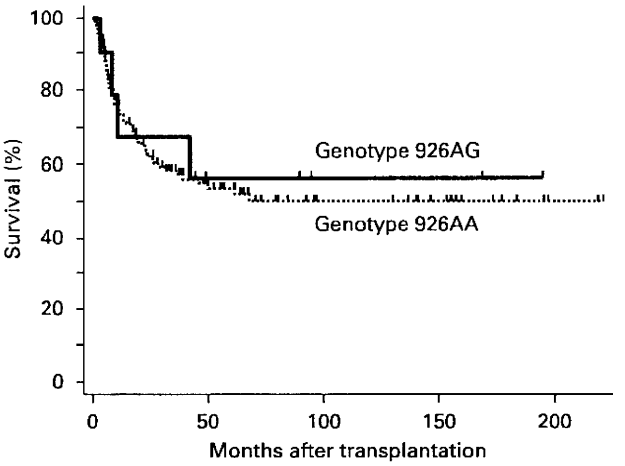

Figure 1 Overall survival between G926A genotypes. At a median follow-up of 42 months (range: 2–220 months), the estimated 4-year overall survival was not statistically different between G926A genotypes (56 vs 55%, $P=0.78$).

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