Table 2 Univariate analysis of pharmacological variates affecting transplantation outcome

	Patients, *n*	Median follow-up days (range)	% at 3 years after transplantation (95% CI)								
			n	AUC < 30 μg h/ml	*n*	AUC > 30 μg h/ml	*p*	*n*	C_{2h} < 2.5 μg/ml	*n*	C_{2h} > 2.5 μg/ml	*p*
OS												
BMT	15	646 (22–1633)	10	40 (9.6–70.4)	5	80 (44.9–100)		9	44.4 (13.6–71.9)	6	66.7 (19.5–90.4)	
							0.243					0.360
CBT	21	277 (11–1442)	5	80 (44.9–100)	16	30 (6.9–53.1)		7	28.6 (4.1–61.1)	14	49.0 (21.6–71.7)	
							0.054					0.172
DFS												
BMT	15	646 (22–1633)	10	40 (9.6–70.4)	5	80 (44.9–100)		9	44.4 (13.6–71.9)	6	66.7 (19.5–90.4)	
							0.243					0.360
CBT	21	179 (11–1442)	5	80 (44.9–100)	16	18.8 (0–37.9)		7	28.6 (4.1–61.1)	14	35.7 (13.0–59.4)	
							0.046					0.290
Relapse												
BMT	15	646 (22–1633)	10	22.2 (6.1–63.5)	5	0		9	12.5 (1.9–61.3)	6	20 (3.1–79.6)	
							0.331					0.774
CBT	20	188 (50–1442)	5	0	15	72 (46.0–92.8)		6	58.3 (23.3–94.4)	14	52.4 (27.6–81.8)	
							0.031					0.429
NRM												
BMT	15	646 (22–1633)	10	48.6 (21.4–84)	5	20 (3.1–79.6)		9	49.2 (21.9–84.3)	6	16.7 (2.5–72.7)	
							0.440					0.220
CBT	21	277 (11–1442)	5	20 (3.1–79.6)	16	25.5 (10.4–54.6)		7	28.6 (8.0–74.2)	14	21.4 (7.2–52.8)	
							0.816					0.607

p values were calculated with Kaplan–Meier method

Values in italics represent statistically significant difference ($p < 0.05$)

such as steroids, should be considered for preemptive therapy against GVHD.

4.2 Unrelated CBT

We have reported the usefulness of MMF for CBT as a substitute for MTX for avoidance of graft failure [7]. In addition, the incidence of acute and chronic GVHD was very low even in the low AUC_{0-24h} group (Figs. 3c, 4c). Thus, there is no doubt that MMF is quite useful to avoid early complications after CBT. Patients with $AUC_{0-24h} > 30$ μg h/ml showed lower survival due to higher incidence of relapse (Table 2). However, there was no difference in survival and relapse rate if patients were divided into two groups, lower and higher C_{2h} at day 16 (Table 2). It is important to examine whether the excessive immunosuppression by MMF might result in the inhibition of graft-versus-leukemia effects in CBT or not. In contrast to unrelated allo-BMT, AUC_{0-24h} lower than 30 μg h/ml is good enough for GVHD prophylaxis and this condition may even be critical to avoid the relapse in CBT. Dose reduction may be required in some cases with high AUC_{0-24h}. In this study, all CBTs were performed with single unit cord blood. Because the incidence of acute GVHD is relatively high in double unit CBT [22], the optimal strategy of MMF administration might be close to that for unrelated allo-BMT.

The incidence of CMV pneumonia might be unexpectedly high despite the preemptive administration of anti-CMV agents; three were CBT cases. A previous report also demonstrated higher unbound MPA concentration steady state (Css) associated with CMV reactivation [13]. Therefore, we should carefully deal with CMV antigenemia-positive patients, especially in a CBT setting. Appropriate tapering of MMF after engraftment and relatively intense preemptive treatment could be required.

Recently, it has been pointed out that the Kaplan–Meier method, which treats the competing events as censored at the time they occurred, was biased when these incidences were relatively high [23]. Therefore, we also reassessed acute and extensive chronic GVHD, and relapse and non-relapse mortality by competing risk analysis using Gray's method (statistical software R version 2.13.0). The incidences of acute and extensive chronic GVHD analyzed by Gray's method are almost identical to those of Kaplan–Meier method. In relapse and non-relapse mortality, the incidences by Gray's method are relatively low but similar to those by Kaplan–Meier method, except for relapse rate according to AUC_{0-24h} in CBT. Subjects with $AUC_{0-24h} < 30$ μg h/ml and $AUC_{0-24h} > 30$ μg h/ml were 0 and 56.7% (95% CI 25.4–79.1%), respectively. Although the incidence was relatively low compared to the result of the analysis using Kaplan–Meier method, the statistical significance was preserved ($p = 0.046$).

Pharmacokinetics monitoring to calculate AUC_{0-24h} is a heavy burden on the patient, is time-consuming and not practical. This study demonstrated that a single point monitoring of MPA concentration at 2 h after administration, C_{2h}, is a good substitute of AUC_{0-24h}, particularly for the assessment of acute and chronic GVHD (Figs. 2, 3, 4). The day on which to check the MPA C_{2h} after allo-SCT may not be necessarily rigid because AUCs on days 2, 9, and 16 were similar in each individual because of the relatively low intra-patient variability [15, 24]. Although day 16 C_{2h} might be much too simplified to extract the high-risk population of relapse in CBT, this study raises the possibility of significantly improving allo-SCT outcome with an individualized MMF administration strategy by checking this simple parameter.

As a strategy to keep the AUC high in each individual, the increase in dosing frequency rather than dose escalation is likely effective in allo-SCT patients (Fig. 1c) [15]. MPA levels in allo-SCT are lower compared with those in the transplantation of solid organs [25]. Gastrointestinal damage induced by prior chemotherapy, preparative regimens, and gut GVHD has been speculated to result in inadequate absorption. However, it was unlikely because MPA/AUC levels by intravenous administration of MMF were as low as those by oral MMF in allo-SCT (data not shown, our pilot study). Recently, de Winter et al. [25] have reported that this difference might originate from different MPA clearance (higher efficiency with much wider inter-individual variations) depending on plasma albumin concentrations and predose concentrations of calcineurin inhibitors. It may be important to consider these factors in cases that need higher AUC_{0-24h}, for example changing the dose of calcineurin inhibitors to adjust the MPA levels. The present study is a retrospective one composed of small sample number and heterogeneous patient characteristics. Thus, prospective randomized study of individualized administration is necessary to confirm the veiled usefulness of MMF.

Acknowledgments This work was supported, in part, by the Grants-in-Aid for Scientific Research from the Ministry of Health, Welfare, and Labor in Japan.

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PLoS one

A Genetic Variant in the IL-17 Promoter Is Functionally Associated with Acute Graft-Versus-Host Disease after Unrelated Bone Marrow Transplantation

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Abstract

Interleukin IL-17 is a proinflammatory cytokine that has been implicated in the pathogenesis of various autoimmune diseases. The single nucleotide polymorphism (SNP), rs2275913, in the promoter region of the IL-17 gene is associated with susceptibility to ulcerative colitis. When we examined the impact of rs2275913 in a cohort consisting of 438 pairs of patients and their unrelated donors transplanted through the Japan Marrow Donor Program, the donor IL-17 197A allele was found to be associated with a higher risk of acute graft-versus-host disease (GVHD; hazard ratio [HR], 1.46; 95% confidence interval [CI], 1.00 to 2.13; $P = 0.05$). Next, we investigated the functional relevance of the rs2275913 SNP. *In vitro* stimulated T cells from healthy individuals possessing the 197A allele produced significantly more IL-17 than those without the 197A allele. In a gene reporter assay, the 197A allele construct induced higher luciferase activity than the 197G allele, and the difference was higher in the presence of T cell receptor activation and was abrogated by cyclosporine treatment. Moreover, the 197A allele displayed a higher affinity for the nuclear factor activated T cells (NFAT), a critical transcription factor involved in IL-17 regulation. These findings substantiate the functional relevance of the rs2275913 polymorphism and indicate that the higher IL-17 secretion by individuals with the 197A allele likely accounts for their increased risk for acute GVHD and certain autoimmune diseases.

Citation: Espinoza JL, Takami A, Nakata K, Onizuka M, Kawase T, et al. (2011) A Genetic Variant in the IL-17 Promoter Is Functionally Associated with Acute Graft-Versus-Host Disease after Unrelated Bone Marrow Transplantation. PLoS ONE 6(10): e26229. doi:10.1371/journal.pone.0026229

Editor: Eliana Saul Furquim Werneck Abdelhay, Instituto Nacional de Câncer, Brazil

Received August 11, 2011; **Accepted** September 22, 2011; **Published** October 20, 2011

Funding: This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan, and the Ministry of Education, Culture, Sports and Technology of Japan, and Funds from the Mitani Research and Development Assistance Organization (Kanazawa, Japan), by the Japan Leukemia Research Fund (Tokyo, Japan), and by Hokkoku Gan Kikin Fund (Kanazawa, Japan). This work was supported in part by the Research on Allergic Disease and Immunology (H23-010) in Health and Labor Science Grant from the Ministry of Health, Labor and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Interleukin 17 (IL-17), also known as IL-17A, plays an important role in tissue inflammation, and is involved in the pathophysiology of autoimmune diseases and organ allograft rejection [1,2,3,4,5,6,7,8,9,10,11]. Moreover, several reports have shown that Th17 cells and IL-17 have a significant impact on the development of acute graft-versus-host disease (GVHD) in mouse models [12,13,14,15,16,17]. The 197A allele, which is the result of a single nucleotide polymorphism (SNP) rs2275913 (G197A) in the promoter region of the IL-17 gene, has been reported to be associated with the susceptibility to rheumatoid arthritis [18] and ulcerative colitis [19]. In our previous study, we demonstrated that the 197A allele was also implicated in the development of acute GVHD in patients who underwent unrelated myeloablative bone marrow transplantation (BMT) [20]. In the present study, we extended this investigation to a validation cohort of patients who received an unrelated BMT, including patients who underwent reduced intensity transplantation.

Interestingly, the rs2275913 SNP is located within a binding motif for the nuclear factor activated T cells (NFAT), which is a critical regulator of the IL-17 promoter [21]. Therefore, it is conceivable that the rs2275913 SNP exerts an effect on the transcriptional regulation of IL-17. The present study shows that allele 197A correlates with more efficient IL-17 secretion, and that this resulted from its higher affinity for NFAT.

Materials and Methods

Patients

IL-17 genotyping was performed on 438 recipients with hematological malignancies and their unrelated donors who underwent BMT through the Japan Marrow Donor Program (JMDP) with T-cell-replete marrow from HLA-A, -B, -C, -DRB1,

-DQB1, and -DPB1 allele-matched donors between January 1993 and December 2007. The HLA genotypes of patients and donors were determined by the Luminex microbead method as described previously (Luminex 100 System; Luminex, Austin, TX) [22,23]. Although the Luminex microbead method does not provide unambiguous HLA 4-digit typing for all genotypes, the JMDP has confirmed that this method can identify all HLA alleles with >0.1% frequency among the Japanese population [24].

None of the present patients had a history of any prior transplantation. The final clinical survey of these patients was completed by November 1, 2008. The diagnoses were acute myeloid leukemia (AML) in 149 (34%), acute lymphoblastic leukemia (ALL) in 109 (25%), myelodysplastic syndrome (MDS) in 78 (18%), malignant lymphoma (ML) in 55 (15%), chronic myeloid leukemia in 42 (10%), and multiple myeloma (MM) in 5 (1%; **Tables 1** and **2**). The recipients were defined as having standard risk disease if they had AML or ALL in the first complete remission, ML in any complete remission, CML in any chronic phase, or MDS. All others were designated as having high-risk disease. The myeloid malignancies include AML, MDS and CML, and the lymphoid malignancies included ALL, ML and MM. Cyclosporine- or tacrolimus-based regimens were used in all patients for GVHD prophylaxis, and anti-T cell therapy, such as anti-thymocyte globulin and *ex vivo* T cell depletion were not in any of the patients. All patients and donors gave their written informed consent at the time of transplantation to participate in molecular studies of this nature according to the declaration of Helsinki. This project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

IL-17 genotyping

Genotyping of IL-17 was performed using the TaqMan-Allelic discrimination method with the Assay ID C__15879983_10 (Applied Biosystems) as described in a previous report [20].

Cells and reagents

Primers and oligonucleotides were obtained from Hokkaido Science Systems (Sapporo, Japan). The GST-NFATc1 construct [25] was a generous gift from Dr Shoichiro Miyatake. An NFATc binding consensus oligonucleotide (sc-2577) was purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Table 1. The donor and recipient characteristics (first part).

Variable	No.	Ratio
No. of cases	438	
Recipient age, years		
Median	39	
Range	1–70	
Donor age, years		
Median	35	
Range	20–57	
Year of transplant		
Median	2003	
Range	1993–2007	
Recipient IL-17 genotype		
G/G	180	41%
A/G	200	46%
A/A	58	13%
Donor IL-17 genotype		
G/G	166	38%
A/G	200	46%
A/A	66	15%
Recipient sex		
Male	281	64%
Female	157	36%
Donor sex		
Male	296	68%
Female	142	32%
Donor/recipient sex		
Sex matched	299	68%
Female/male	62	14%
Male/female	77	18%

doi:10.1371/journal.pone.0026229.t001

Table 2. Donor and recipient characteristics (second part).

Variable	No.	Ratio
Disease		
Acute myeloid leukemia	149	34%
Acute lymphoblastic leukemia	109	25%
Myelodysplastic syndrome	78	18%
Malignant lymphoma	55	13%
Chronic myeloid leukemia	42	10%
Multiple myeloma	5	1%
Disease stage		
Standard risk	178	41%
High risk	260	59%
ABO matching		
Major or/and minor mismatch	160	37%
Major mismatch	91	21%
Minor mismatch	86	20%
Bidirectional	17	4%
Missing	8	2%
Conditioning regimen		
Myeloablative	325	74%
Reduced intensity	113	26%
With total body irradiation	333	76%
Pretransplant CMV serostatus		
CMV positive recipient	324	74%
Missing	40	9%
GVHD prophylaxis		
With cyclosporine	190	43%
With tacrolimus	248	57%
TNC, $\times 10^8$ per kg		
Median	4.6	
Range	0.1–316.8	

Abbreviations: TNC: total nucleated cell count harvested.

doi:10.1371/journal.pone.0026229.t002

Cell preparation, cell culture and measurement of IL-17

Heparinized blood samples were collected from 54 healthy Japanese volunteers. The ages of the subjects (30 males and 24 females) ranged from 20 and 56 years (median, 32 years). Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden) and were induced to secrete IL-17 by culturing the PBMCs (10^6/well) in 24 well plates for 48 hours in RPMI 1640 supplemented with 10% fetal bovine serum in the presence or absence of 5 μg/ml phytohemagglutinin (PHA; Sigma) at 37°C in 5% CO_2. In some experiments, PBMCs (10^6 cells/well) were seeded in 48 well plates coated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) monoclonal antibodies (Miltenyi Biotec, Gladbach, Germany) to activate T cells selectively, and then were cultured for 48 hrs. The concentrations of IL-17 in collected supernatants were measured by an enzyme-linked immunosorbent assay (ELISA; Mabtech, Nacka Strand, Sweden). For some functional assays, PBMCs (6×10^6 cells/well) were cultured in six well plates for 72 hrs in the presence of 5 μg /ml of PHA and 100 U/ml of IL-2, and are hereafter designated as PHA-PBMCs.

Quantitative RT- PCR

RNA was extracted from resting or PHA-activated PBMCs using the high pure RNA isolation kit (Roche). Reverse transcription was carried out with the PrimeScript RT reagent/gDNA eraser kit (Catalog RR047A, Takara). Quantitative real time PCR was performed in a StepOne Plus PCR system (Applies Biosystems) using the SYBR premix ExTaq perfect Real Time (Catalog RR041A, Takara) with the IL-17 primers described previously [26] and a set of primers for human GAPDH (Takara). The relative IL-17 mRNA levels normalized to GAPDH were calculated by the ΔΔCT method using the relative expression function included in the StepOne v2.2 software program. The specificity of the PCR products was monitored by a melting curves analysis.

Luciferase assay

The promoter region of the IL-17 gene was amplified from the genomic DNA of individuals homozygous for the rs2275913 SNP (A197A or G197G) by polymerase chain reaction (PCR) with forward 5′-ACGCGTGGATCTCAGGACAAACAGGTTC-3′ and reverse 5′-AAGCTTGACTCACCACCAATGAGGT-CTT-3′ primers as described previously [21]. The resultant fragments IL-17/197A or IL-17/197G were subcloned into the pGL3-enhancer vector at the MluI and HindIII sites (Promega, Madison, WI) to generate pGL3-197A-enhancer or pGL3-197G-enhancer constructs. The fragments were inserted with the same orientation, and their nucleotide sequences were confirmed by DNA sequencing. Equimolar amounts of the following reporter plasmids: pGL3-enhancer, pGL3-197A-enhancer and pGL3-197G enhancer designated thereafter as pGL3-Luc, IL-17/A-Luc IL-17/G-Luc respectively, were transfected into PHA-PBMCs using the Exfect transfection reagent following the manufacturer instructions (Takara Bio, Japan). To control for differences in the transfection efficiency, cells were cotransfected with a renilla reporter plasmid, pRL-TK. In some experiments, the cells were treated with anti-CD3 and anti-CD28 mAbs or with Cyclosporine A (CsA) 24 hours after the transfection, and were cultured for other 24 hours. The activity of both luciferase and renilla in the transfected cells was measured with the Dual Luciferase Reporter Assay System (Promega).

Electrophoresis motility shift assay (EMSA)

Double stranded IL-17 probes, including those harboring G197A, were generated by annealing the following oligonucleotides to their complementary oligonucleotides: CAT TTT CCT TCA GAA G**A**A GAG ATT CTT CTA (197A allele) and CAT TTT CCT TCA GAA G**G**A GAG ATT CTT CTA (197G allele). These oligomers encompass nucleotides −180 to −210 upstream of the transcriptional start site, based on data in the human genomic DNA Gene bank accession number AY460616.1. Before annealing, both complementary oligonucleotides were separately biotin-labeled at their 3′ ends, using the 3′ end DNA labeling kit (Thermo Fisher Scientific, Suwanee, USA) following the manufacturer's recommendations. Nuclear extracts from PHA-PBMCs were prepared using a nuclear extraction kit (Thermo Fisher Scientific). The DNA/protein binding assay was performed with 10 μg of nuclear extracts using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's recommendations with minor modifications as follows: In the DNA/NFAT recombinant protein assay 0.5% bovine serum albumin was included in the binding reaction and purified GST-NFAT-recombinant proteins were desalted using Zeba spin desalting columns (Pierce). The DNA/protein complexes were detected by streptavidin peroxidase and visualized in a Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

Data management and statistical analysis

The 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 cytomegalovirus (CMV) serostatus was routinely tested for only patients, but not the donors. Engraftment was confirmed by an absolute neutrophil count of more than 0.5×10^9/L for at least 3 consecutive days. After collecting the data, acute and chronic GVHD were diagnosed and graded based on the classically defined criteria [27,28], namely, acute GVHD develops within the first 100 days post-transplant while the manifestation of GVHD occurring after day 100 is classified as chronic GVHD. Data using the updated criteria for assessment of GVHD [29,30] were not available in our cohort. The overall survival (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. Transplant-related mortality (TRM) was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. The data about the causative microbes of infections and postmortem changes in the cause of death, as well as the data on supportive care, including prophylaxis for infections and therapy for GVHD, which were given on an institutional basis, were not available for this cohort.

The analysis was performed using the Excel 2007 software program (Microsoft Corp, Redmond, WA, USA) and modified R (The R Foundation for Statistical Computing, Perugia, Italy) software programs [31,32], as described in a previous report [33,34]. 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 engraftment were compared using the Grey test [35] and analyzed using a cumulative incidence analysis [31], while considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD, and death without engraftment as respective competing risks. The variables included the recipient age at the time of transplantation, sex, pretransplant CMV serostatus, disease characteristics (disease type, disease lineage and disease risk at transplantation), donor characteristics (age, sex, sex compatibility, and ABO compatibility), transplant characteristics (conventional or reduced-intensity conditioning [36], total body irradiation-containing regimen, tacrolimus versus

Table 3. The results of the univariate analysis of the association of IL-17 polymorphisms with the clinical outcomes after transplantation.

Variable	No.	5-year OS	*P*	5-year TRM	*P*	5-year relapse	*P*	II–IV acute GVHD	*P*	Chronic GVHD	*P*
Recipient IL-17 genotype											
G/G	180	41%		29%		37%		35%		41%	
A/G or A/A	258	50%	0.59	28%	0.48	30%	0.10	30%	0.21	40%	0.78
Donor IL-17 genotype											
G/G	166	50%		29%		31%		27%		37%	
A/G or A/A	272	43%	0.22	28%	0.71	33%	0.77	38%	0.03	42%	0.18

doi:10.1371/journal.pone.0026229.t003

cyclosporine, and total nucleated cell count harvested per recipient weight [TNC]), and the year of transplantation. The median was used as the cutoff point for continuous variables. The chi-square test and the Mann-Whitney U test were used to compare the two groups. The Hardy-Weinberg equilibrium for the IL-17 gene polymorphism was determined using the Haploview software program [37].

Multivariate Cox models were used to evaluate the hazard ratio associated with the IL-17 polymorphism. The covariates found to be $P \leq 0.10$ according to univariate analyses were used to adjust the hazard ratio.

For both the univariate and multivariate analyses, the P values were two sided, and the outcomes were considered to be significant for $P \leq 0.05$.

Results

Transplant outcome according to the IL-17 genotype

The genotype frequencies of 197G/G, 197A/G and 197A/A were 41%, 46% and 13% in recipients, and 38%, 46% and 15% in donors. These were similar to previous reports [18,38], and were in accord with the Hardy-Weinberg equilibrium ($P = 0.88$).

The transplant outcomes according to the IL-17 genotype are summarized in **Table 3**. The presence of the 197A allele in the 197A/G or 197A/A genotype in the donor was associated with a significantly higher incidence of grades II to IV acute GVHD (38% vs. 27%, $P = 0.03$; **Fig. 1**), while no significant differences between the 197A/G genotype and the 197A/A genotype in the recipient were seen in the incidence of grades II to IV acute GVHD (38% vs. 36%, $P = 0.78$). The acute GVHD-related mortality did not differ between the donor 197A/G or 197A/A genotypes and the donor 197G/G genotype (2% vs. 2%, $P = 0.83$).

All of the factors found to be significant in the univariate analyses were included in the model. The 197A/G or 197A/A genotype in donors remained statistically significant in the multivariate analyses for the development of grades II to IV acute GVHD (**Table 4**). The 197A/G or 197A/A genotype in the donor resulted in a higher incidence of grades II to IV acute GVHD (hazard ratio [HR], 1.46; 95% confidence interval [CI], 1.00 to 2.13; $P = 0.05$) even when adjusted for the other factors in the models. The IL-17 genotype showed no significant effects on the OS, TRM or relapse (**Table 5**).

The impact of the rs2275913 SNP on the secretion of IL-17

To substantiate the biological significance of the rs2275913 SNP, we first examined whether the different genotypes correlated with IL-17 secretion. PBMCs from a total of 54 healthy individuals (197G/G in 24, 197A/G in 24 and 197A/A in 6) were stimulated *in vitro* with PHA and the levels of secreted IL-17 were determined by ELISA. As shown in **Fig. 2A**, the 197A allele positive (197A/G or 197A/A genotype) PBMCs secreted significantly higher levels of IL-17 than the 197A allele negative cells (197G/G genotype). Similar results were obtained when T cells were selectively stimulated with anti-CD3 and anti-CD28 mAbs (data not shown). The quantitative RT-PCR analysis showed that PHA-stimulated PBMCs from donors harboring the 197A allele had a significantly higher IL-17 mRNA level than those from 197A allele negative donors (**Fig. 2B**). Of note, the IL-17 mRNA levels in unstimulated cells were very low, irrespective of 197A allele positivity, and resulted in no differences between the two groups (data not shown). Together, these results suggested that the sequence variant rs2275913 influences the response of the IL-17 gene promoter to factors released in response to T cell activation, thus leading to a differential IL-17 production.

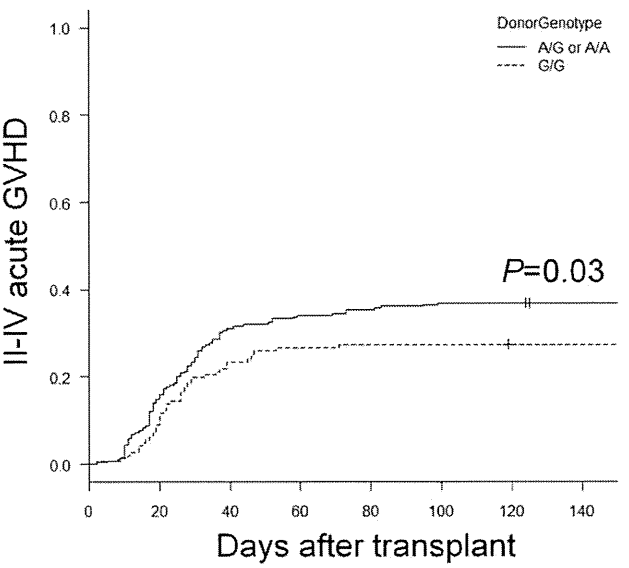

Figure 1. The estimated cumulative incidence curve of grades II–IV acute GVHD according to the donor IL-17 genotype. The solid line represents the donor 197A/G or A/A genotype, and the dashed line represents the donor 197G/G genotype.
doi:10.1371/journal.pone.0026229.g001

Table 4. The results of the multivariate analysis of the association of IL-17 polymorphisms with the GVHD after transplantation.

	II–IV acute GVHD			Chronic GVHD		
Variable	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**
Recipient IL-17 genotype, A/G or A/A	0.80	0.56–1.13	0.20	1.32	0.86–1.03	0.21
Donor IL-17 genotype, A/G or A/A	1.46	1.00–2.13	0.05	1.08	0.70–1.67	0.72

doi:10.1371/journal.pone.0026229.t004

Functional relevance of the rs227513 SNP in the IL-17 gene promoter region

To address the functional significance of the rs2275913 SNP, reporter gene constructs containing the 197A and 197G alleles were prepared and used to transfect PHA-PBMCs. The results revealed that the insertion of the IL-17 promoter fragment consistently resulted in an augmentation of the luciferase activity compared with the construct without the fragments, however, the cells transfected with the IL-17/A-Luc construct had significantly higher luciferase activity than cells transfected with the IL-17/G-Luc construct (**Fig. 3A**). The differences in luciferase expression induced by these two constructs were more evident when the transfected cells were treated with anti-CD3 and anti-CD28 mAbs (**Fig. 3B**). Notably, treatment of the transfected cells with CsA abrogated the differences in luciferase activity induced by the two alleles, thus suggesting that the effects of the rs2275913 SNP on the regulation of the IL-17 promoter function are dependent on T cell activation.

The 197A allele has a stronger interaction with NFAT than the 197G allele

To substantiate the functional relevance of the rs2275913 SNP, an EMSA assay was performed. Oligomers containing the 197A or 197G variants were biotin-labeled and allowed to interact with nuclear extracts derived from PHA-PBMCs. Despite the fact that the probes differed in just one nucleotide (A/G), the shift band corresponding to 197A probe-protein complexes was significantly more intense than that corresponding to 197G probe-protein complexes (**Fig. 4B**), thus suggesting that the two alleles have different affinities for some transcription factor in the nuclear extracts. A 50-fold excess of unlabeled IL-17 probes abrogated the formation of DNA-protein complexes, confirming the specificity of these interactions. Since NFAT has been demonstrated to play a crucial role in the regulation of IL-17 production [21] and the rs2275913 SNP maps to within the NFAT binding motif (**Fig. 4A**), DNA-protein interactions were subsequently carried out using recombinant NFAT instead of the nuclear extracts. The 197A probe-NFAT complexes displayed more a intense band than 197G probe-NFAT complexes (**Figs. 4C, D**), which were both completely eliminated by adding a competitor with a 50-fold excess of unlabeled IL-17 probes or an oligonucleotide containing a known NFAT target consensus in the binding reaction, thus suggesting that NFAT is the transcription factor which binds with differential affinities to the IL-17 probes.

Discussion

The present study showed that the 197A allele of the IL-17 gene in the donors was associated with a higher risk of acute GVHD after unrelated fully HLA-matched BMT through the JMDP. The reason that this association did not significantly influence the TRM and OS might have resulted from the low incidence of acute GVHD-related mortality, regardless of the donor IL-17 genotype in the present cohort. Of note we have found that 197A allele positive PBMCs can produce IL-17 more efficiently than 197A allele negative PBMCs, which has not been reported so far, thus implying that the high inducibility of IL-17 might be correlated with the development of acute GVHD.

The role of IL-17 in the pathogenesis of acute GVHD remains unclear. In several mouse model experiments transfer of IL-17 producing cells induced acute GVHD [15,16,17], while in contrast there is a report [13] showing that donor IL-17 producing cells ameliorated acute GVHD. Host dendritic cells (DCs) are critical in the initiation of acute GVHD [39,40,41], thus leading to a hypothesis that IL-17 producing cells could modify the function of host DCs through unknown mechanisms. Direct interaction between IL-17 and host DCs may be supported by the fact that DCs expressed IL-17 receptors [1].

The IL-17 197A allele, which was associated with the higher production of IL-17 in comparison with the 197G allele, exhibited a higher promoter activity, as well as a higher affinity to transcriptional factor NFAT. The functional relevance of rs2275913 SNP was supported by the findings in our gene reporter assay showing that the higher promoter activity induced by 197A allele was stronger in the presence of T cell receptor activation by anti-CD3 and anti-CD28 treatment which is an upstream event in NFAT induction whereas in conditions leading to NFAT inactivation, namely CsA treatment, the differences in promoter activity induced by the 197A and 197G constructs were completely abrogated. Consistent with these observations, EMSA assay using recombinant NFAT proteins directly demonstrated a higher in affinity of 197A

Table 5. The results of the multivariate analysis of the association of IL-17 polymorphisms with the clinical outcomes after transplantation.

	OS			TRM			Relapse		
Variable	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**
Recipient IL-17 genotype, A/G or A/A	1.01	0.71–1.42	0.97	1.43	0.84–2.41	0.87	0.75	0.49–1.16	0.19
Donor IL-17 genotype, A/G or A/A	1.29	0.90–1.84	0.16	1.31	0.75–2.31	0.34	1.24	0.79–1.93	0.35

doi:10.1371/journal.pone.0026229.t005

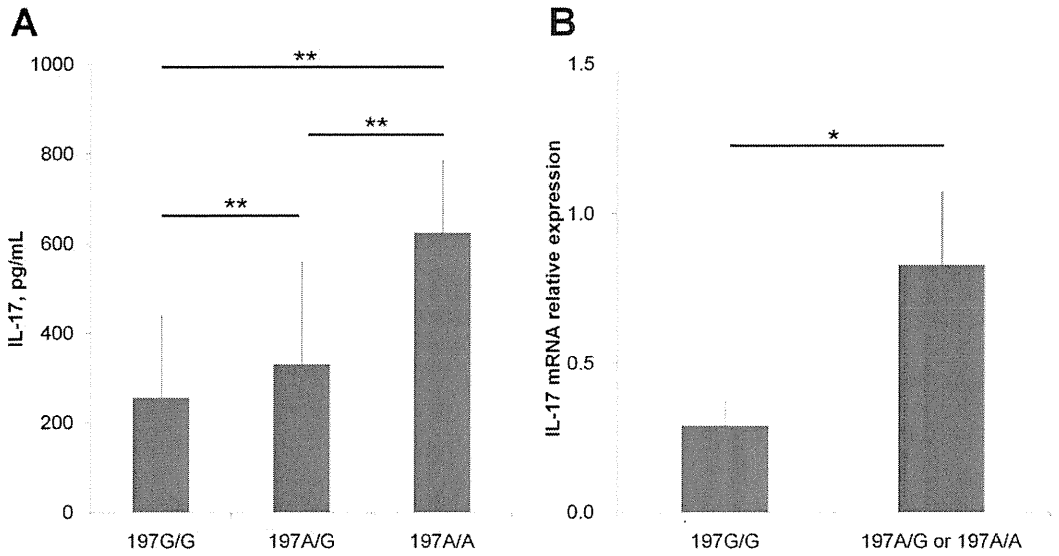

Figure 2. The influence of the IL-17 SNP on IL-17 secretion by *in vitro* stimulated PBMCs. (A) PBMCs from healthy individuals were stimulated for 48 hours in the presence of PHA or in 48 well plates coated with anti-CD3 and anti-CD28 mAbs. The values are expressed as the means +/− SD. (B) PBMCs from healthy donors (197A allele positive, n = 12 and 197A allele negative, n = 10) were cultured for 24 hrs in the presence of PHA. Total RNA was extracted, and the IL-17 mRNA levels were determined by quantitative RT-PCR and normalized to GAPDH. The data are the means +/−SD of triplicate measurements in each donor. *indicates $P<0.05$ and **$P<0.01$.
doi:10.1371/journal.pone.0026229.g002

allele. NFAT is a transcription factor crucial for the regulation of T cell-mediated IL-17 gene transcription [21], and the rs2275913 SNP is located in the promoter adjacent to the NFAT binding region (**Fig. 4A**). These findings suggest that the rs2275913 SNP plays a functional role in the promoter activity of the IL-17 gene through influencing the transcriptional activity of NFAT, affecting the production of IL-17 from T cells.

Previous studies have reported an association between the G197A SNP in the IL-17 promoter region and the susceptibility of the Japanese population to ulcerative colitis [19], as well as to rheumatoid arthritis in the Caucasian population [18]. The present study demonstrated that the 197A genotype is related to high IL-17 production, and the results of a previous Japanese study [19] showed that the 197A genotype was a risk factor for the

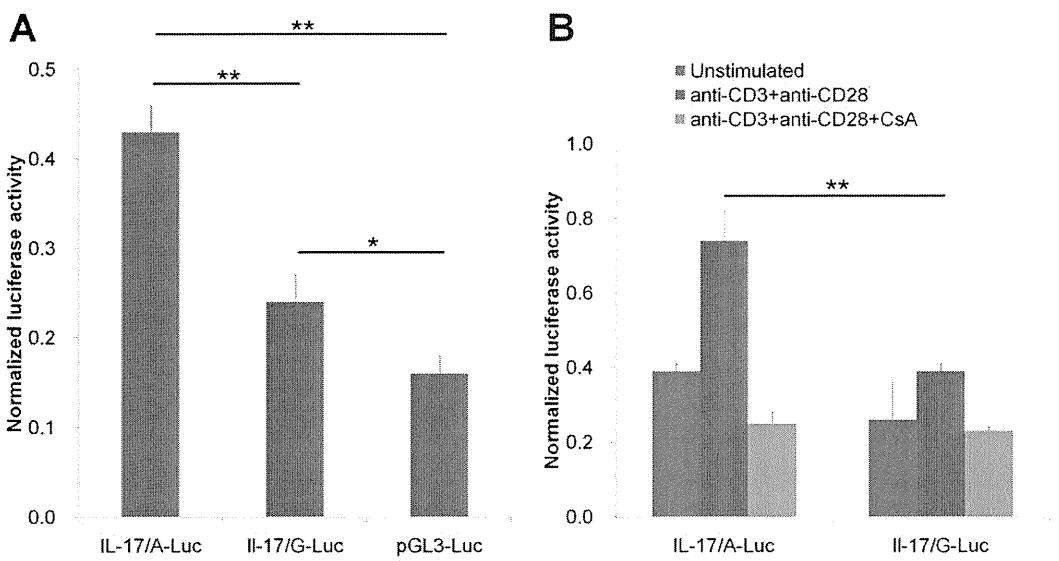

Figure 3. The modulation of the reporter gene expression by the rs2275913 SNP. (A) PHA-PBMCs were transfected with a luciferase expression vector alone (pGL3-Luc) or with a luciferase expression vector containing fragments of the IL-17 promoter with the 197A or 197G alleles (IL-17/A-Luc and IL-17/G-Luc). The transfected cells were cultured for 48 hr, and firefly luciferase activities were measured and normalized to Renilla luciferase. (B) The PHA-PBMCs were transfected as described above. Twenty four hours after transfection, the cells were treated with anti-CD3 and anti-CD28 mAbs or with CsA, and cultured for other 24 hr. The firefly luciferase activities were measured and normalized to Renilla luciferase. The values represent the normalized levels +/− S.E.M. from five independent experiments. *indicates $P<0.05$ and **$P<0.01$.
doi:10.1371/journal.pone.0026229.g003

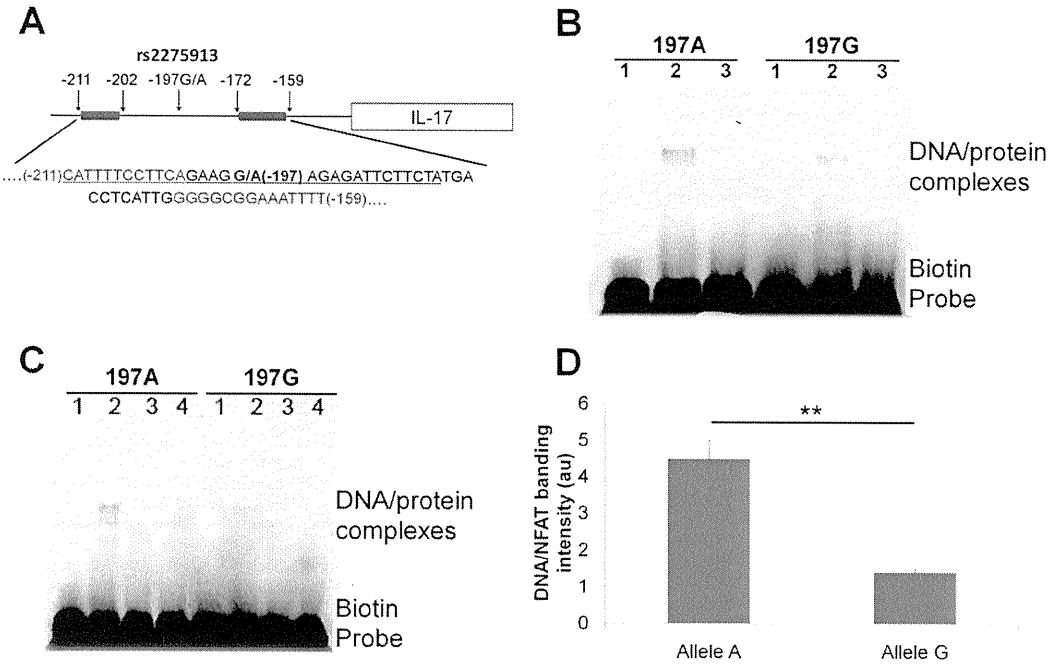

Figure 4. The rs2275913 SNP results in differential binding of NFAT. (A) The location of the rs2275913 SNP within the promoter region of the IL-17 gene. The underlined sequence corresponds to the oligomers used in EMSA assay. The NFAT binding sites [21] are indicated in red and by red boxes. (B) IL-17 probes were allowed to interact with nuclear extracts from PHA-PBMCs in an EMSA assay. Lane 1, free probes; lane 2, biotin-labeled probes plus nuclear extracts; lane 3, biotin-labeled probes plus nuclear extracts plus a 50-fold molar excess of unlabeled probes. The figure shown is representative of five independent experiments. (C) IL-17 probes were allowed to interact with recombinant NFAT proteins in an EMSA assay. Lane 1, free probes; lane 2, biotin-labeled probes plus GST-NFAT; lane 3, biotin-labeled probes plus GST-NFAT plus a 50-fold molar excess of unlabeled probes; lane 4, biotin-labeled probes plus GST-NFAT plus a 50-fold molar excess of unlabeled oligomers containing a NFAT consensus site. The figure shown is representative of five independent experiments. (D) The intensity of the bands corresponding to the DNA/protein interaction (lane 2 in **Fig. 4C**) were evaluated by densitometry to compare the binding affinity of the 197A allele and 197G allele for recombinant NFAT. The values are represented as arbitrary units (au). **indicates $P<0.01$.
doi:10.1371/journal.pone.0026229.g004

development of ulcerative colitis. Together, these results may explain the previous observations of increased expression of IL-17 in patients with inflammatory bowel disease such as ulcerative colitis, which promotes the recruitment of inflammatory cells into the intestinal mucosa through an increase in chemoattractants and the expression of adhesion molecules [4,42,43,44]. However, another study from Norway [18] suggested an association between the 197A genotype and resistance to developing rheumatoid arthritis Since many studies have demonstrated higher levels of IL-17 in patients with rheumatoid arthritis and the essential roles of IL-17 in mediating joint damage [45,46,47], the G197A SNP might affect the initiation of rheumatoid arthritis, but not disease progression and severity. This issue should thus be clarified using larger cohort studies in the future.

Our earlier report [34] showed an association with the IL-17 197A genotype in the recipient, but not the donor, as in the present study, with a higher incidence of acute graft-versus-host disease. However, unlike in the previous study [34], the current cohort mainly consisted of patients receiving relatively recent transplants, including reduced-intensity transplantation. The reason for these discrepancies is unclear, because the year of the transplant and conditioning intensity were considered as co-factors in the multivariate analysis. This issue should be clarified by further investigations in patients at higher risk for acute GVHD, including those receiving peripheral blood stem cell or HLA-mismatched transplants.

In conclusion, we have reported that the G197A SNP in the IL-17 promoter predicts the development of acute GVHD and plays a functionally important role in the regulation of IL-17 production. Given that the 197A allele is significantly associated with the higher production of IL-17, G197A genotyping may be used to predict the susceptibility and severity of other IL-17-related diseases and complications including rheumatoid arthritis, periodontal disease, multiple sclerosis, allergic rhinitis, psoriasis, inflammatory bowel disease, and organ allograft rejection [11]. Furthermore, a better understanding of the molecular mechanism by which this promoter SNP controls the production of IL-17 may therefore offer some novel therapeutic insights into the mechanisms of such diseases.

Acknowledgments

We thank all of the Japan Marrow Donor Program (JMDP) transplant teams who have contributed patients and donors to this study. We are indebted to Dr. Shoichiro Miyatake at the Tokyo Metropolitan Institute of Medical Science for providing the plasmid encoding GST-NFAT.

Author Contributions

Conceived and designed the experiments: JLE AT. Performed the experiments: JLE KN. Analyzed the data: AT. Contributed reagents/materials/analysis tools: AT MO HA KM YM TF YK SN TK. Wrote the paper: AT JLE.

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Related transplantation with HLA-1 Ag mismatch in the GVH direction and HLA-8/8 allele-matched unrelated transplantation: a nationwide retrospective study

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To clarify which is preferable, a related donor with an HLA-1 Ag mismatch at the HLA-A, HLA-B, or HLA-DR loci in the graft-versus-host (GVH) direction (RD/1AG-MM-GVH) or an HLA 8/8-allele (HLA-A, HLA-B, HLA-C, and HLA-DRB1)–matched unrelated donor (8/8-MUD), we evaluated 779 patients with acute leukemia, chronic myelogenous leukemia, or myelodysplastic syndrome who received a T cell–replete graft from an RD/1AG-MM-GVH or 8/8-MUD. The use of an RD/1AG-MM-GVH donor was significantly associated with a higher overall mortality rate than the use of an 8/8-MUD in a multivariate analysis (hazard ratio, 1.49; $P < .001$), and this impact was statistically significant only in patients with standard-risk diseases ($P = .001$). Among patients with standard-risk diseases who received transplantation from an RD/1AG-MM-GVH donor, the presence of an HLA-B Ag mismatch was significantly associated with a lower overall survival rate than an HLA-DR Ag mismatch because of an increased risk of treatment-related mortality. The HLA-C Ag mismatch or multiple allelic mismatches were frequently observed in the HLA-B Ag-mismatched group, and were possibly associated with the poor outcome. In conclusion, an 8/8-MUD should be prioritized over an RD/1AG-MM-GVH donor during donor selection. In particular, an HLA-B Ag mismatch in the GVH direction has an adverse effect on overall survival and treatment-related mortality in patients with standard-risk diseases. (*Blood.* 2012;119(10):2409-2416)

Introduction

An HLA-matched unrelated donor (MUD) is considered to be an alternative donor in hematopoietic stem cell transplantation (SCT) for patients who lack an HLA-identical sibling. However, it is difficult to find an MUD for patients with rare HLA haplotypes. SCT from a related donor with 1 Ag mismatch at HLA-A, HLA-B, or HLA-DR loci in the graft-versus-host (GVH) direction results in a higher but acceptable incidence of acute GVHD and outcomes comparable to that of SCT from a matched related donor (MRD) in patients with high-risk diseases because it reduces the risk of relapse via a graft-versus-leukemia (GVL) effect.[1-3] In previous studies, HLA mismatches in the host-versus-graft (HVG) direction were associated with higher graft failure and lower overall survival (OS).[1,2,4] However, strategies to reduce the risk of graft failure might have been improved by the use of conditioning regimens that strongly suppress recipient immune system.[5] Therefore, in current clinical practice in Japan, SCT from a related donor with 1 Ag mismatch in the GVH direction and accepting multiple Ag mismatches in the HVG direction without specific stem cell manipulation is being performed,[1,2] although such an approach has not yet been evaluated in a large cohort.

Our previous study showed that SCT from an HLA-1 Ag-mismatched donor in the GVH or HVG direction is comparable to that from an HLA-A, HLA-B, or HLA-DR Ag-MUD.[1] However, this study is relatively old (1991-2000) and may not reflect current practice. Furthermore, the analysis was mainly performed based on serological matching, because information on HLA allele matching in unrelated transplantation was insufficient at that time. The importance of allele matching at the HLA-A, HLA-B, and HLA-DRB1 loci in unrelated donor transplantation has been established previously.[6-8] In addition, the importance of allele matching at the HLA-C locus has been highlighted in several recent studies of unrelated transplantation, although HLA-C matching is, in general,

Submitted August 8, 2011; accepted October 20, 2011. Prepublished online as *Blood* First Edition paper, October 31, 2011; DOI 10.1182/blood-2011-08-372573.

There is an Inside *Blood* commentary on this article in this issue.

The online version of this article contains a data supplement.

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still not considered in related transplantation.[9-12] Therefore, we conducted a nationwide retrospective study to compare the clinical outcomes of transplantation from a related donor with an HLA-1 Ag mismatch at the HLA-A, HLA-B, or HLA-DR loci in the GVH direction (RD/1AG-MM-GVH) with an HLA 8/8-allele (HLA-A, HLA-B, HLA-C, and HLA-DRB1)–MUD (8/8-MUD).

Methods

Data collection

Data for patients 16-70 years of age with acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), or chronic myelogenous leukemia (CML) who received a first allogeneic transplantation from a related donor or HLA-6/6–Ag-MUD between January 1, 2001 and December 31, 2008 were obtained from the Transplant Registry Unified Management Program,[13] which includes data from the Japan Society for Hematopoietic Cell Transplantation and the Japan Marrow Donor Program. Our analysis included 344 patients who received a graft from an RD/1AG-MM-GVH donor and 453 patients who received a graft from an 8/8-MUD. The following patients were excluded: 11 patients who lacked data on survival status, survival date, sex of recipient and donor, stem cell source, GVHD prophylaxis, or performance status; 2 patients who received both BM and peripheral blood in related transplantation; and 5 patients who received stem cells manipulated by ex vivo T-cell depletion or CD34 selection. Finally, 327 patients who received a graft from an RD/1AG-MM-GVH donor and 452 patients who received a graft from an 8/8-MUD fulfilled the criteria. The data on 2318 patients who received transplantation from an MRD were also collected on the basis of similar inclusion and exclusion criteria to compare the OS rate. The study was approved by the data management committees of Transplant Registry Unified Management Program and by the institutional review board of Saitama Medical Center (Jichi Medical University, Saitama, Japan), where this study was organized.

Histocompatibility

Histocompatibility data for serological and genomic typing for the HLA-A, HLA-B, HLA-C, and HLA-DR loci were obtained from reports obtained from the institution at which the transplantation was performed. To reflect current practice in Japan, HLA matching in RD/1AG-MM-GVH donors was assessed by serological data for HLA-A, HLA-B, and HLA-DR loci, whereas that in 8/8-MUD was assessed by genomic data for HLA-A, HLA-B, HLA-C, and HLA-DR loci. When the recipient's Ags or alleles were not shared by the donor, this was considered an HLA mismatch in the GVH direction; when the donor's Ags or alleles were not shared by the recipient, this was considered a mismatch in the HVG direction. SCT from a related donor with 1 Ag mismatch in the GVH direction has been performed by accepting multiple Ag mismatches in the HVG direction,[1,2] and therefore was included in this study.

End points and statistical analyses

The primary end point of the study was to compare OS rates between the RD/1AG-MM-GVH and 8/8-MUD groups. For exploratory purposes, OS, treatment-related mortality (TRM), relapse, acute and chronic GVHD, and cumulative incidences of neutrophil engraftment were analyzed in a subset of cohorts. The physicians who performed transplantation at each center diagnosed and graded acute and chronic GVHD according to standard criteria.[14,15] The incidence of chronic GVHD was evaluated in patients who survived for at least 100 days. Neutrophil recovery was considered to have occurred when the absolute neutrophil count exceeded 0.5×10^9/L for 3 consecutive days after transplantation.

Descriptive statistics were used to summarize variables related to patient characteristics. Comparisons between groups were performed with the χ^2 statistic or extended Fisher exact test as appropriate for categorical variables and the Mann-Whitney U test or the Kruskal-Wallis test as appropriate for continuous variables. The probability of OS was estimated according to the Kaplan-Meier method, and the groups were compared with the log-rank test. The probabilities of TRM, relapse, acute and chronic GVHD, and neutrophil engraftment were estimated on the basis of cumulative incidence curves to accommodate the following competing events[16]: death for relapse, relapse for TRM, death without GVHD for acute and chronic GVHD, and death without engraftment for neutrophil engraftment; the groups were compared with a Gray test.[17] Cox proportional-hazards regression was used to evaluate variables that may affect OS, whereas the Fine and Gray proportional-hazard model was used to evaluate variables that may affect TRM, relapse, acute and chronic GVHD, and neutrophil engraftment.[18] For patients for whom conditioning intensity (myeloablative or reduced-intensity) was not reported, we reclassified the conditioning regimen as either myeloablative or reduced-intensity according to the National Marrow Donor Program/Center for International Blood and Marrow Transplant Research operational definitions.[19] To be consistent with our previous study, acute leukemia in the first or second remission, CML in the first or second chronic phase, and MDS without leukemic transformation were defined as standard-risk diseases, and others were defined as high-risk diseases.[1] The following variables were considered: the recipient's age group (≤ 50 years or > 50 years at transplantation), recipient's sex, presence of female (donor) to male (recipient) sex mismatch, performance status (0-1 or 2-4), disease (AML, ALL, CML, or MDS), disease status before transplantation (standard- or high-risk), type of conditioning regimen (myeloablative or reduced-intensity), type of GVHD prophylaxis (cyclosporine-based, tacrolimus-based, or other), use of antithymocyte globulin or alemtuzumab, and the time from diagnosis to transplantation (< 6 months or ≥ 6 months). In addition, a variable of graft source (BM or peripheral blood) was also considered in the analysis specific to related donors. Factors with $P < .10$ in the univariate analysis were used in the first multivariate model without donor type and deleted in a stepwise manner from the model by backward selection. We added donor type to the final model. All tests were 2-sided, and $P < .05$ was considered to indicate statistical significance. All statistical analyses were performed with STATA Version 11 software (StataCorp) and R Version 2.12.0 software (The R Foundation for Statistical Computing).

Results

Patient characteristics

Compared with recipients of an 8/8-MUD, recipients of an RD/1AG-MM-GVH were more likely to be younger, to be male receiving a transplantation from a female donor, to have a shorter duration from diagnosis to transplantation, to have a high-risk disease, to receive cyclosporine for GVHD prophylaxis, to receive antithymocyte globulin or alemtuzumab, and to have a longer follow-up period (Table 1). Approximately half of the recipients in the RD/1AG-MM-GVH group received peripheral blood stem cells, whereas during this period in Japan, the source of transplantation from an MUD was restricted to BM. In the RD/1AG-MM-GVH group, the number of Ag mismatches in the HVG direction was 0 in 11%, 1 in 67%, 2 in 20%, and 3 in 2%. HLA-A, HLA-B, and HLA-DRB1 allelic information in both recipients and donors was available in 148 of 327 transplantations from an RD/1AG-MM-GVH donor and information on HLA-C Ag mismatch in either the GVH or HVG direction was available in 123 of 327.

OS

The 2-year OS rates in the 8/8-MUD and RD/1AG-MM-GVH groups were 0.59 (95% confidence interval [CI], 0.53-0.64) and 0.44 (95% CI, 0.38-0.49), respectively (log-rank test; $P < .001$; Figure 1A). Multivariate analysis revealed that, compared with the use of an 8/8-MUD, the use of an RD/1AG-MM-GVH was a significant adverse factor for OS (hazard ratio [HR], 1.49; 95% CI,

Table 1. Patient characteristics

Variable	RD/1AG-MM-GVH (n = 327)	8/8 MUD (n = 452)	P
Median age at transplantation, y (range)	45 (16-69)	48 (16-68)	.043
Recipient sex, n (%)			
Male	184 (56%)	267 (59%)	.434
Female	143 (44%)	185 (41%)	
Sex combination of donors and recipients, n (%)			
Female to male	91 (28%)	73 (16%)	< .001
Other combinations	236 (72%)	379 (84%)	
Performance status, n (%)			
0/1	298 (91%)	415 (92%)	.736
2/3/4	29 (9%)	37 (8%)	
Disease, n (%)			
AML	167 (51%)	249 (55%)	.512
ALL	90 (28%)	107 (24%)	
CML	19 (6%)	21 (5%)	
MDS	51 (16%)	75 (17%)	
Duration from diagnosis to transplantation, n (%)			
< 6 mo	124 (38%)	102 (23%)	< .001
≥ 6 mo	191 (58%)	350 (77%)	
Unknown	12 (4%)	0 (0%)	
Disease risk, n (%)			
Standard	175 (54%)	317 (70%)	< .001
High	133 (41%)	129 (29%)	
Unknown	19 (6%)	6 (1%)	
Source of stem cells, n (%)			
BM	142 (43%)	452 (100%)	< .001
Peripheral blood	185 (57%)		
HLA compatibility in the HVG direction, n (%)*			
Matched	36 (11%)	452 (100%)	< .001
1-antigen mismatch	218 (67%)		
2-antigen mismatch	65 (20%)		
3-antigen mismatch	8 (2%)		
HLA compatibility in the GVH direction, n (%)*			
Matched	0 (0%)	452 (100%)	< .001
1-allele mismatch	111 (34%)		
2-allele mismatch	36 (11%)		
3-allele mismatch	1 (0%)		
Uncertain/missing	179 (55%)		
Conditioning regimen, n (%)			
Myeloablative	243 (74%)	338 (75%)	.883
Reduced-intensity	84 (26%)	114 (25%)	
GVHD prophylaxis, n (%)			
Cyclosporine-based	113 (35%)	108 (24%)	0.004
Tacrolimus-based	209 (64%)	338 (75%)	
Others	5 (2%)	6 (1%)	
Use of ATG/alemtuzumab, n (%)			
Yes	33 (10%)	13 (3%)	< .001
No	294 (90%)	439 (97%)	
Median follow-up of survivors, mo (range)	36.2 (3.0-95.7)	13.5 (1.7-62.8)	< .001

*HLA compatibility was defined according to the HLA-A, HLA-B, and HLA-DR loci.

1.19-1.86; $P < .001$; Table 2). Age > 50 years, performance status ≥ 2, and high-risk disease were also found to be significant adverse factors, whereas other variables, such as the time from diagnosis to transplantation, were not.

Because our previous study showed that the impact of an HLA-1 Ag mismatch in a related transplantation on OS differed according to whether patients had standard-risk or high-risk diseases,[1] the survival rates were compared separately in each disease-risk group. The OS rates of patients with standard-risk diseases in the 8/8-MUD group were significantly higher than those in the RD/1AG-MM-GVH group ($P = .003$), whereas there was no significant difference in high-risk patients ($P = .090$; Figure 1B-C). Although the interaction between the donor type and disease risk did not reach statistical significance ($P = .140$), multivariate analyses in each disease-risk group showed that the adverse impact of the use of an RD/1AG-MM-GVH donor was significant in standard-risk patients (HR, 1.72; 95% CI, 1.24-2.39; $P = .001$), but not in high-risk patients (Table 2).

To visually compare MRDs and other stem-cell sources, the OS rate for MRDs was layered on those for MUDs and RD/1AG-MM-GVHs (Figure 1). The OS curve of transplantation from an MRD

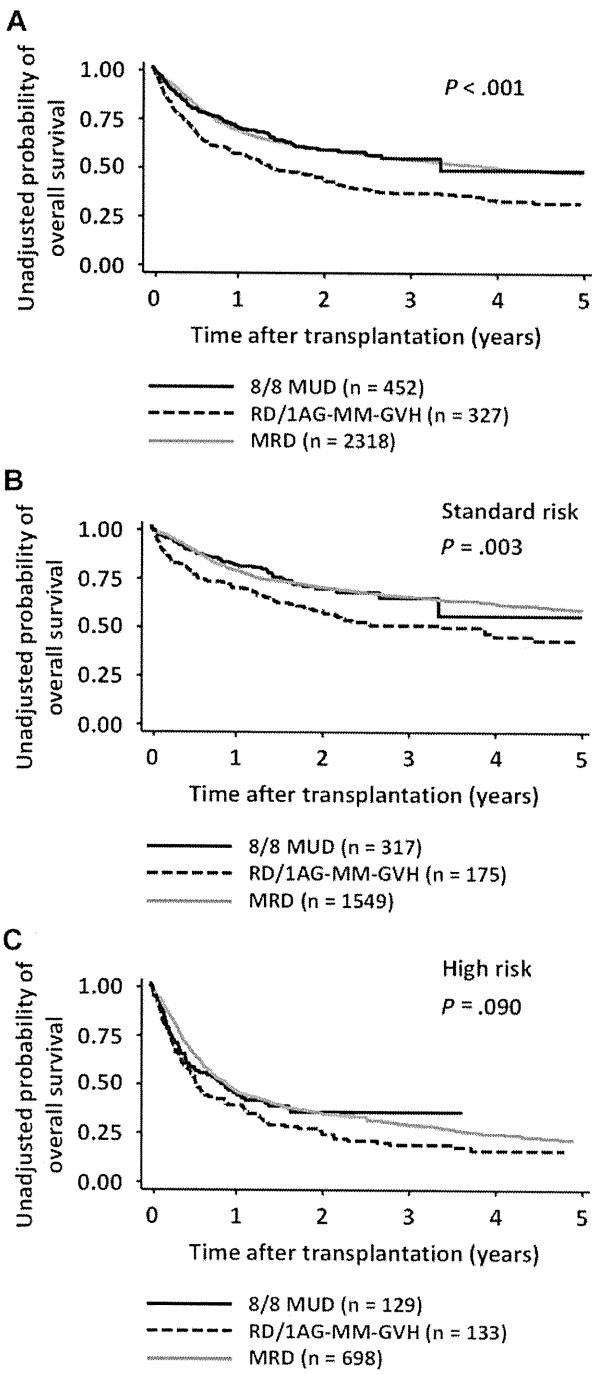

Figure 1. OS according to donor type and risk of disease. OS after transplantation from an RD/1AG-MM-GVH donor, an 8/8-MUD, and HLA-MRD in patients with both-risk (A), standard-risk (B), or high-risk diseases (C). Survival rates in the 8/8-MUD and RD/1AG-MM-GVH groups were compared with the log-rank test.

Table 2. Multivariate analysis of OS

	Total (n = 779)		Standard-risk (n = 492)		High-risk (n = 262)	
Variable	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Donor type						
8/8 MUD	1.00		1.00		1.00	
RD/1AG-MM-GVH	1.49 (1.19-1.86)	< .001	1.72 (1.24-2.39)	.001	1.30 (0.96-1.76)	.095
Age, y						
≤ 50	1.00		1.00			
> 50	1.44 (1.16-1.79)	.001	1.55 (1.13-2.15)	.007		
Performance status						
0/1	1.00				1.00	
2/3/4	1.79 (1.30-2.48)	< .001			1.76 (1.24-2.52)	.002
Disease risk						
Standard	1.00					
High	2.41 (1.92-3.03)	< .001				
Unknown	1.38 (0.82-2.33)	.227				

Only variables that remained after backward selection in the multivariate analysis are shown.

was superimposed on that from an MUD in both standard- and high-risk patients (MRD vs MUD: standard-risk group, $P = .895$, and high-risk group, $P = .581$). Multivariate analysis confirmed that OS in the MRD group was comparable to the MUD group (MRD vs MUD: standard-risk group, HR, 1.02; 95% CI, 0.79-1.32; $P = .878$; high-risk group, HR, 0.98; 95% CI, 0.76-1.26; $P = .865$).

Effect of HLA mismatches on OS

To identify factors that may contribute to the inferior OS in standard-risk patients in the RD/1AG-MM-GVH group compared with those in the 8/8-MUD group, we evaluated the impact of each HLA-A, HLA-B, or HLA-DR Ag mismatch in the GVH direction and the number of Ag mismatches in the HVG direction on OS rates in the RD/1AG-MM-GVH group.

In the RD/1AG-MM-GVH group, the OS rate for patients who received a transplantation from a related donor with an HLA-B Ag mismatch in the GVH direction and that from a donor with 2 or 3 Ag mismatches in the HVG direction were significantly lower than those in other groups (log-rank test for HLA-A Ag mismatch vs HLA-B Ag mismatch vs HLA-DR Ag mismatch in the GVH direction, $P < .001$, and 0-1 mismatches vs 2-3 mismatches in the HVG direction, $P = .003$; Figure 2). However, multivariate analysis revealed that only the presence of an HLA-B Ag mismatch in the GVH direction (HR, 1.57; 95% CI, 1.13-2.18; $P = .007$) was significantly associated with a lower OS (Table 3).

The OS rates were also compared separately in the standard-risk and high-risk disease groups (Figure 2). Although the interaction between the presence of an HLA-B Ag mismatch and disease risk did not reach statistical difference ($P = .232$), the adverse impact of an HLA-B Ag mismatch in the GVH direction was observed in the standard-risk group (HR, 1.86 95% CI, 1.14-3.01; $P = .012$), but not in the high-risk group (Table 3). Conversely, the survival curve for the HLA-A Ag or HLA-DR Ag-mismatched group was almost superimposed on that for 8/8-MUDs (Figure 2; standard-risk group: for the HLA-A Ag-mismatched group vs the 8/8-MUD group, HR, 1.26; 95% CI, 0.73-2.19; $P = .411$; for the HLA-DR Ag-mismatched group vs the 8/8-MUD group, HR, 1.37; 95% CI, 0.89-2.11; $P = .154$; high-risk group: for the HLA-A Ag-mismatched group vs the 8/8-MUD group, HR, 1.26; 95% CI, 0.80-2.00; $P = .320$; and for the HLA-DR Ag-mismatched group vs the 8/8-MUD group, HR, 1.03; 95% CI, 0.67-1.59]; $P = .880$). The impact of 2 or 3 Ag mismatches in the HVG direction was not significant in either the standard-risk or high-risk group (Table 3).

Effect of an HLA-B mismatch on TRM, relapse, GVHD, and neutrophil engraftment in patients with standard-risk diseases

Our findings showed that an HLA-B Ag mismatch in the GVH direction strongly contributed to the low survival rate in standard-risk patients, which can explain the inferior survival rates in the RD/1AG-MM-GVH group compared with the 8/8-MUD group. Therefore, we evaluated the impact of an HLA-B Ag mismatch in the GVH direction on other outcomes in patients with standard-risk diseases in the RD/1AG-MM-GVH group.

First, we compared the characteristics of patients with standard-risk diseases who received transplantation from a related donor with an HLA-A, HLA-B, and HLA-DR Ag mismatch in the GVH direction (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Two or 3 Ag mismatches in the HVG direction were observed more frequently in the HLA-B Ag-mismatched group (28%) than in the HLA-A Ag-mismatched group (2%) or the HLA-DR Ag-mismatched group (17%). Although there was no information available on allelic mismatch or HLA-C Ag mismatch in more than half of the patients, an HLA-C Ag mismatch in either the GVH or HVG direction was observed more frequently in the HLA-B Ag-mismatched group (61% among the available data) than in the HLA-A Ag-mismatched group (25%) or the HLA-DR Ag-mismatched group (17%).

The incidence of TRM was higher in the HLA-B Ag-mismatched group (3-year mortality rate: HR, 0.47; 95% CI, 0.32-0.60) than in the HLA-A Ag-mismatched group (HR, 0.28; 95% CI, 0.14-0.44) or the HLA-DR Ag-mismatched group (HR, 0.27; 95% CI, 0.17-0.38; Figure 3A; log-rank test, $P = .030$). The presence of an HLA-B Ag mismatch in the GVH direction was an independent significant adverse factor that affected TRM in the RD/1AG-MM-GVH group (Table 4). Conversely, the incidence of relapse did not significantly differ among the 3 groups (Figure 3B and Table 4).

The incidence of grade 2-4 acute GVHD in the HLA-B Ag-mismatched group was higher than that in the HLA-A Ag-mismatched group, but comparable to that in the HLA-DR Ag-mismatched group (supplemental Figure 1 and supplemental Table 2). There was no significant difference in the incidence of grade 3-4 acute GVHD among the 3 groups. Regarding neutrophil engraftment, multivariate analysis showed that an HLA-B Ag mismatch was significantly associated with inferior neutrophil engraftment and 2 or 3 Ag mismatches in the HVG direction were

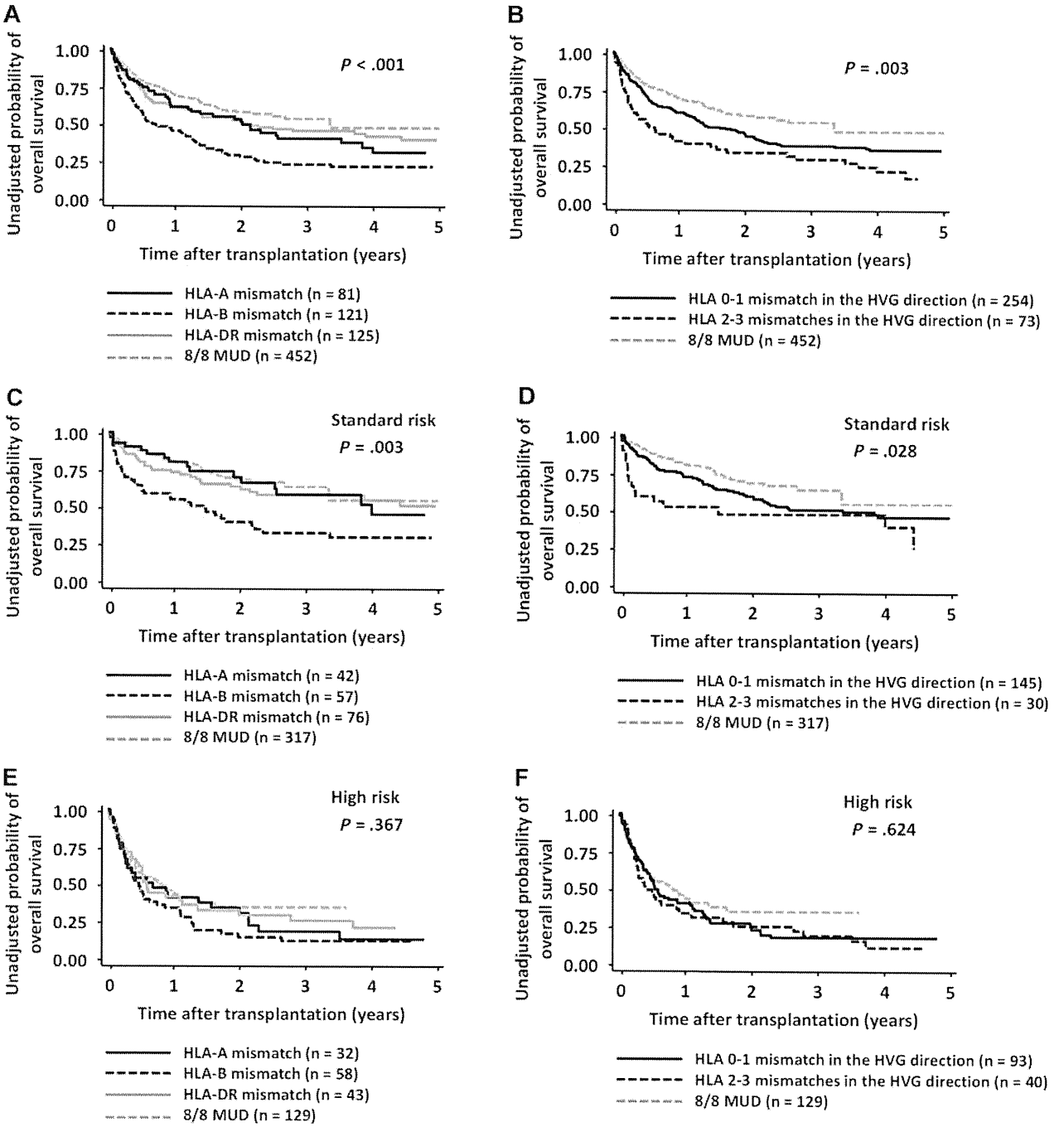

Figure 2. OS in patients with both-risk, standard-risk, or high-risk diseases according to the locus of HLA mismatch in the GVH direction and the number of mismatches in the HVG direction. Survival rates in patients with HLA-A, HLA-B, and HLA-DR Ag mismatches in the GVH direction were compared with the log-rank test (A,C,E). Survival rates in patients with 0-1 and 2-3 mismatches in the HVG direction were compared with the log-rank test (B,D,F). Survival rates of the 8/8-MUD group are shown for visual comparison.

associated with inferior neutrophil engraftment, with marginal significance (supplemental Table 2).

Discussion

In this nationwide retrospective study, we found that the survival rate of the RD/1AG-MM-GVH group was significantly inferior to that of the 8/8-MUD group, and this significant difference was observed only in patients with standard-risk diseases, although the interaction between donor type and disease risk did not reach statistical significance. We reported previously that transplantation from a related donor with 1 Ag mismatch in the GVH or HVG direction gave a clinical outcome comparable to that of transplantation from a 6/6-Ag-MUD in patients with either standard-risk or high-risk diseases.[1] However, because HLA matching at the allelic level in unrelated transplantation significantly reduces the risk of GVHD, in the present study, the survival curve of transplantation from an 8/8-MUD was substantially improved, and could be superimposed on a curve corresponding to that from an MRD.

Table 3. Multivariate analysis of OS in patients receiving transplantation from a related donor with a 1-antigen mismatch in the GVH direction

	Total (n = 327)		Standard-risk (n = 175)		High-risk (n = 133)	
Variable	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
HLA mismatch in the GVH direction						
HLA-DR mismatch	1.00		1.00		1.00	
HLA-A mismatch	1.07 (0.73-1.56)	.737	0.98 (0.54-1.81)	.966	1.11 (0.65-1.89)	.701
HLA-B mismatch	1.57 (1.13-2.18)	.007	1.86 (1.14-3.01)	.012	1.36 (0.86-2.17)	.193
HLA mismatch in the HVG direction						
0-1 mismatches	1.00		1.00		1.00	
2-3 mismatches	1.27 (0.91-1.76)	.154	1.67 (0.98-2.85)	.061	1.06 (0.69-1.61)	.799
Age, y						
≤ 50	1.00		1.00			
> 50	1.52 (1.14-2.03)	.004	1.87 (1.21-2.91)	.005		
Disease risk						
Standard	1.00					
High	2.06 (1.53-2.78)	< .001				
Unknown	1.00 (0.53-1.89)	.989				

Only variables that remained after backward selection in the multivariate analysis are shown.

Consistent with our findings, several studies have shown that the clinical outcomes of transplantation from an 8/8-10/10 MUD are comparable to those from an MRD.[20,21] The significant difference in survival rates between transplantation from an RD/1AG-MM-GVH donor and an 8/8-MUD disappeared in patients with high-risk diseases, probably because the adverse impact of acute GVHD on survival might be offset by the potential GVL effect in transplantation from an RD/1AG-MM-GVH donor.[1,2,22]

We evaluated factors that may contribute to the inferior OS in patients with standard-risk diseases in the RD/1AG-MM-GVH group and found that, compared with the presence of an HLA-DR Ag mismatch, the presence of an HLA-B Ag mismatch in the GVH direction was significantly associated with lower OS and higher TRM. Conversely, the rates of OS and TRM in the HLA-A Ag- or HLA-DR Ag-mismatched group were superimposed on those in the MUD group. However, HLA-A, HLA-B, and HLA-DR Ag mismatches had similar effects on the incidence of severe acute GVHD; consequently, the causal relationship between an HLA-B Ag mismatch in the GVH direction and higher TRM remains unknown. In contrast to our findings, Valcarcel et al reported that there was no significant difference in OS between the use of 1-Ag–mismatched related donors (n = 89) and 8/8-MUDs (n = 700) in transplantation for AML and ALL during the first or second complete remission.[23] This difference from our results can be partly

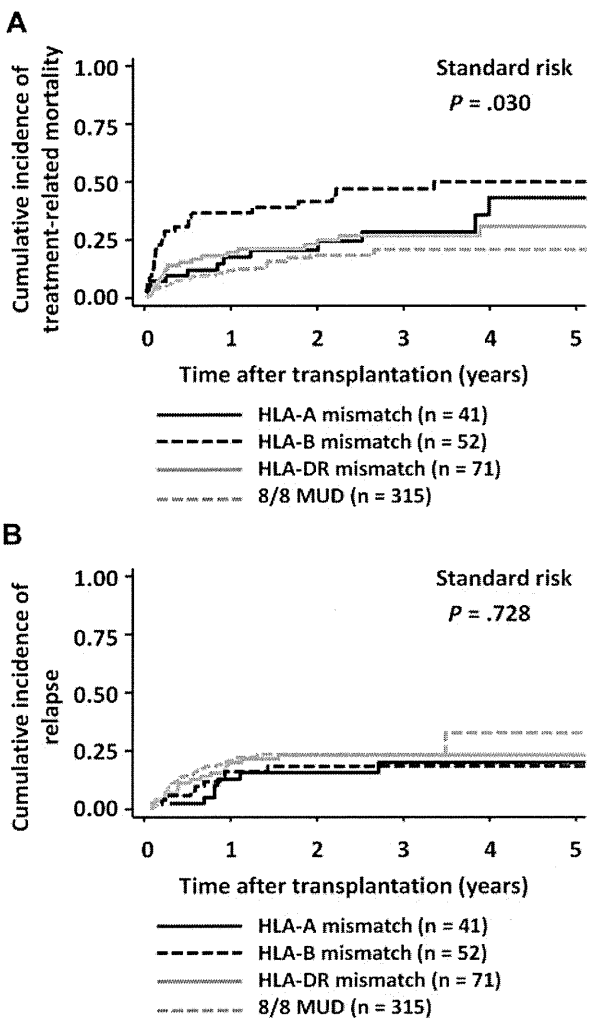

Figure 3. Cumulative incidence according to the locus of HLA mismatch in the GVH direction in patients with standard-risk diseases. Cumulative incidences in the related transplantation groups were compared with the Gray test. (A) TRM. (B) Relapse.

Table 4. Multivariate analysis of TRM and relapse in patients with standard-risk diseases receiving transplantations from a related donor with a 1-antigen mismatch in the GVH direction

	TRM (n = 164)		Relapse (n = 164)	
Variable	HR (95% CI)	P	HR (95% CI)	P
HLA mismatch in the GVH direction				
HLA-DR mismatch	1.00		1.00	
HLA-A mismatch	1.22 (0.59-2.52)	.587	0.70 (0.29-1.67)	.418
HLA-B mismatch	2.00 (1.09-3.65)	.025	0.80 (0.34-1.87)	.605
HLA mismatch in the HVG direction				
0-1 mismatches	1.00		1.00	
2-3 mismatches	2.21 (1.14-4.28)	.019	0.67 (0.23-1.98)	.467
Age, y				
≤ 50	1.00			
> 50	2.08 (1.18-3.65)	.011		
Duration from diagnosis to transplantation				
< 6 mo	1.00			
≥ 6 mo	2.40 (1.19-4.82)	.014		
Unknown	2.23 (0.77-6.48)	.140		

Only variables that remained after backward selection in the multivariate analysis are shown.

explained by the fact that the MUD group in their study included a significantly smaller number of ALL patients with low-risk cytogenetics. In addition, in our study, genetic homogeneity in the Japanese population might affect the lower incidence of severe acute GVHD in MUD transplantation because of the less frequent mismatches in minor histocompatibility Ags.[24,25]

The frequency of an HLA-C Ag mismatch was substantially higher in the HLA-B Ag-mismatched group than in the HLA-A or HLA-DR Ag-mismatched groups. This finding may represent linkage disequilibrium between the HLA-B and HLA-C genes, which are located at a very close physical proximity within the major histocompatibility complex.[26,27] Therefore, the impact of HLA-B-Ag might be affected by the co-presence of HLA-C Ag mismatch. We could not evaluate the impact of HLA-C Ag mismatch on OS rates because of the limited information on HLA-C Ag mismatch; therefore, an analysis with larger cohorts with complete HLA-C Ag information will be needed to evaluate the impact of HLA-C and/or HLA-B mismatch in transplantation from an RD/1AG-MM-GVH donor. Accordingly, we could not evaluate the impact of the KIR ligand mismatch. Although the impact of KIR ligand mismatch is still controversial, several studies analyzing T cell–replete transplantation showed that KIR ligand mismatch is associated with lower OS.[12,28,29] The analysis of KIR matching would be helpful in elucidating the mechanism underlying the adverse effect of HLA-B mismatch in T cell–replete transplantation from an RD/1AG-MM-GVH donor.

Whether the presence of allelic mismatches in addition to the 1-Ag mismatch (2 or more allelic mismatches in total) affects transplantation outcome is also an important clinical question in transplantation from an RD/1AG-MM-GVH donor. A high frequency of 2-allele mismatches in the GVH direction was seen in the HLA-B Ag-mismatched group, suggesting a possible association between 2-allele mismatches and low OS. However, we did not observe a significant effect of the number of allelic mismatches on OS after transplantation from an RD/1AG-MM-GVH donor, possibly because of the small sample size.

Our study has several limitations. First, because several months are required to arrange unrelated transplantations, patients at low risk for relapse may more often be selected for these procedures. To minimize this bias, we included the duration from diagnosis to transplantation in the multivariate analysis; however, this variable did not have a significant effect in the multivariate analysis. Second, heterogeneous backgrounds may have resulted in a bias. In particular, the stem-cell source in unrelated transplantation was exclusively BM. However, the analysis of OS in the subgroup of patients who received a BM graft from an RD/1AG-MM-GVH donor or an 8/8-MUD showed similar results. Third, because we have incomplete Ag and allele information on the HLA-C and -DQB1 loci, we may have underestimated the degree of mismatching in transplantation from an RD/1AG-MM-GVH donor. Fourth, the difference in the impact of donor type between standard- and high-risk diseases should be cautiously interpreted, because the interaction between the donor type and disease risk did not reach statistical significance. This may be partly because of the lower statistical power to detect the interaction than the main effect.

In conclusion, our findings suggest that an 8/8-MUD, if available, should be prioritized over an RD/1AG-MM-GVH donor for patients without an MRD if an immediate transplantation is not necessary. In particular, the presence of an HLA-B Ag mismatch in the GVH direction has an adverse effect on OS because of treatment-related complications. This may be because of the high frequencies of additional mismatches of HLA-C Ag or allele in the HLA-B Ag-mismatched group. To elucidate the mechanism of the adverse outcomes in RD/1AG-MM-GVH donors with an HLA-B Ag mismatch, HLA Ag/allele matching including HLA-C should be performed in transplantations from an RD/1AG-MM-GVH donor.

Acknowledgments

The authors are indebted to all of the physicians and data managers at the centers who contributed valuable data on transplantation to the Japan Society for Hematopoietic Cell Transplantation and the Japan Marrow Donor Program. They also thank all of the members of the data management committees of the Japan Society for Hematopoietic Cell Transplantation and the Japan Marrow Donor Program for their management of data.

J.K. is a Research Fellow of the Japan Society for the Promotion of Science. This work was supported in part by a grant-in-aid for Japan Society for the Promotion of Science (to J.K.).

Authorship

Contribution: Y.K. designed the research and organized the project; J.K., H. Saji, and Y.K. reviewed and analyzed the data and wrote the manuscript; J.K. and Y.K. performed the statistical analysis; H. Sakamaki, J.T., R.S., and Y.A. collected data from Japan Society for Hematopoietic Cell Transplantation; K.K. and Y.M. collected data from Japan Marrow Donor Program; and all authors interpreted the data and reviewed and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Leukemia (2011) 25, 259–265

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ORIGINAL ARTICLE

A decision analysis of allogeneic hematopoietic stem cell transplantation in adult patients with Philadelphia chromosome-negative acute lymphoblastic leukemia in first remission who have an HLA-matched sibling donor

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Clinical studies using genetic randomization cannot accurately answer whether adult patients with Philadelphia chromosome-negative acute lymphoblastic leukemia (ALL) who have a human leukocyte antigen (HLA)-matched sibling should undergo allogeneic hematopoietic stem cell transplantation (HSCT) or chemotherapy in first remission, as, in these studies, patients without a sibling donor undergo alternative donor transplantation or chemotherapy alone after a relapse. Therefore, we performed a decision analysis to identify the optimal strategy in this setting. Transition probabilities and utilities were estimated from prospective studies of the Japan Adult Leukemia Study Group, the database of the Japan Society for Hematopoietic Cell Transplantation and the literature. The primary outcome measure was the 10-year survival probability with or without quality of life (QOL) adjustments. Subgroup analyses were performed according to risk stratification on the basis of white blood cell count and cytogenetics, and according to age stratification. In analyses without QOL adjustments, allogeneic HSCT in first remission was superior in the whole population (48.3 vs 32.6%) and in all subgroups. With QOL adjustments, a similar tendency was conserved (44.9 vs 31.7% in the whole population). To improve the probability of long-term survival, allogeneic HSCT in first remission is recommended for patients who have an HLA-matched sibling.

Leukemia (2011) **25,** 259–265; doi:10.1038/leu.2010.260; published online 12 November 2010

Keywords: decision analysis; acute lymphoblastic leukemia; allogeneic hematopoietic stem cell transplantation; HLA-matched sibling donor; first remission

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Received 21 June 2010; revised 25 August 2010; accepted 1 October 2010; published online 12 November 2010

Introduction

With modern intensive chemotherapy, 74–93% of adult patients with acute lymphoblastic leukemia (ALL) achieve complete remission. However, the overall survival rate is only 27–48% because of the high rate of relapse.[1] Therefore, the establishment of optimal postremission therapy is important. The efficacy of allogeneic hematopoietic stem cell transplantation (HSCT) for adult patients with ALL in first remission has been demonstrated through clinical studies using genetic randomization, in which patients with a human leukocyte antigen (HLA)-matched sibling donor were allocated to the allogeneic HSCT arm, and those without a donor were placed in the chemotherapy or autologous transplantation arm. First, the LALA-87 trial showed that overall survival in patients with a donor was better than that in patients without a donor in a subgroup analysis of patients with high-risk characteristics.[2] A meta-analysis of seven similar studies confirmed that the donor group was superior to the non-donor group in patients with high-risk ALL in first remission.[3] However, such genetic randomization studies cannot accurately answer the question of whether patients with an HLA-matched sibling should undergo allogeneic HSCT or chemotherapy in first remission. In these studies, patients without a sibling donor had to choose transplantation from an alternative donor or chemotherapy alone once they had a relapse. The outcome of these treatments has been reported to be inferior to that of HSCT from an HLA-matched sibling donor in patients with relapsed ALL; therefore, the expected survival after the decision to continue chemotherapy in first remission in patients without a sibling donor is assumed to be originally poorer than that in patients with a sibling donor. However, it is practically difficult to perform a clinical trial in which patients with an HLA-matched sibling in first remission are randomly assigned to receive allogeneic HSCT or chemotherapy alone. Another important problem has been poor compliance with the assigned treatment in some studies. In addition, previous genetic