Table 2
Multivariate Analysis to Evaluate the Impact of Single HLA Allele Mismatches on the Incidence of Grade III to IV Acute GVHD Stratified according to the Transplantation Time Period

Year	Factor		Hazard Ratio	*P* Value
1993-2001				
	Donor age		1.02 (1.00-1.03)	.082
	Donor sex	Female	1.00	
		Male	1.65 (1.05-2.60)	.031
	Female to male transplantation	No	1.00	
		Yes	1.52 (.91-2.55)	.11
	Disease	AML	1.00	
		ALL	1.15 (.79-1.68)	.47
		CML	1.62 (1.11-2.36)	.012
		MDS	.65 (.32-1.35)	.25
	Disease risk	Low	1.00	
		High	1.30 (.93-1.83)	.13
		Others	.80 (.23-2.85)	.74
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.83 (.61-1.14)	.25
	HLA	Low-risk mismatch	1.00	
		Match	.89 (.65-1.21)	.44
		High-risk mismatch	2.74 (1.73-4.32)	<.0001
2002-2007				
	Donor age		1.03 (1.01-1.05)	.0028
	Donor sex	Female	1.00	
		Male	1.50 (.96-2.33)	.076
	Female to male transplantation	No	1.00	
		Yes	1.53 (.89-2.64)	.13
	Disease	AML	1.00	
		ALL	1.36 (.95-1.96)	.094
		CML	1.27 (.74-2.20)	.38
		MDS	1.25 (.77-2.02)	.37
	Disease risk	Low	1.00	
		High	1.76 (1.25-2.48)	.0011
		Others	1.65 (.82-3.34)	.16
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.86 (.63-1.19)	.37
	HLA	Low-risk mismatch	1.00	
		Match	.64 (.46-.89)	.008
		High-risk mismatch	1.06 (.58-1.93)	.85
2008-2011				
	Donor age		1.03 (1.01-1.06)	.0016
	Donor sex	Female	1.00	
		Male	1.28 (.78-2.12)	.33
	Female to male transplantation	No	1.00	
		Yes	.98 (.52-1.88)	.96
	Disease	AML	1.00	
		ALL	1.18 (.80-1.74)	.42
		CML	1.53 (.69-3.37)	.3
		MDS	.66 (.36-1.20)	.17
	Disease risk	Low	1.00	
		High	1.53 (1.08-2.17)	.018
		Others	NA (NA-NA)	NA
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.82 (.55-1.24)	.34
	HLA	Low-risk mismatch	1.00	
		Match	.56 (.39-.80)	.0014
		High-risk mismatch	.40 (.10-1.64)	.21

AML indicates acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus.

these patients were obtained from the TRUMP [8]. We excluded patients who lacked data on survival status, those with more than 1 allele or antigen mismatch, those who received a reduced-intensity conditioning regimen, and those who received ex vivo or in vivo T cell depletion, such as antithymocyte globulin or alemtuzumab. Finally, 3718 patients were included in the main part of this study. As a post hoc analysis, 415 patients with 2 LR-MMs and 66 patients with 2 allele mismatches including at least 1 HR-MM were added to compare the impact of 1 HR-MM and 2 LR-MMs and to analyze the statistical interaction between HR-MM and the presence of an additional allele mismatch. The study was approved by the data management committee of TRUMP and by the institutional review board of Saitama Medical Center, Jichi Medical University.

Histocompatibility

Histocompatibility data for serological and genetic typing for the HLA-A, HLA-B, HLA-C, and HLA-DR loci were obtained from the TRUMP database, which includes HLA allele data determined retrospectively by the Japan Marrow Donor Program using frozen samples [7,9]. In this study, the following donor-recipient HLA-mismatch combinations were regarded as HR-MMs: A*02:06-A*02:01, A*02:06-A*02:07, A*26:02-A*26:01, A*26:03-A*26:01, B*15:01-B*15:07, C*03:03-C*15:02, C*03:04-C*08:01, C*04:01-C*03:03, C*08:01-C*03:03, C*14:02-C*03:04, C*15:02-C*03:04, C*15:02-C*14:02, DR*04:05-DR*04:03, and DR*14:03-DR*-DR1401, as we did not have enough data on HLA-DP and -DQ [7]. In HR-MM pairs, the donor and the recipient must have the HLA allele as shown above, and at the same time, these donor and recipient HLA alleles should not be shared by the recipient and the donor, respectively. For example, if the donor has HLA-A*02:06/02:06 and the recipient has HLA-A*02:01/02:06, this pair was not regarded as HR-MM pair, as the donor's HLA-A*02:06 was shared by the recipient. Other HLA mismatch pairs were regarded as LR-MM pairs. Only the HLA-C mismatch group included HLA mismatch at a serological (antigen) level.

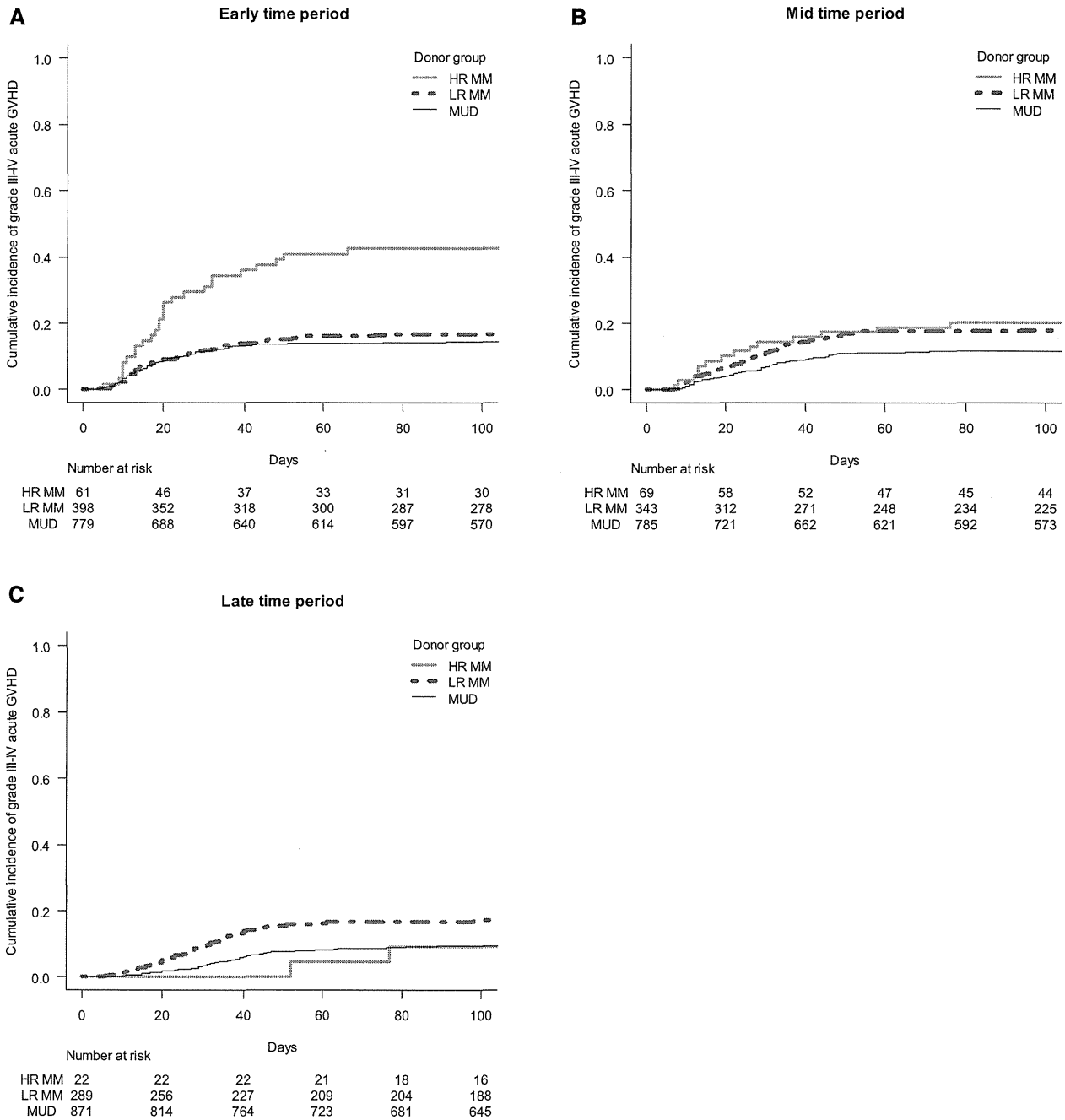

Figure 1. The cumulative incidence of grade III to IV acute GVHD grouped according to the HLA mismatch between the donor and recipient in the early (A), mid (B), and late time periods (C). HR-MM indicates high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

Statistical Analyses

We divided the patients into 3 groups according to the time period when HSCT was performed to evaluate whether the impact of HR-MM changed over time periods: the early, mid, and late groups included HSCT performed from 1993 through 2001, 2002 through 2007, and 2008 through 2011, respectively. The break points among groups were determined to make the number of patients in each group equivalent (n = 1278, 1236, and 1204, respectively). To avoid making misleading conclusions by arbitrary grouping, we confirmed that there was a statistically significant interaction between the presence of HR-MMs and transplantation year as a continuous variable, both for overall survival ($P = .0098$) and the incidence of grade III to IV acute GVHD ($P < .001$). The following analyses were performed separately in each group. However, in post hoc analyses to evaluate the impact of HR-MMs at each locus and to compare 1 HR-MM and 2 LR-MMs, the mid and late groups were combined to increase the statistical power, after confirming that similar results were obtained in the 2 groups.

The primary endpoint was the incidence of grade III to IV acute GVHD. Overall survival was evaluated as a secondary endpoint. The chi-square test or Fisher exact test was used to compare categorical variables and Student *t*-test or an analysis of variance test was used for continuous variables to evaluate the homogeneity of background characteristics of the HR-MM, LR-MM, and HLA-matched (MUD) groups. *P* values were adjusted using the Bonferroni's method and Tukey's method for multiple comparisons between each pair. Overall survival was estimated according to the Kaplan-Meier method, and compared among groups with the log-rank test. The incidence of acute GVHD was calculated treating death without GVHD as a competing event, and it was compared using Gray's test [10].

The impact of HR-MMs was evaluated using multivariate models: the Cox proportional hazards model was used for overall survival and Fine and Gray's proportional hazards model was used for acute GVHD [11]. The LR-MM group was regarded as the reference group. Potential confounding factors that were considered in these analyses included recipient/donor age, recipient/donor sex, sex mismatch, ABO major/minor mismatch, the use of

Table 3
Multivariate Analysis to Evaluate the Impact of Single High-Risk Allele Mismatches on Overall Survival Stratified According to the Transplantation Time Period

Year	Factor		Hazard Ratio	*P* Value
1993-2001				
	Age		1.02 (1.01-1.03)	<.0001
	Sex	Female	1.00	
		Male	1.06 (.90-1.23)	.51
	Disease	AML	1.00	
		ALL	1.20 (.99-1.45)	.065
		CML	.89 (.72-1.10)	.29
		MDS	.61 (.45-.83)	.0015
	Disease risk	Low	1.00	
		High	2.72 (2.30-3.23)	<.0001
		Others	2.03 (1.27-3.23)	.0029
	ABO major mismatch	Absent	1.00	
		Present	1.25 (1.06-1.47)	.0092
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.85 (.72-1.00)	.049
	HLA	Low-risk mismatch	1.00	
		Match	.86 (.73-1.01)	.063
		High-risk mismatch	1.46 (1.06-2.01)	.019
2002-2007				
	Age		1.01 (1.00-1.02)	.0025
	Sex	Female	1.00	
		Male	1.20 (1.02-1.41)	.0027
	Disease	AML	1.00	
		ALL	1.16 (.96-1.39)	.13
		CML	.84 (.62-1.12)	.23
		MDS	.56 (.43-.73)	<.0001
	Disease risk	Low	1.00	
		High	2.87 (2.41-3.40)	<.0001
		Others	2.23 (1.58-3.15)	<.0001
	ABO major mismatch	Absent	1.00	
		Present	.97 (.81-1.16)	.77
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.97 (.83-1.15)	.76
	HLA	Low-risk mismatch	1.00	
		Match	.83 (.69-.98)	.032
		High-risk mismatch	1.06 (.75-1.48)	.75
2008-2011				
	Age		1.02 (1.01-1.03)	<.0001
	Sex	Female	1.00	
		Male	1.08 (.89-1.31)	.42
	Disease	AML	1.00	
		ALL	.97 (.76-1.25)	.83
		CML	.97 (.57-1.64)	.9
		MDS	.65 (.48-.87)	.004
	Disease risk	Low	1.00	
		High	2.73 (2.23-3.35)	<.0001
		Others	NA (NA-NA)	NA
	ABO major mismatch	Absent	1.00	
		Present	1.14 (.92-1.41)	.22
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.95 (.75-1.21)	.69
	HLA	Low-risk mismatch	1.00	
		Match	.86 (.69-1.06)	.15
		High-risk mismatch	.82 (.42-1.62)	.58

AML indicates acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus.

total body irradiation in the conditioning regimen, cell dose in the bone marrow graft, the use of cyclosporine or tacrolimus as GVHD prophylaxis, background disease, and disease risk. Acute leukemia in first or second remission, CML in first or second chronic phase, CML in accelerated phase, and myelodysplastic syndrome of refractory anemia or refractory anemia with excess blasts were considered low-risk diseases, and other conditions were considered high-risk diseases. All of these potential confounding factors were included in the multivariate analyses and then deleted in a stepwise fashion from the model to exclude factors with a *P* value of .05 or higher. Finally, HLA mismatch was added to the model. Different multivariate models were compared using the likelihood ratio test. The quantity of interest was the deviance difference between the 2 models, under the null hypothesis that 2 models fit the data equally well and the deviance difference has an approximate chi-square distribution with degrees of freedom equal to the difference in the number of independent variables between the compared models.

All *P* values were 2 sided and *P* values of .05 or less were considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University) [12], which is a graphical user interface for R (The R Foundation for Statistical Computing). More precisely, it is a modified version of R commander that was designed to add statistical functions frequently used in biostatistics.

RESULTS

Patients

The patient characteristics are summarized in Table 1. HR-MMs were observed in 64 of 1278, 71 of 1236, and 22 of 1204 donor-recipient pairs in the early, mid, and late time periods, respectively. On the other hand, 412, 351, and 294 pairs had LR-MMs, respectively. With regard to the

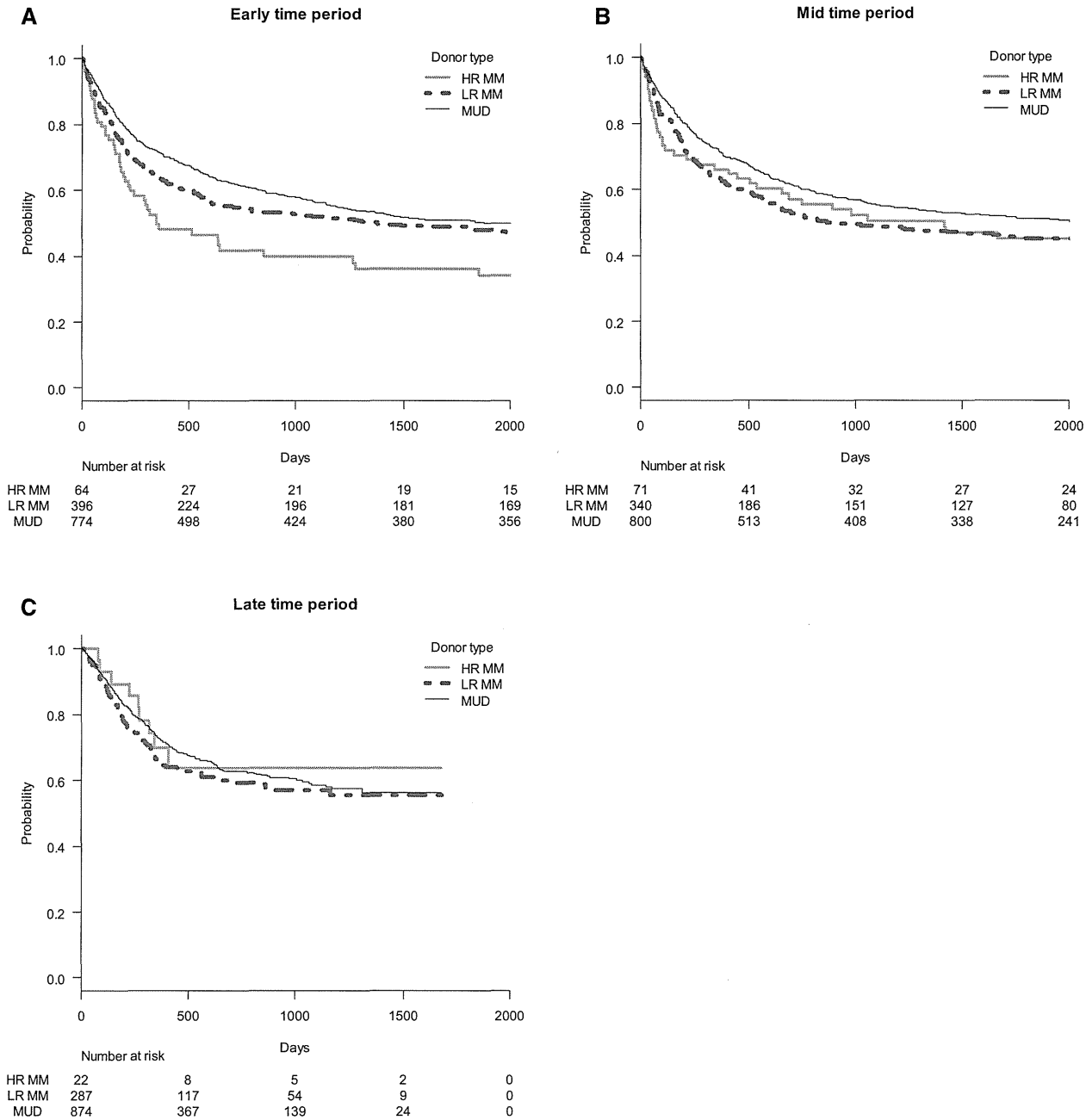

Figure 2. Overall survival grouped according to the HLA mismatch between the donor and recipient in the early (A), mid (B), and late time periods (C). The survival curves were adjusted for other significant factors by the mean of covariates method, in which average values of covariates are entered into the Cox proportional hazards model. HR-MM, high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

differences among transplantation time periods, the numbers of LR-MMs and HR-MMs decreased in the late time periods, ie, after the introduction of routine typing for HLA-C and the publication of a paper about HR-MMs [7]. The proportion of HSCTs for CML also dramatically decreased over time periods (30.7%, 10.4%, and 3.6% in the early, mid, and late periods, respectively). With regard to the difference among HLA mismatch groups, the proportion of patients with high-risk underlying disease in the MUD group (29.9%) was significantly lower than those in the HR-MM (37.6%) and LR-MM groups (34.4%). In addition, the proportion of HSCTs for CML was significantly higher in the HR-MM group in the early time period (29.6%, 30.3%, and 46.9% in the MUD, LR-MM, and HR-MM groups, respectively).

Incidence of Grade III to IV Acute GVHD

To adjust the impact of HLA mismatch for possible confounding factors, we identified the following independently significant factors for the incidence of grade III to IV acute GVHD: donor age, donor sex, sex mismatch, disease, disease risk, and GVHD prophylaxis. After we adjusted for these factors, we confirmed that the incidence of grade III to IV acute GVHD in the HR-MM group was significantly higher than that in the LR-MM group (hazard ratio [HR], 2.74; 95% confidence interval [CI], 1.73 to 4.32; $P < .0001$) in the early time period, whereas the difference between the MUD and LR-MM groups was not significant (HR, .89; 95% CI, .65 to 1.21; $P = .44$) (Table 2, Figure 1). On the other hand, in the mid and late time periods, the difference in the incidence of

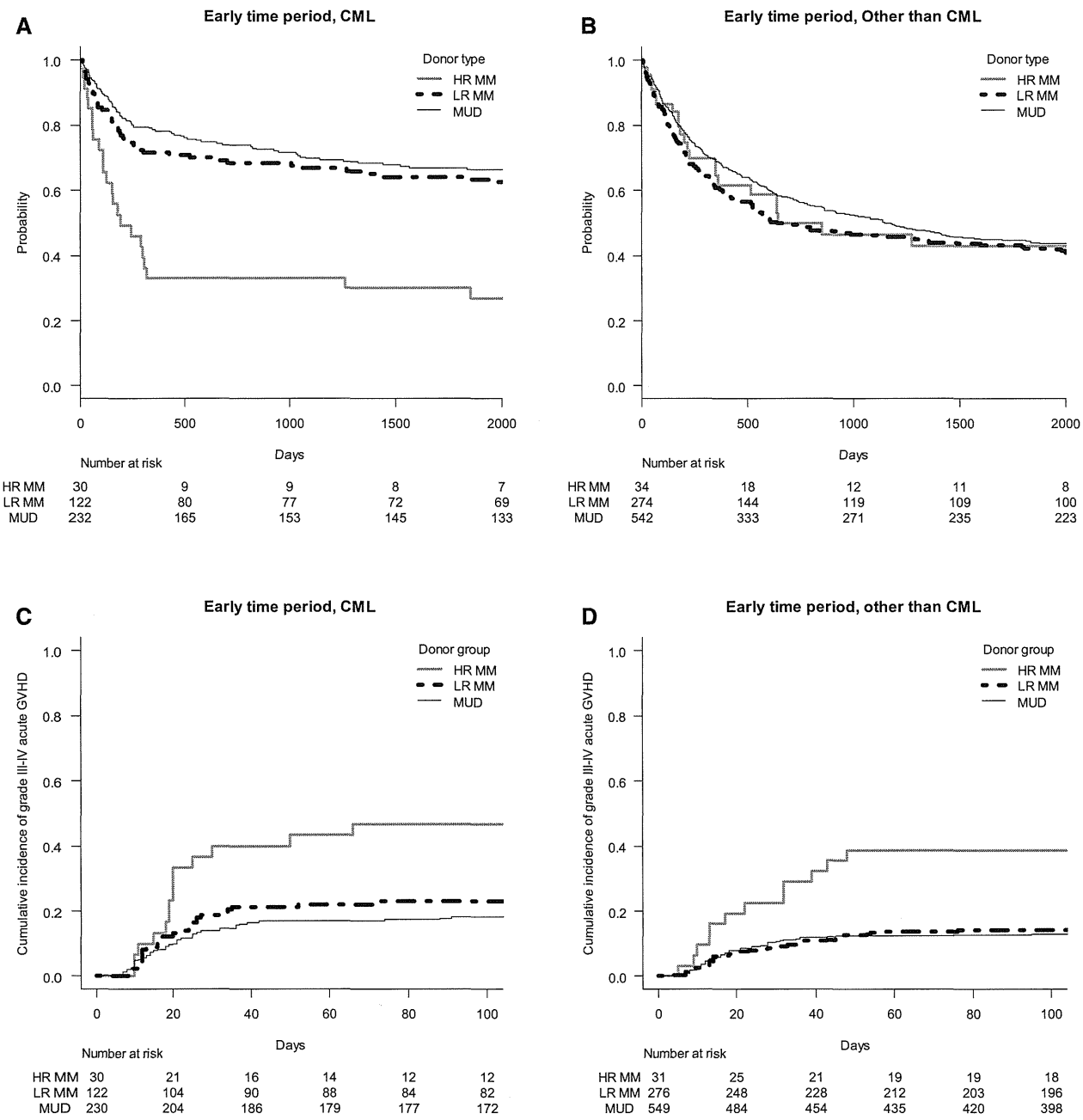

Figure 3. Adjusted overall survival (A,B) and the cumulative incidence of grade III to IV acute GVHD (C,D) grouped according to the underlying disease in the early time period. CML, chronic myelogenous leukemia; HR-MM, high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

grade III to IV acute GVHD between the HR-MM and LR-MM groups was not statistically significant (HR, 1.06; 95% CI, .58 to 1.93; $P = .85$ and HR, .40; 95% CI; .10 to 1.64; $P = .21$, respectively). The presence of LR-MM significantly adversely affected the incidence of grade III to IV acute GVHD in the mid and late periods (HR, .64; 95% CI, .46 to .89; $P = .008$ and HR, .56; 95% CI, .39 to .80; $P = .0014$, respectively, for the MUD group).

Similarly, the presence of HR-MM significantly affected the incidence of grade II to IV acute GVHD compared with LR-MM only in the early time period (HR, 1.53; 95% CI, 1.05 to 2.24; $P = .028$), and not in the mid and late periods (HR, .92; 95% CI, .61 to 1.37; $P = .67$ and HR, .79; 95% CI, .40 to 1.58; $P = .51$, respectively).

Overall Survival

After adjusting for recipient age, recipient sex, presence of ABO-major mismatch, disease, disease risk, and GVHD prophylaxis, we again confirmed that survival in the HR-MM group was significantly inferior to that in the LR-MM group (HR, 1.46; 95% CI, 1.06 to 2.01; $P = .019$) in the early time period, whereas there was no significant difference between the MUD and LR-MM groups (HR, .86; 95% CI, .73 to 1.01; $P = .063$) (Table 3). On the other hand, the difference in survival between the HR-MM and LR-MM groups was not statistically significant in the mid and late time periods (HR, 1.06; 95% CI, .75 to 1.48; $P = .75$ and HR, .82; 95% CI, .42 to 1.62; $P = .58$, respectively). The difference in survival between the MUD and LR-MM groups was consistent among

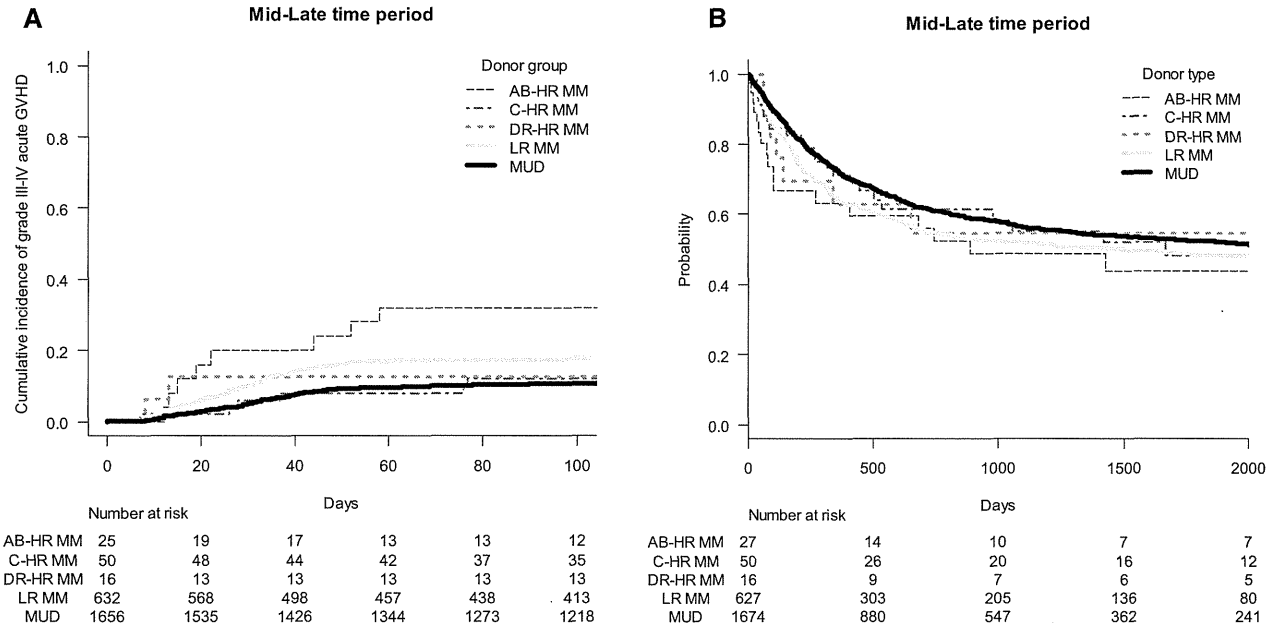

Figure 4. The cumulative incidence of grade III to IV acute GVHD (A) and adjusted overall survival (B) grouped according to the HLA mismatch loci between the donor and recipient in the mid or late time period. AB-HR MM, high-risk mismatch at the HLA-A or -B locus; C-HR MM, high-risk mismatch at the HLA-C locus; DR-HR MM, high-risk mismatch at the DRB1 locus; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

the 3 time periods but statistically significant only in the mid period (HR, .83; 95% CI, .69 to .98; $P = .032$). Figure 2 shows the overall survival curves grouped according to the HLA-mismatch groups in each time period, adjusted for other significant factors by the mean of covariates method.

Disease-specific Effects of HR-MM in the Early Period

The number of patients with CML was significantly higher in the early period than in the mid and late periods. Therefore, we evaluated the disease-specific impact of HR-MM in the early period. As shown in Figures 3A and B, the presence of HR-MM had an adverse impact on overall survival only in patients with CML, although HR-MM showed a similar adverse impact on the incidence of grade III to IV acute GVHD regardless of the underlying disease (Figure 3C, D). Of the 24 CML patients who died after HSCT with HR-MM, 23 died without relapse of CML, and 10 of these patients died without grade III to IV acute GVHD.

Impact of HR-MM at Each Locus

To evaluate the impact of HR-MM at each locus in the mid and early periods, we combined the 2 periods together to

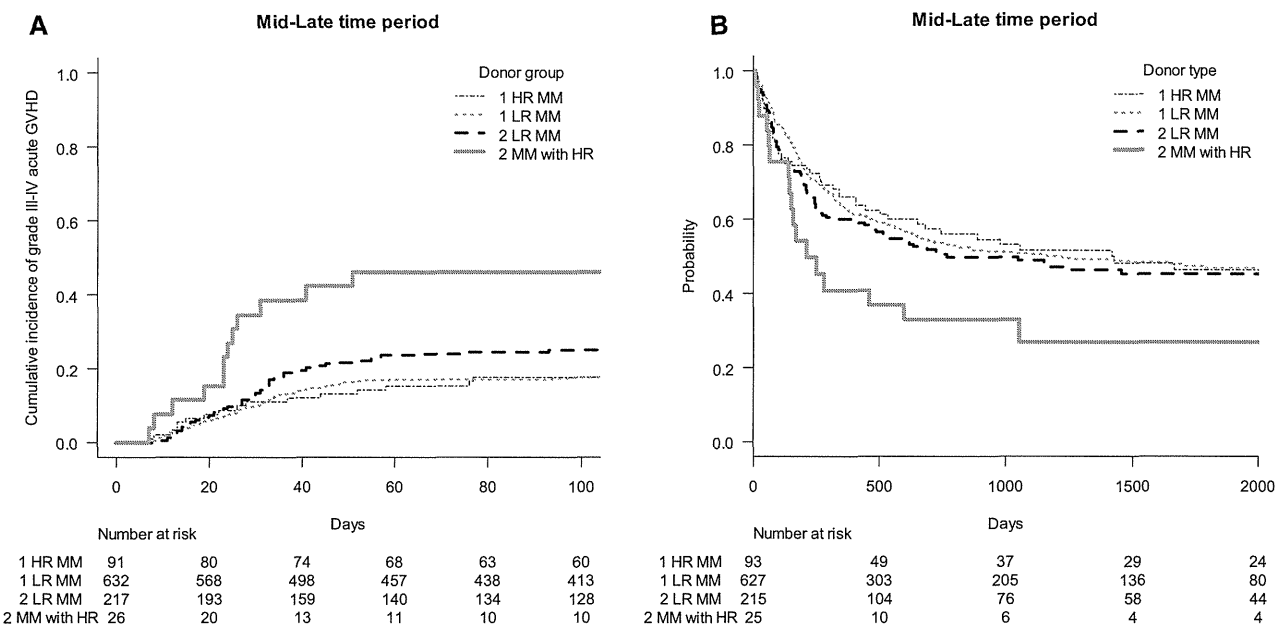

Figure 5. The cumulative incidence of grade III to IV acute GVHD (A) and adjusted overall survival (B) grouped according to the HLA mismatch between the donor and recipient in the mid or late time period. 1HR-MM, 1 high-risk mismatch; 1LR-MM, 1 low-risk mismatch; 2LR-MM, 2 low-risk mismatches; 2MM with HR, 2 allele mismatches including at least 1 HR-MM.

increase statistical power because the impact of HR-MM on acute GVHD and survival tended to be similar in these 2 time periods. The presence of HR-MMs at the HLA-A/B (HLA-A or -B), HLA-C, and HLA-DRB1 loci was not associated with significantly different survival compared with the LR-MM group (HR, 1.23; 95% CI, .76 to 1.98; $P = .41$; HR, .96; 95% CI, .65 to 1.44; $P = .86$; and HR, .95; 95% CI, .45 to 2.02; $P = .89$, respectively. Figure 4A). However, the incidence of grade III to IV acute GVHD was higher in patients who had HR-MM at the HLA-A/B locus than in those with LR-MM, although this difference was not statistically significant (HR, 1.78; 95% CI, .86 to 3.66; $P = .12$; HR, .63; 95% CI, .28 to 1.41; $P = .26$; and HR, .69; 95% CI, .15 to 3.12; $P = .63$ for HLA-A/B, HLA-C, and HLA-DRB1, respectively.) (Figure 4B).

Comparison of One HR-MM and Two LR-MMs

To evaluate whether a donor with 1 HR-MM or a donor with 2 LR-MMs should be preferred, we added patients with 2 LR-MMs and those with 2 allele mismatches including at least 1 HR-MM to the dataset, and we compared the outcome of HSCT from these donors with that of HSCT from a donor with 1 LR-MM as a reference in the combined mid and late periods.

The presence of 2 LR-MMs was associated with a significantly higher incidence of grade III to IV acute GVHD (HR, 1.44; 95% CI, 1.04 to 2.00; $P = .030$), but the impact of 1 HR-MM was not statistically significant (HR, .94; 95% CI, .56 to 1.59; $P = .83$) (Figure 5A). However, the impact of 2 LR-MMs was not associated with inferior survival. The HR for survival of 1 HR-MM and 2 LR-MMs were 1.05 (95% CI, .78 to 1.42; $P = .75$) and 1.12 (95% CI, .90 to 1.39; $P = .33$), respectively (Figure 5B).

On the other hand, the presence of 2 allele mismatches including at least 1 HR-MM was associated with an extremely poor outcome; HR, 3.61 (95% CI, 1.96 to 6.66; $P < .001$) for grade III to IV acute GVHD and HR, 2.02 (95% CI, 1.25 to 3.26; $P = .0040$) for overall survival. These results suggested that the impact of HR-MM may change according to the presence or absence of an additional allele mismatch. In fact, there was a statistically significant interaction between the presence of HR-MM and the presence of an additional allele mismatch ($P = .020$). The likelihood ratio test revealed that the prognostic value of Fine and Gray's proportional hazards model for acute GVHD was significantly improved by adding the interaction term to the model ($P = .024$).

DISCUSSION

In this study, we reevaluated the clinical impact of HR-MMs in unrelated HSCT. We confirmed that the presence of HR-MMs was associated with a significantly higher incidence of grade III to IV acute GVHD and significantly inferior survival in the early transplantation time period. However, in the mid and late periods, ie, after 2002, there was no statistically significant difference in overall survival or the incidence of grade III to IV acute GVHD between patients with HR-MMs and those with LR-MMs. The methods used for the statistical analyses were somewhat different than those in a previous study, but this is not the major reason for the different results, as the significant impact of HR-MMs on survival and acute GVHD was reproduced in the early time period. Another possible explanation is a bias caused by the availability of information about HR-MMs. After the publication of a paper that reported the importance of HR-MM, physicians may have tended to intensify prophylaxis against GVHD in unrelated HSCT with HR-MMs, and, thereby, the impact of HR-MMs might have become less significant. However, this is not the case because the impact of HR-MMs was already not apparent in the mid time period, before the paper was published. We also considered that the difference in the underlying disease might have influenced the effect of HR-MMs. The proportion of patients with CML decreased from 30.7% in the early period to 10.4% and 3.6% in the mid and late periods, respectively. Therefore, we analyzed the impact of HR-MMs grouped according to the underlying disease in the early period. The effect of HR-MMs on survival was observed only in patients with CML (Figure 3A,B). However, HR-MMs had an adverse effect on the incidence of grade III to IV acute GVHD regardless of the underlying disease (Figure 3C,D). Therefore, the different effects of HR-MMs on the incidence of grade III to IV acute GVHD among the time periods could not be explained solely by the underlying diseases. We could not clarify the reason for this different effect, but the changes in the transplantation procedure, including prophylaxis against GVHD, might have reduced the clinical impact of HR-MM. In fact, the incidence of grade III to IV acute GVHD decreased from 42.6%, 16.8%, and 14.5% in the HR-MM, LR-MM, and MUD groups, respectively, in the early time period to 17.6%, 17.7%, and 10.6% in the mid or late period. Improved survival in patients who developed severe acute GVHD might also reduce the effect of HR-MMS on survival. The 1-year survival in patients who developed grade III to IV acute GVHD improved from 32.1% in the early period to 44.4% in the mid and late time periods. This change may have resulted from the progress in supportive care, including strategies against fungal or viral infections.

Another important finding is that the impact of HR-MM was significantly enhanced by the presence of an additional allele mismatch in the mid and late time periods. This fact may be explained by a hypothesis that the HR-MM biologically increases the graft-versus-host (GVH) reaction, but the recent improvement in GVHD prophylaxis has masked its effect, if HR-MM exists as a single allele mismatch, whereas the adverse impact of HR-MM is not suppressed even by recent methods of GVHD prophylaxis when an additional allele mismatch is present. Based on these findings, interaction terms should be incorporated into the statistical model when the impact of HR-MMs is analyzed in datasets that include HSCT with multiple allele mismatches.

A major limitation of this study is the small number of patients with HR-MMs, especially in the late time period. We cannot deny the possibility that an important effect of HR-MMs might be overlooked because of the poor statistical power. The lack of a significant difference in the incidence of grade III to IV acute GVHD between unrelated HSCT with HR-MMs at the HLA-A/B locus and HSCT with LR-MM should be interpreted with caution, because of the small number of patients. Furthermore, it was impossible to evaluate the effect of each mismatch combination, as the number of patients with each mismatch combination was most often fewer than 10. HR-MMs associated with at least a 20% incidence of grade III to IV acute GVHD in the mid and late periods included A*0206-A*0201 (4 of 14), A*0206-A*0207 (3 of 4), B*1501-B*1507 (1 of 1), C*0801-C*0303 (4 of 15), and C*1402-C*0304 (1 of 5), but the number of patients in each pair was too small to draw any definitive conclusions.

When we consider the impact of HR-MMs, especially at the HLA-C locus, we should also consider the effect of a killer immunoglobulin-like receptor ligand (KIR) mismatch [13,14]. Among the 50 patients with HR-MMs at the HLA-C locus in the mid and late periods, 20 had a KIR mismatch in the GVH direction, whereas 30 did not. The incidence of grade III to IV acute GVHD was 5% and 16.7%, respectively, but this

difference was not statistically significant ($P = .24$). The incidence of grade III to IV acute GVHD in the 21 patients who had LR-MMs and a KIR mismatch in the GVH direction was 15.0%. We could not conclude that a KIR mismatch had an impact in this study because of the small number of patients with a KIR mismatch in the GVH direction.

We should note that the results of the current study are applicable to patients who receive bone marrow graft after a myeloablative conditioning regimen. The impact of HR-MMs may change according to the stem cell source or the conditioning regimen. Therefore, further analyses are required to evaluate the impact of HR-MMs in peripheral blood stem cell transplantation and reduced-intensity conditioning transplantation.

In conclusion, this retrospective study revealed that the clinical impact of HR-MMs became less significant after 2002. Although HR-MMs may have a biological impact, their effect may be controlled by recent methods for GVHD prophylaxis when they exist as a single allele mismatch. It may still be prudent to avoid a donor with HR-MMs, especially at the HLA-A or -B locus, if a donor with the other mismatch combination is available. However, in the absence of MUD or an unrelated donor with a LR-MM, a donor with a single HR-MM could be a viable option for unrelated HSCT, and it is preferred over a donor with 2 LR-MMs. In addition, we should be aware that the clinical impact of risk factors may change over time periods, and therefore, we should repeatedly confirm the validity of risk factors.

ACKNOWLEDGMENTS

Financial disclosure: This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

Conflict of interest statement: There are no conflicts of interest to report.

Authorship statement: Y.K. designed the study. Y.K. and J.K. analyzed the data. Y.A., S.F., Y.M., T.I., M.T., K.O., T.F., K.M., T.M., C.K., N.K., K.I., A.S., and S.M. gathered the data. Y.K. wrote the first draft of the paper and all other authors contributed to the final version.

REFERENCES

1. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood*. 2004;104:1923-1930.
2. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110:4576-4583.
3. Petersdorf EW, Anasetti C, Martin PJ, et al. Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood*. 2004;104:2976-2980.
4. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. *N Engl J Med*. 1998;339:1177-1185.
5. Kanda Y, Kanda J, Atsuta Y, et al. Impact of a single human leucocyte antigen (HLA) allele mismatch on the outcome of unrelated bone marrow transplantation over two time periods. A retrospective analysis of 3003 patients from the HLA Working Group of the Japan Society for Blood and Marrow Transplantation. *Br J Haematol*. 2013;161:566-577.
6. Zino E, Frumento G, Marktel S, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. *Blood*. 2004;103:1417-1424.
7. Kawase T, Morishima Y, Matsuo K, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. *Blood*. 2007;110:2235-2241.
8. Atsuta Y, Suzuki R, Yoshimi A, et al. Unification of hematopoietic stem cell transplantation registries in Japan and establishment of the TRUMP System. *Int J Hematol*. 2007;86:269-274.
9. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood*. 2002;99:4200-4206.
10. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. *Ann Stat*. 1988;16:1141-1154.
11. Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. *J Am Stat Assoc*. 1999;94:456-509.
12. Kanda Y. Investigation of the freely available easy-to-use software "EZR" (Easy R) for medical statistics. *Bone Marrow Transplant*. 2013;48:452-458.
13. Morishima Y, Yabe T, Matsuo K, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. *Biol Blood Marrow Transplant*. 2007;13:315-328.
14. Leung W. Use of NK cell activity in cure by transplant. *Br J Haematol*. 2011;155:14-29.

Bone Marrow Transplantation (2013) **48,** 56–62

www.nature.com/bmt

ORIGINAL ARTICLE

Allogeneic hematopoietic stem cell transplantation for intermediate cytogenetic risk AML in first CR

N Imahashi[1], R Suzuki[2], T Fukuda[3], K Kakihana[4], H Kanamori[5], T Eto[6], T Mori[7], N Kobayashi[8], K Iwato[9], T Sakura[10], K Ikegame[11], M Kurokawa[12], T Kondo[13], H Iida[14], H Sakamaki[4], J Tanaka[15], K Kawa[16], Y Morishima[17], Y Atsuta[2] and K Miyamura[1]

Allogeneic hematopoietic SCT (allo-HCT) from matched sibling donor (MSD) is recommended for younger patients with intermediate cytogenetic risk AML in first CR (CR1), whereas the role of alternative donor transplants in these patients is unknown. We retrospectively analyzed 605 patients with intermediate-risk AML, who received myeloablative allo-HCT in CR1. The 4-year OS for MSD ($n=290$) and matched unrelated donor (MUD; $n=141$) was 65% and 68% ($P=0.50$), respectively. In multivariate analysis, MUD had a similar risk of overall mortality as MSD (hazard ratio = 0.90; 95% confidence interval, 0.62–1.30; $P=0.58$), whereas older age, female donor/male recipient (FDMR) combination, and requiring more than one course of induction chemotherapy to achieve CR1 were poor prognostic factors for OS. Thus, OS after MUD HCT with sex combinations other than FDMR was significantly higher than that after MSD HCT from female donors to male recipients (4-year OS 72% versus 55%, $P=0.04$). These results suggest that HCT, not only from MSD, but also from MUD, should be considered in younger patients with intermediate-risk AML in CR1, and that the donor–recipient sex combination is more important than the donor type in donor selection.

Bone Marrow Transplantation (2013) **48,** 56–62; doi:10.1038/bmt.2012.84; published online 18 June 2012

Keywords: AML; first CR; allogeneic hematopoietic SCT

INTRODUCTION

The current standard treatment strategy for young patients with AML consists of induction chemotherapy and subsequent post-remission therapy. The post-remission therapy includes intensive consolidation chemotherapy and allogeneic hematopoietic SCT (allo-HCT). Although the toxicity of consolidation chemotherapy is relatively low, a substantial proportion of patients relapse, and the risk of relapse depends on cytogenetic risk.[1,2] On the other hand, allo-HCT as a post-remission therapy is associated with the lowest relapse rates. However, this benefit is limited by the high nonrelapse mortality (NRM) and the donor type has a significant impact on NRM.[3] The risk of NRM associated with allo-HCT needs to be balanced with the risk of relapse, and hence, the indication for allo-HCT among patients with AML in the first CR (CR1) depends on the cytogenetic risk and available donor type.[4]

Regarding those patients with favorable cytogenetic risk AML, who achieved CR1, the long-term disease-free survival after intensive consolidation chemotherapy of approximately 60% is reported, and they did not benefit from allo-HCT in CR1.[5–7] Thus, these patients are not considered candidates for allo-HCT in CR1.[8]

As for patients with unfavorable cytogenetic risk AML in CR1, previous prospective studies that assigned allo-HCT versus alternative post-remission therapies, on an intent-to-treat donor versus no-donor basis showed significant disease-free survival and OS benefit with allo-HCT, not only from a matched sibling donor (MSD), but also from a matched unrelated donor (MUD).[5–7,9] Accordingly, allo-HCT in CR1 from MSD or MUD is recommended for unfavorable risk AML.[8]

The indication for allo-HCT in CR1 depends on the available donor type in patients with intermediate cytogenetic risk AML. As meta-analyses of prospective studies showed that allo-HCT in CR1 from MSD offered significant disease-free survival and OS benefit,[5,6] allo-HCT in CR1 from MSD is recommended. In contrast, the indication for allo-HCT from alternative donors among these patients is unknown, because higher NRM may offset therapeutic benefits.[3] Although several studies reported comparable outcome after MUD or MSD transplantation,[10–13] these studies included only a small number of patients with intermediate-risk AML in CR1, and information regarding the outcome of allo-HCT from alternative donors in this group of patients is limited. Collectively, further investigation of the outcome of allo-HCT from alternative donors in patients with intermediate-risk AML in CR1 is warranted. In the present study, we retrospectively analyzed the impact of donor type on

[1]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; [2]Department of Hematopoietic Stem Cell Transplantation Data Management/Biostatistics, Nagoya University Graduate School of Medicine, Nagoya, Japan; [3]Department of Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan; [4]Department of Hematology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan; [5]Department of Hematology, Kanagawa Cancer Center, Yokohama, Japan; [6]Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan; [7]Department of Medicine, Division of Hematology, Keio University School of Medicine, Tokyo, Japan; [8]Department of Hematology, Sapporo Hokuyu Hospital, Sapporo, Japan; [9]Department of Hematology, Hiroshima Red Cross Hospital and Atomic-bomb Survivors Hospital, Hiroshima, Japan; [10]Department of Hematology, Saiseikai Maebashi Hospital, Gunma, Japan; [11]Department of Internal Medicine, Divison of Hematology, Hyogo College of Medicine, Nishinomiya, Japan; [12]Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [13]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [14]Department of Hematology, Meitetsu Hospital, Nagoya, Japan; [15]Department of Hematology, Hokkaido University Hospital, Sapporo, Japan; [16]Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan and [17]Department of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan. Correspondence: Dr N Imahashi, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.
E-mail: nimahashi@med.nagoya-u.ac.jp
Received 31 October 2011; revised 3 April 2012; accepted 10 April 2012; published online 18 June 2012

transplant outcomes among patients with intermediate-risk AML in CR1.

MATERIALS AND METHODS

Collection of data and data source

The recipients' clinical data were provided by the Japan Society for Hematopoietic Cell Transplantation (JSHCT) and the Japan Marrow Donor Program (JMDP). The registry data is managed using the 'Transplant Registry Unified Management Program' system.[14] Both JSHCT and JMDP collect recipients' clinical data at 100 days after allo-HCT. The patient's data on survival, disease status and long-term complications, including chronic GVHD and second malignancies, are renewed annually by follow-up forms. This study was approved by the data management committees of JSHCT. Informed consent was provided according to the Declaration of Helsinki.

Patients

Between January 1996 and December 2008, a total of 682 adult patients aged 16 to 70 years, with intermediate cytogenetic risk AML in CR1, received first BM or PBSC transplantation with myeloablative conditioning regimens. Excluding 66 patients without complete HLA data and 11 patients whose follow-up data were not available, we analyzed 605 patients. Only BM grafts were used in unrelated HCT, because the PBSC donation from unrelated donors was not permitted in Japan. HLA compatibility was determined by serological typing for HLA-A, -B and -DR in related donor (RD) HCT, and by high-resolution typing for HLA-A, -B, -C and -DRB1 in unrelated donor HCT. A MSD was defined as a serologically MSD, whereas other RDs were defined as RDs other than MSD. A MUD was defined as an eight/eight identical unrelated donor, whereas a mismatched unrelated donor (MMUD) was defined as an unrelated donor who had at least one locus mismatch.

Definitions

Neutrophil recovery was defined by an ANC of at least 500 cells per mm^3 for three consecutive points. Acute and chronic GVHD were diagnosed and graded according to defined criteria.[15,16] Relapse was defined as a recurrence of underlying hematological malignant diseases. NRM was defined as death during continuous remission. For OS, failure was death due to any cause, and surviving patients were censored at the last follow-up. The date of transplantation was the starting time point for calculating all outcomes. Cytogenetic risk-group assignment was done according to the Southwest Oncology Group/Eastern Cooperative Oncology Group classification.[2]

Statistical analysis

The two-sided χ^2-test was used for categorical variables, and the two-sided Wilcoxon rank sum test was used for continuous variables. OS was calculated using the Kaplan–Meier method. The log-rank test was used for group comparisons. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of acute and chronic GVHD, relapse and NRM.[17] For GVHD, death without GVHD and relapse were the competing events; for relapse, death without relapse was the competing event; and for NRM, relapse was the competing event. Gray's test was used for group comparison of cumulative incidence.[18] The Cox proportional hazards regression model was used to test the statistical significance of several potential prognostic factors for relapse, NRM and OS. Variables with a significance level less than 0.1 in univariate analysis were entered into multivariable models and sequentially eliminated in a stepwise backward fashion. Each step of model building contained the main effect of donor type. Factors with a significance level less than 0.05 were kept in the final model. The median value was used as a cut-off point for year of transplant. For WBC counts at diagnosis, 50×10^9/L was used as a cut-off point according to the previous report.[10] All *P*-values were two-sided, and *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Characteristics of the patients are summarized in Table 1. Among the 605 patients analyzed, 290 had MSD HCT, 53 had other RD HCT, 141 had MUD HCT and 121 had MMUD HCT. Of 53 patients with other RD, HLA was matched in 14 and mismatched in 39 patients. Of 121 patients with MMUD, 69 were one locus mismatched and 52 were two or more loci mismatched. The median age of patients was 37 (range, 16–59) years, and median time from diagnosis to HCT was 7.43 (range, 0.43–54.3) months. The median follow-up period of survivors was 4.2 (range, 0.1–13) years. The proportions of male patients, normal karyotype, conditioning regimens, including TBI, and BMT were significantly higher, whereas those of M1/M2/M3/M4/M5 FAB classification and CYA-based GVHD prophylaxis were significantly lower in the unrelated HCT than in the related HCT. The time from diagnosis to HCT was longer in the unrelated HCT compared with related HCT. Other characteristics were not significantly different between related and unrelated HCT.

Acute and chronic GVHD

The unadjusted cumulative incidences of grade II–IV acute GVHD for the MSD and MUD HCT were 26% and 25% at 100 days ($P = 0.89$), respectively, and those of grade III–IV acute GVHD were 10% and 7% at 100 days ($P = 0.46$), respectively (Table 2). The unadjusted cumulative incidences of chronic GVHD for the MSD and MUD HCT were 45% and 44% at 2 years ($P = 0.98$), respectively, and those of extensive chronic GVHD were 28% and 23% at 2 years ($P = 0.37$), respectively (Table 2).

Survival

OS rates for the MSD and MUD HCT were 65% and 68% at 4 years, respectively ($P = 0.50$; Table 2, Figure 1a). Univariate analysis of risk factors for overall mortality showed that the following factors were significant at the 0.1 level: patient age $\geqslant 40$ years, female donor/male recipient (FDMR) combination, and requiring more than one course of induction chemotherapy to achieve CR1 (Table 3). In multivariate analysis, MUD was not a significant factor for overall mortality (hazard ratio (HR) = 0.90; 95% confidence interval (CI), 0.62–1.30; $P = 0.58$). Significant factors for overall mortality were patient age $\geqslant 40$ years (HR = 1.55; 95% CI, 1.17–2.06; $P < 0.01$), FDMR combination (HR = 1.42; 95% CI, 1.03–1.95; $P = 0.03$) and requiring more than one course of induction chemotherapy to achieve CR1 (HR = 1.81; 95% CI, 1.36–2.41; $P < 0.01$) (Table 4). As the donor–recipient sex combination, but not donor type, was a significant factor for overall mortality, OS after MUD HCT with sex combinations other than FDMR was significantly higher than that after MSD HCT from female donors to male recipients (4-year OS 72% versus 55%, $P = 0.04$) (Figure 1b).

Nonrelapse mortality

The cumulative incidences of NRM for the MSD and MUD HCT were 17% and 19% at 4 years, respectively ($P = 0.52$) (Table 2, Figure 2a). Univariate analysis of risk factors for NRM showed that the following factors were significant at the 0.1 level: patient age $\geqslant 40$ years, FDMR combination and MMUD (Table 3). In multivariate analysis, MUD HCT was not a significant factor for NRM compared with MSD HCT (HR = 1.26; 95% CI, 0.77–2.06; $P = 0.35$; Table 4). Significant factors for higher NRM were patient age $\geqslant 40$ years (HR = 1.71; 95% CI, 1.17–2.50; $P < 0.01$), FDMR combination (HR = 1.68; 95% CI, 1.12–2.52; $P = 0.01$) and MMUD (HR = 1.83; 95% CI, 1.16–2.86; $P < 0.01$).

Relapse

The cumulative incidences of relapse for the MSD and MUD HCT were 24% and 19% at 4 years, respectively ($P = 0.25$; Table 2, Figure 2b). Univariate analysis of risk factors for relapse showed that the following factors were significant at the 0.1 level: longer interval between diagnosis and transplantation, peripheral blood

Table 1. Patient characteristics

Characteristics	*MSD*	*Other RD*	*MUD*	*MMUD*	P-*values*[a]
No. of patients	290	53	141	121	
Median patient age at HCT, years	39	36	35	37	0.09
Range	16–58	17–58	16–59	16–59	
Patient sex, n *(%)*					0.02
Male	155 (53)	24 (45)	86 (61)	75 (62)	
Female	135 (47)	29 (55)	55 (39)	46 (38)	
Sex matching, n *(%)*					0.61
Others	202 (77)	45 (87)	112 (79)	98 (81)	
Female to male	61 (23)	7 (13)	29 (21)	23 (19)	
Not available	27	1	0	0	
FAB classification, n *(%)*					<0.01
M1–M5	227 (82)	39 (80)	90 (70)	83 (74)	
M0, M6, M7	51 (18)	10 (20)	39 (30)	29 (26)	
Others, not available	12	4	12	9	
Prior myelodysplastic syndrome, n *(%)*					0.52
No	279 (97)	49 (92)	134 (98)	116 (96)	
Yes	10 (3)	4 (8)	3 (2)	5 (4)	
Not available	1	0	4	0	
Cytogenetics, n *(%)*					0.03
Normal	272 (94)	49 (92)	138 (98)	117 (97)	
+8, +6, -Y, del(12p)	18 (6)	4 (8)	3 (2)	4 (3)	
Conditioning regimen					<0.01[b]
CY + TBI	94 (32)	25 (47)	65 (46)	64 (53)	
CY + CA + TBI	40 (14)	3 (6)	18 (13)	10 (8)	
CY + BU + TBI	12 (4)	1 (2)	13 (9)	5 (4)	
Other TBI regimen	36 (12)	8 (15)	12 (9)	16 (13)	
BU + CY	102 (35)	12 (23)	31 (22)	17 (14)	
Other non-TBI regimen	6 (2)	4 (8)	2 (1)	9(7)	
GVHD prophylaxis, n *(%)*					<0.01[c]
CsA-based	268 (94)	29 (55)	55 (39)	40 (34)	
FK-based	9 (3)	21 (40)	79 (56)	69 (59)	
Others[d]	9 (3)	3 (6)	7 (5)	8 (9)	
Not available	4	0	0	4	
Time from diagnosis to HCT[e]					
Median	5.79	7.60	8.62	10.2	<0.01
Range	0.43–47.6	2.83–27.6	2.50–54.3	3.49–27.7	
<6 months	153 (54)	17 (33)	20 (14)	10 (8)	<0.01
6 to < 9 months	97 (34)	21 (41)	53 (38)	35 (29)	
9 months or longer	34 (12)	13 (25)	68 (48)	75 (63)	
Not available	6	2	0	1	
Year of transplant, n *(%)*					0.76
1996–2003	156 (54)	23 (43)	74 (52)	66 (55)	
2004–2008	134 (46)	30 (57)	67 (48)	55 (45)	
Stem cell source, n *(%)*					<0.01
BM	175 (60)	33 (62)	141 (100)	121 (100)	
Peripheral blood	115 (40)	20 (38)	0 (0)	0 (0)	
WBC counts at diagnosis, $\times 10^9$*/L*					
<50	196 (71)	36 (75)	108 (79)	82 (75)	0.14
⩾50	79 (29)	12 (25)	29 (21)	27 (25)	
Not available	15	5	4	12	
No. of induction courses to achieve CR, n *(%)*					0.43
1	187 (68)	31 (62)	88 (67)	68 (60)	
⩾2	88 (32)	19 (38)	43 (33)	45 (40)	
Not available	15	3	10	8	

Abbreviations: CA = cytarabine; FK = tacrolimus; HCT = hematopoietic SCT; MMUD = mismatched unrelated donor; MSD = matched sibling donor; MUD = matched unrelated donor; RD = related donor. [a]*P*-value between related and unrelated donors. [b]*P*-value between TBI regimen and non-TBI regimen. [c]*P*-value between CsA-based prophylaxis and FK-based prophylaxis. [d]Others include T-cell depletion. [e]The median time from diagnosis to transplant was 7.43 months for the whole group.

Table 2. Clinical outcomes

	MSD	*Other RD*		*MUD*		*MMUD*	
	% (95% CI)	*% (95% CI)*	*P-values*[a]	*% (95% CI)*	*P-values*[a]	*% (95% CI)*	*P-values*[a]
Acute GVHD, grades II–IV at 100 days	26 (21–31)	38 (25–51)	0.04	25 (18–32)	0.89	51 (42–59)	<0.01
Acute GVHD, grades III–IV at 100 days	10 (6–13)	15 (7–26)	0.19	7 (4–12)	0.46	14 (9–21)	0.16
Chronic GVHD at 2 years	45 (39–51)	48 (33–62)	0.75	44 (35–53)	0.98	41 (32–51)	0.55
Extensive chronic GVHD at 2 years	28 (23–34)	31 (18–44)	0.73	23 (16–31)	0.37	23 (15–31)	0.25
OS at 4 years	65 (59–71)	53 (37–68)	0.26	68 (59–76)	0.50	61 (51–70)	0.25
Nonrelapse mortality at 4 years	17 (12–22)	18 (9–30)	0.73	19 (13–27)	0.52	25 (18–34)	<0.01
Relapse at 4 years	24 (19–29)	29 (17–42)	0.45	19 (13–27)	0.25	12 (7–19)	0.02

Abbreviations: CI = confidence interval; MSD = matched sibling donor; RD = related donor; MUD = matched unrelated donor; MMUD = mismatched unrelated donor. [a]*P*-values for comparison with MSD.

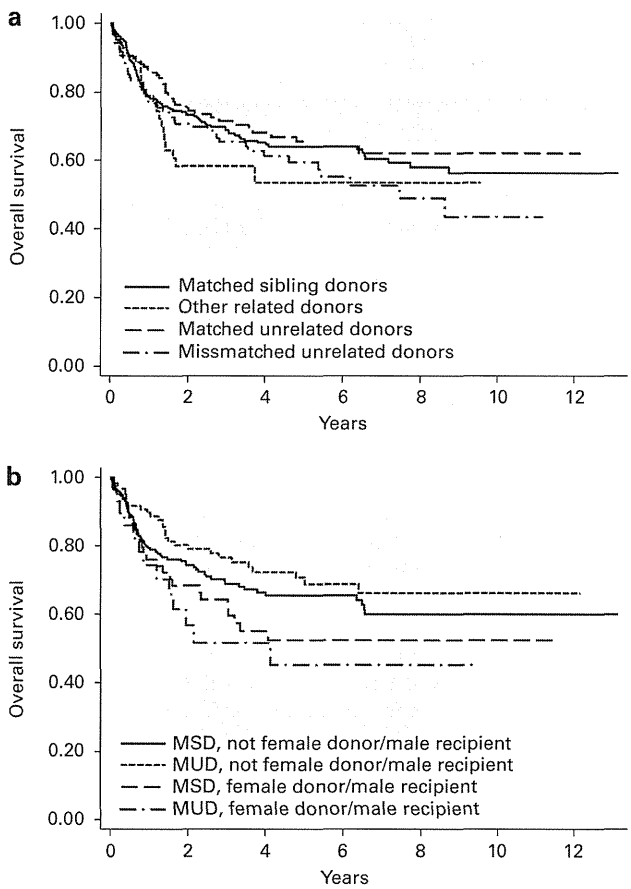

Figure 1. OS. (**a**) Comparison of MSD, other RD, MUD and MMUD transplantation. (**b**) Comparison according to the donor–recipient sex combination and donor type among patients with MSD and MUD.

as stem cell source, WBC counts at diagnosis $\geqslant 50 \times 10^9$/L, requiring more than one course of induction chemotherapy to achieve CR1, and MMUD (Table 3). In multivariate analysis, MUD HCT was not a significant factor for relapse compared with MSD HCT (HR = 0.98; 95% CI, 0.58–1.64; $P = 0.93$; Table 4). Significant factors for relapse were WBC counts at diagnosis $\geqslant 50 \times 10^9$/L (HR = 1.77; 95% CI, 1.20–2.63; $P < 0.01$) and requiring more than one course of induction chemotherapy to achieve CR1 (HR = 2.24; 95% CI, 1.54–3.27; $P < 0.01$), and 9 months or longer interval between diagnosis and transplantation (HR = 0.56; 95% CI, 0.32–0.98; $P = 0.04$).

DISCUSSION

We retrospectively analyzed the impact of donor type on transplant outcomes among patients with intermediate-risk AML in CR1. We observed comparable survival after MSD or MUD HCT, but the donor–recipient sex combination had a significant impact on transplant outcomes. The prognosis of older patients was poorer than that of younger patients because of higher NRM. These findings have important implications for the treatment of intermediate-risk AML in CR1.

The prognosis of younger patients with intermediate-risk AML could be improved by performing allo-HCT in CR1 when MSD is available.[5,6] On the other hand, it is unknown whether these patients without MSD may benefit from alternative donor transplantation, because higher NRM associated with alternative donor transplantation may offset therapeutic benefits.[3] In our study, NRM for a MUD HCT was 19% at 4 years, which was similar to that for a MSD HCT and appeared acceptable. The comparable outcomes after a MSD or a MUD HCT observed in our study suggest that HCT, not only from MSD, but also from MUD, should be considered in younger patients with intermediate-risk AML in CR1.

The FDMR combination had a crucial negative impact on transplant outcome in the present study, whereas it had no or a modest effect on transplant outcome in other studies.[19–21] We suggest two possible explanations for this discrepancy. First, it has been reported that the negative effect of the FDMR combination on survival was more pronounced in the standard-risk disease group than in the high-risk disease group, because the negative impact of the FDMR combination on NRM was stronger in the former than in the latter group, whereas the GVL effect associated with the FDMR combination becomes less important in the standard-risk disease group.[21,22] In the current study, subjects were restricted to patients with intermediate-risk AML in CR1. This may have resulted in a pronounced impact of the FDMR combination on transplant outcome in the current study. Second, as the impact of the FDMR combination on NRM is reported to be at least partially independent from that of GVHD on NRM,[21] and Japanese patients have lower incidence of GVHD,[23] the impact of sex combination on transplant outcome may be more evident in the Japanese than in the western populations.[22] The results of the present study suggest that the donor–recipient sex combination is a more important factor than the donor type in donor selection, in a certain subgroup of patients. As this may alter the current strategies in donor selection, verification in future studies is warranted.

Regarding older patients with intermediate-risk AML, a recent retrospective study showed that patients who underwent allo-HCT in CR1 had better survival than those who were treated with conventional chemotherapy alone, because the latter patients were associated with high relapse rates.[24] On the other hand, previous prospective studies, including patients with AML of all

Table 3. Univariate analysis of OS, nonrelapse mortality and relapse

Variables	N	*OS*		*NRM*		*Relapse*	
		HR (95% CI)	*P-values*	*HR (95% CI)*	*P-values*	*HR (95% CI)*	*P-values*
Patient age							
20–39	290	1.00		1.00		1.00	
<20	45	0.83 (0.47–1.46)	0.52	0.67 (0.29–1.57)	0.36	1.05 (0.53–2.06)	0.89
⩾40	270	1.47 (1.11–1.95)	<0.01	1.65 (1.14–2.41)	<0.01	1.13 (0.78–1.65)	0.52
Sex matching							
Others	457	1.00		1.00		1.00	
Female to male	120	1.39 (1.01–1.91)	0.04	1.68 (1.12–2.53)	0.01	0.80 (0.49–1.31)	0.38
FAB classification							
M1–M5	439	1.00		1.00		1.00	
M0, M6, M7	129	0.89 (0.63–1.25)	0.51	1.01 (0.65–1.56)	0.97	0.87 (0.56–1.37)	0.55
Prior MDS							
No	578	1.00		1.00		1.00	
Yes	22	0.67 (0.28–1.64)	0.39	0.46 (0.11–1.86)	0.28	0.70 (0.22–2.19)	0.54
Cytogenetics							
Normal	576	1.00		1.00		1.00	
+8, +6, -Y, del(12p)	29	0.72 (0.35–1.46)	0.36	1.11 (0.52–2.38)	0.80	0.31 (0.08–1.25)	0.10
TBI							
Yes	422	1.00		1.00		1.00	
No	183	1.06 (0.80–1.42)	0.68	1.01 (0.69–1.50)	0.94	1.01 (0.68–1.49)	0.97
GVHD prophylaxis							
CsA-based	392	1.00		1.00		1.00	
FK-based	178	1.13 (0.84–1.53)	0.42	1.14 (0.77–1.71)	0.51	1.10 (0.73–1.64)	0.65
Others	27	1.19 (0.63–2.27)	0.59	1.06 (0.43–2.63)	0.89	1.48 (0.68–3.20)	0.32
Time from diagnosis to HCT							
< 6 months	200	1.00		1.00		1.00	
6 to <9 months	206	0.86 (0.62–1.20)	0.37	0.92 (0.58–1.48)	0.74	0.77 (0.51–1.17)	0.23
9 months or longer	190	0.88 (0.63–1.22)	0.45	1.26 (0.81–1.96)	0.31	0.48 (0.29–0.77)	<0.01
Year of transplant							
2004–2008	286	1.00		1.00		1.00	
1996–2003	319	0.91 (0.69–1.21)	0.53	1.08 (0.73–1.59)	0.69	0.83 (0.57–1.19)	0.31
Stem cell source							
BM	470	1.00		1.00		1.00	
Peripheral blood	135	1.08 (0.78–1.49)	0.64	0.76 (0.47–1.23)	0.27	1.64 (1.11–2.42)	0.01
WBC counts at diagnosis							
$<50 \times 10^9$/L	422	1.00		1.00		1.00	
$\geqslant 50 \times 10^9$/L	147	1.15 (0.84–1.57)	0.38	0.77 (0.49–1.24)	0.28	1.86 (1.27–2.74)	<0.01
No. of induction courses							
1	374	1.00		1.00		1.00	
⩾2	195	1.76 (1.32–2.33)	<0.01	1.36 (0.92–2.01)	0.12	2.25 (1.55–3.26)	<0.01
Donor							
MSD	290	1.00		1.00		1.00	
Other RD	53	1.34 (0.84–2.15)	0.23	1.17 (0.58–2.39)	0.66	1.31 (0.73–2.33)	0.36
MUD	141	0.88 (0.61–1.26)	0.49	1.12 (0.69–1.79)	0.65	0.77 (0.48–1.23)	0.28
MMUD	121	1.21 (0.86–1.71)	0.27	1.73 (1.11–2.67)	0.02	0.56 (0.32–0.99)	0.046

Abbreviations: CI = confidence interval; FK = tacrolimus; HCT = hematopoietic SCT; HR = hazard ratio; MDS = myelodysplastic syndrome; MSD = matched sibling donor; MMUD = mismatched unrelated donor; MUD = matched unrelated donor; NRM = nonrelapse mortality; RD = related donor.

cytogenetic risk groups, showed that the beneficial effect of allo-HCT in CR1 on OS was absent in patients older than 35–40 years, because the benefits of the reduced relapse rate were offset by a higher NRM.[6,25] In accordance with these prospective studies, older patients had higher NRM and overall mortality than younger patients in the current study. Our study revealed that a substantial number of older patients received allo-HCT in CR1, but the results of our study and others indicate that prospective studies to evaluate the efficacy of allo-HCT in CR1 for older patients with intermediate-risk AML are necessary before it becomes a general practice.

The proportion of patients who received TBI regimens tended to be lower in the older patients than in the younger patients in the current study (data not shown), perhaps in an attempt to

Table 4. Significant factors in multivariate analysis for OS, nonrelapse mortality and relapse

Variables	N	*OS*		*NRM*		*Relapse*	
		HR (95% CI)	P-*values*	*HR (95% CI)*	P-*values*	*HR (95% CI)*	P-*values*
Patient age							
20–39	290	1.00		1.00		—	—
<40	45	0.85 (0.48–1.50)	0.58	0.67 (0.28–1.57)	0.35	—	—
⩾40	270	1.55 (1.17–2.06)	<0.01	1.71 (1.17–2.50)	<0.01	—	—
Sex matching							
Others	457	1.00		1.00		—	—
Female to male	120	1.42 (1.03–1.95)	0.03	1.68 (1.12–2.52)	0.01	—	—
WBC counts at diagnosis							
$<50 \times 10^9$/L	422	—	—	—	—	1.00	
$\geqslant 50 \times 10^9$/L	147	—	—	—	—	1.77 (1.20–2.63)	<0.01
No. of induction courses							
1	374	1.00	—	—	—	1.00	
⩾2	195	1.81 (1.36–2.41)	<0.01	—	—	2.24 (1.54–3.27)	<0.01
Time from diagnosis to HCT							
<6 months	200	—	—	—	—	1.00	
6 to <9 months	206	—	—	—	—	0.85 (0.55–1.31)	0.45
9 months or longer	190	—	—	—	—	0.56 (0.32–0.98)	0.04
Donor							
MSD	290	1.00		1.00		1.00	
Other RD	53	1.35 (0.84–2.18)	0.21	1.31 (0.64–2.68)	0.47	1.44 (0.80–2.61)	0.22
MUD	141	0.90 (0.62–1.30)	0.58	1.26 (0.77–2.06)	0.35	0.98 (0.58–1.64)	0.93
MMUD	121	1.17 (0.83–1.67)	0.37	1.83 (1.16–2.86)	<0.01	0.71 (0.38–1.32)	0.28

Abbreviations: CI = confidence interval; HCT = hematopoietic SCT; HR = hazard ratio; MMUD = mismatched unrelated donor; MSD = matched sibling donor; MUD = matched unrelated donor; NRM = nonrelapse mortality; RD = related donor.

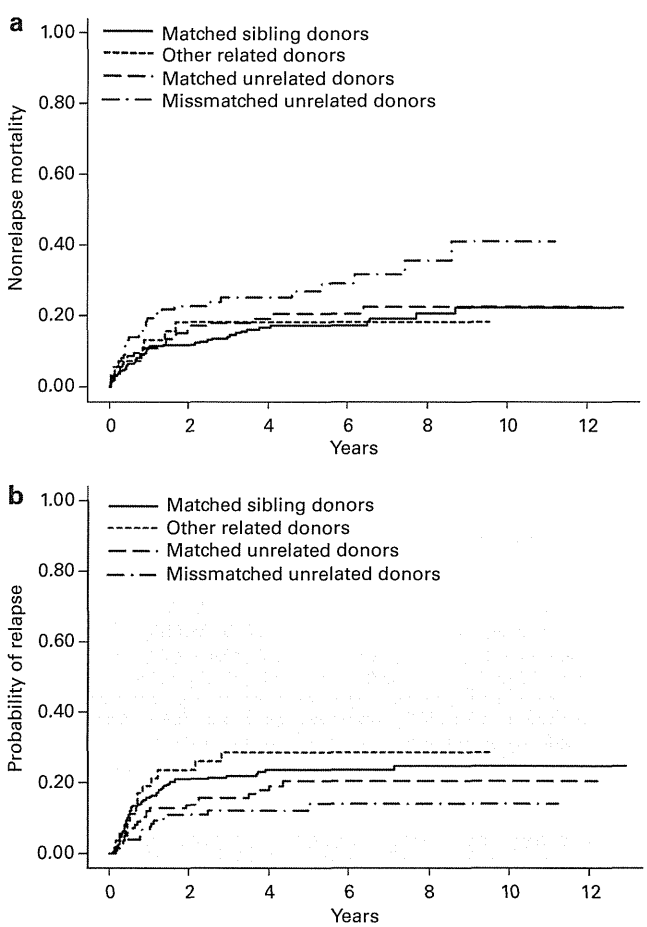

Figure 2. Comparison of MSD, other RD, MUD, and MMUD transplantation. (**a**) Cumulative incidence of NRM. (**b**) Cumulative incidence of relapse.

reduce toxicity. However, there was no significant difference in NRM between TBI and non-TBI regimens among older patients (data not shown). Recently, reduced toxicity myeloablative regimens, such as the combination of fludarabine with myeloablative doses of BU, were developed with an aim to decrease toxicity without compromising antileukemic effects.[26] These regimens might be beneficial for older patients, especially for those with standard-risk disease.[27] The optimal conditioning regimens for older patients need to be determined in the future studies.

OS after other RD and MMUD HCT did not differ significantly from that after MSD HCT in the current study, but these results need to be interpreted with caution. First, the small number of patients with other RD limited the power to detect significant differences in survival between MSD and other RD HCT. Second, other RD and MMUD included donors with various degrees of HLA incompatibilities. Thus, it is difficult to draw firm conclusions regarding the role of other RD and MMUD HCT from this study. Nonetheless, considering that other RD and MMUD HCT yielded a 4-year OS of 53% and 61%, respectively, allo-HCT from these donors might be an option for patients with unfavorable features. For example, as patients who required more than one course of induction therapy to achieve CR1 have poor outcomes with conventional chemotherapy,[8] they might benefit from allo-HCT from other RD or MMUD, when MSD and MUD are not available.

Our study has several limitations. First, this is a non-randomized, retrospective observational study using registry data, which would allow for the introduction of bias. To minimize bias, we conducted multivariate analyses to adjust for baseline differences. However, some factors which might have influenced transplant outcomes (such as performance score and extramedullary disease) could not be included in the Cox proportional hazards regression model due to a high frequency of missing values. Second, a time-censoring effect might have influenced the results.[28] Patients who undergo transplantation late after achievement of CR may be at a lower risk of relapse, by virtue of having remained in remission a time long enough for a transplantation to be performed.[28] This effect might have favorably affected the outcome of unrelated donor HCT. However, there was no significant difference in OS between MSD

and MUD HCT, even when the time from diagnosis to transplantation was included in the final model of multivariate analyses (data not shown). Third, although the role of allo-HCT according to genetic mutations, such as *FLT3-ITD*, *NPM1* and *CEBPA*, is now being explored,[29] the information about these mutations was not available and this was beyond the scope of the present study. However, the results of our study do support the inclusion of not only MSD HCT, but also MUD HCT, in the prospective studies, which evaluate the role of allo-HCT according to these genetic mutations.

In conclusion, the results of our study suggest that HCT, not only from MSD, but also from MUD ,should be considered in younger patients with intermediate-risk AML in CR1, and that the donor–recipient sex combination is more important than the donor type in donor selection. Prospective studies to evaluate the role of allo-HCT in CR1 for older patients are warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Health, Labor and Welfare of Japan Grant-in-Aid (KM). We thank all of the staff of the participating institutions of the Japan Society for Hematopoietic Cell Transplantation and the Japan Donor Marrow Program. We thank Dr Y Kuwatsuka for thoughtful discussion.

REFERENCES

1 Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G *et al.* The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* 1998; **92**: 2322–2333.
2 Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A *et al.* Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood* 2000; **96**: 4075–4083.
3 Ringden O, Pavletic SZ, Anasetti C, Barrett AJ, Wang T, Wang D *et al.* The graft-versus-leukemia effect using matched unrelated donors is not superior to HLA-identical siblings for hematopoietic stem cell transplantation. *Blood* 2009; **113**: 3110–3118.
4 Rowe JM. Optimal induction and post-remission therapy for AML in first remission. *Hematol Am Soc Hematol Educ Program* 2009 396–405.
5 Koreth J, Schlenk R, Kopecky KJ, Honda S, Sierra J, Djulbegovic BJ *et al.* Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA* 2009; **301**: 2349–2361.
6 Cornelissen JJ, van Putten WL, Verdonck LF, Theobald M, Jacky E, Daenen SM *et al.* Results of a HOVON/SAKK donor versus no-donor analysis of myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: benefits for whom? *Blood* 2007; **109**: 3658–3666.
7 Suciu S, Mandelli F, de Witte T, Zittoun R, Gallo E, Labar B *et al.* Allogeneic compared with autologous stem cell transplantation in the treatment of patients younger than 46 years with acute myeloid leukemia (AML) in first complete remission (CR1): an intention-to-treat analysis of the EORTC/GIMEMAAML-10 trial. *Blood* 2003; **102**: 1232–1240.
8 Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK *et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European Leukemia Net. *Blood* 2010; **115**: 453–474.
9 Schlenk RF, Dohner K, Mack S, Stoppel M, Kiraly F, Gotze K *et al.* Prospective evaluation of allogeneic hematopoietic stem-cell transplantation from matched related and matched unrelated donors in younger adults with high-risk acute myeloid leukemia: German-Austrian trial AMLHD98A. *J Clin Oncol* 2010; **28**: 4642–4648.
10 Gupta V, Tallman MS, He W, Logan BR, Copelan E, Gale RP *et al.* Comparable survival after HLA-well-matched unrelated or matched sibling donor transplantation for acute myeloid leukemia in first remission with unfavorable cytogenetics at diagnosis. *Blood* 2010; **116**: 1839–1848.
11 Walter RB, Pagel JM, Gooley TA, Petersdorf EW, Sorror ML, Woolfrey AE *et al.* Comparison of matched unrelated and matched related donor myeloablative hematopoietic cell transplantation for adults with acute myeloid leukemia in first remission. *Leukemia* 2010; **24**: 1276–1282.
12 Schetelig J, Bornhauser M, Schmid C, Hertenstein B, Schwerdtfeger R, Martin H *et al.* Matched unrelated or matched sibling donors result in comparable survival after allogeneic stem-cell transplantation in elderly patients with acute myeloid leukemia: a report from the cooperative German Transplant Study Group. *J Clin Oncol* 2008; **26**: 5183–5191.
13 Moore J, Nivison-Smith I, Goh K, Ma D, Bradstock K, Szer J *et al.* Equivalent survival for sibling and unrelated donor allogeneic stem cell transplantation for acute myelogenous leukemia. *Biol Blood Marrow Transplant* 2007; **13**: 601–607.
14 Atsuta Y, Suzuki R, Yoshimi A, Gondo H, Tanaka J, Hiraoka A *et al.* Unification of hematopoietic stem cell transplantation registries in Japan and establishment of the TRUMP System. *Int J Hematol* 2007; **86**: 269–274.
15 Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J *et al.* Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant* 1995; **15**: 825–828.
16 Sullivan KM, Shulman HM, Storb R, Weiden PL, Witherspoon RP, McDonald GB *et al.* Chronic graft-versus-host disease in 52 patients: adverse natural course and successful treatment with combination immunosuppression. *Blood* 1981; **57**: 267–276.
17 Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med* 1999; **18**: 695–706.
18 Scrucca L, Santucci A, Aversa F. Competing risk analysis using R: an easy guide for clinicians. *Bone Marrow Transplant* 2007; **40**: 381–387.
19 Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M *et al.* High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood* 2007; **110**: 4576–4583.
20 Randolph SS, Gooley TA, Warren EH, Appelbaum FR, Riddell SR. Female donors contribute to a selective graft-versus-leukemia effect in male recipients of HLA-matched, related hematopoietic stem cell transplants. *Blood* 2004; **103**: 347–352.
21 Stern M, Brand R, de Witte T, Sureda A, Rocha V, Passweg J *et al.* Female-versus-male alloreactivity as a model for minor histocompatibility antigens in hematopoietic stem cell transplantation. *Am J Transplant* 2008; **8**: 2149–2157.
22 Nannya Y, Kataoka K, Hangaishi A, Imai Y, Takahashi T, Kurokawa M. The negative impact of female donor/male recipient combination in allogeneic hematopoietic stem cell transplantation depends on disease risk. *Transpl Int* 2011; **24**: 469–476.
23 Oh H, Loberiza Jr FR, Zhang MJ, Ringden O, Akiyama H, Asai T *et al.* Comparison of graft-versus-host-disease and survival after HLA-identical sibling bone marrow transplantation in ethnic populations. *Blood* 2005; **105**: 1408–1416.
24 Kurosawa S, Yamaguchi T, Uchida N, Miyawaki S, Usuki K, Watanabe M *et al.* Comparison of allogeneic hematopoietic cell transplantation and chemotherapy in elderly patients with non-M3 acute myelogenous leukemia in first complete remission. *Biol Blood Marrow Transplant* 2011; **17**: 401–411.
25 Burnett AK, Wheatley K, Goldstone AH, Stevens RF, Hann IM, Rees JH *et al.* The value of allogeneic bone marrow transplant in patients with acute myeloid leukaemia at differing risk of relapse: results of the UK MRC AML 10 trial. *Br J Haematol* 2002; **118**: 385–400.
26 de Lima M, Couriel D, Thall PF, Wang X, Madden T, Jones R *et al.* Once-daily intravenous busulfan and fludarabine: clinical and pharmacokinetic results of a myeloablative, reduced-toxicity conditioning regimen for allogeneic stem cell transplantation in AML and MDS. *Blood* 2004; **104**: 857–864.
27 Russell JA, Duan Q, Chaudhry MA, Savoie ML, Balogh A, Turner AR *et al.* Transplantation from matched siblings using once-daily intravenous busulfan/fludarabine with thymoglobulin: a myeloablative regimen with low nonrelapse mortality in all but older patients with high-risk disease. *Biol Blood Marrow Transplant* 2008; **14**: 888–895.
28 Bonetti F, Zecca M, Pession A, Messina C, Montagna D, Lanino E *et al.* Total-body irradiation and melphalan is a safe and effective conditioning regimen for autologous bone marrow transplantation in children with acute myeloid leukemia in first remission. The Italian Association for Pediatric Hematology and Oncology-Bone Marrow Transplantation Group. *J Clin Oncol* 1999; **17**: 3729–3735.
29 Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L *et al.* Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med* 2008; **358**: 1909–1918.

Biol Blood Marrow Transplant 20 (2014) 495–500

Bone Marrow Graft-versus-Host Disease: Evaluation of Its Clinical Impact on Disrupted Hematopoiesis after Allogeneic Hematopoietic Stem Cell Transplantation

Yusuke Shono [1,*,†], Souichi Shiratori [1], Mizuha Kosugi-Kanaya [1], Satoshi Ueha [2], Junichi Sugita [1], Akio Shigematsu [1], Takeshi Kondo [1], Daigo Hashimoto [1], Katsuya Fujimoto [1], Tomoyuki Endo [1], Mitsufumi Nishio [1], Satoshi Hashino [1], Yoshihiro Matsuno [3], Kouji Matsushima [2], Junji Tanaka [4], Masahiro Imamura [5], Takanori Teshima [1]

[1] *Department of Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan*
[2] *Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[3] *Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan*
[4] *Department of Hematology, Tokyo Women's Medical University, Tokyo, Japan*
[5] *Department of Hematology, Sapporo Hokuyu Hospital, Sapporo, Japan*

Article history:
Received 4 November 2013
Accepted 20 December 2013

Key Words:
Bone marrow graft-versus-host disease (BM GVHD)
Idiopathic cytopenias
Osteoblasts

A B S T R A C T

Idiopathic cytopenias are frequently observed in patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). We have previously reported the effect of graft-versus-host disease (GVHD) on bone marrow (BM) in murine models, indicating that the osteoblast injury mediated by donor T cells was associated with bone marrow suppression and delayed immune reconstitution. In this study, we prospectively evaluated the relevance of these findings in 51 patients. Patients with chronic GVHD manifested the loss of osteoblasts, contributing to cytopenic symptoms ($P = .0427$ compared with patients without cytopenic symptoms). The loss of osteoblasts was significantly associated with the extensive type of chronic GVHD ($P = .012$), and flow cytometric analyses revealed lower numbers of $CD19^+$ B cells and a significantly increased CD4 to CD8 ratio ($P = .0002$) in these patients. Our data, for the first time to our knowledge, summarize the detailed analyses of the effect of GVHD on BM in the clinical allo-HSCT patients.

INTRODUCTION

Allogeneic stem cell transplantation (allo-HSCT) is currently established as a curative therapy for hematologic malignancies. However, graft-versus-host disease (GVHD) still remains a major complication after allo-HSCT and, therefore, developing better strategies for the prophylaxis and treatment of GVHD is essential to improve outcomes of allo-HSCT. The principal target organs of acute GVHD are the skin, liver, and gastrointestinal tract [1]. However, cytopenias and bone marrow (BM) suppression are often observed in association with GVHD in patients undergoing allo-HSCT, suggesting that BM is a potential target of GVHD. Clinical and experimental data have shown that immunologic reconstitution is impaired by GVHD [2-5], and GVHD-associated myelosuppression and lymphoid hypoplasia have been reported [6-8]. Recently, we demonstrated the destruction of BM hematopoietic niches, especially osteoblasts, by donor T cells in murine models of GVHD, resulting in BM suppression, including B lymphopoiesis. We identified this phenomenon as *BM GVHD* [9]. Here, we report clinical research investigating BM GVHD in patients after allo-HSCT. We analyzed 51 patients undergoing allo-HSCT who were evaluable with BM biopsy samples both before and after allo-HSCT.

METHODS

Study Design and Patients

For our prospective analyses of BM GVHD, we enrolled 57 patients who underwent allo-HSCT from February 2010 to June 2012 in Hokkaido University Hospital. A total of 51 patients were assessed for BM biopsy specimens before and after allo-HSCT (6 patients who did not have BM biopsies at all after allo-HSCT were excluded). The study protocol was approved by the review board of Hokkaido University Graduate School of Medicine on January 29, 2010. Patients provided written informed consent before being enrolled in the protocol. Characteristics of patients, as well as of the transplantation procedures, are summarized in Table 1.

Evaluation of GVHD

Diagnosis and clinical grading of acute and chronic GVHD were performed according to established criteria [10-12].

Bone Marrow Samples

We performed BM biopsies and aspirations for patients before and after allo-HSCT. BM aspirates were analyzed for B and T cell profiles by flow cytometry. Biopsied specimens were stained with hematoxylin and eosin, as well as with CD56 for immunohistochemical assessments of cellularity, morphology, and presence or absence of osteoblasts [13]. We categorized the loss of osteoblasts into 3 groups: (1) not affected, if the osteoblasts were intact or the decrease was moderate, up to 30%; (2) partial loss, if the

Financial disclosure: See Acknowledgments on page 499.

* Correspondence and reprint requests: Yusuke Shono, Department of Hematology, Hokkaido University Graduate School of Medicine, Kita-15 Nishi-7, Kita-ku, Sapporo, Hokkaido, Japan.
E-mail address: yusuke@med.hokudai.ac.jp (Y. Shono).
Yusuke Shono and Souichi Shiratori contributed equally to this work.
† Current affiliation: Yusuke Shono: Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, New York.

1083-8791/$ — see front matter © 2014 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2013.12.568

Table 1
Characteristics of Patients

Characteristics	Value
No. of transplantations	51
Age, median (range), yr	41 (19-66)
Patient sex	
Male	27 (53%)
Female	24 (47%)
Disease	
AML	16 (31%)
MDS	5 (10%)
ALL/LBL	12 (24%)
ML	9 (18%)
ATL	3 (6%)
AA	3 (6%)
Others	3 (6%)
Donor sources	
U-BMT	28 (55%)
R-PBSCT	8 (16%)
R-BMT	5 (10%)
CBT	10 (20%)
Preparative regimen	
CST	20 (39%)
RIST	31 (61%)
Immuno suppression	
CyA based	13 (25%)
FK based	38 (75%)

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndrome; ALL/LBL, acute lymphocytic/lymphoblastic lymphoma; ML, malignant lymphoma; ATL, acute T cell leukemia; AA, aplastic anemia; U-BMT, unrelated bone marrow transplantation; R-PBSCT, related peripheral blood stem cell transplantation; R-BMT, related bone marrow transplantation; CBT, cord blood transplantation; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; CyA, cyclosporin A; FK, tacrolimus.
Data presented are n (%) unless otherwise indicated.

osteoblasts were partially lost, in between 30% to 90% of the bone trabeculae in the pathological sections; and (3) complete loss, if more than 90% of osteoblasts were lost.

Assessment of Cytopenias

We identified the cytopenic condition as *idiopathic cytopenias* after excluding the following conditions. We excluded bacterial, fungal, and viral infections by routine screening tests (serological as well as culture tests). Additionally, thrombo microangiopathy and hemophagocytic syndrome that also cause cytopenias in patients were excluded. Insufficient hematopoiesis after engraftment was also excluded when the patient showed recovery (confirmed retrospectively) in hematopoiesis without any specific treatment for the cytopenias.

Statistical Analysis

Median values and ranges were used for continuous variables and percentages were used for categorical variables (Table 1). Gray's test was used for group comparisons of cumulative incidences of acute and chronic GVHD. Statistical analyses were performed using chi-square test and *t*-test, as appropriate. JMP software version 8.0.2 (SAS Institute, Cary, NC) was used for most of the statistical analyses. Analysis of cumulative incidences was carried out with EZR (Saitama Medical Center, Jichi Medical University, http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) [14]. All *P* values were 2 sided and a value of $P = .05$ was used as a cut off for statistical significance.

RESULTS

Patients' Characteristics, Sample Collections, and Acute and Chronic GVHD after Allo-HSCT

The patients' characteristics are shown in Table 1. The median age at allo-HSCT for the 27 males and 24 females was 41 years (range, 19 to 66). Of 51 patients analyzed, 32 developed acute GVHD (Figure 1A) and 29 developed chronic GVHD (Figure 1B). BM biopsies were performed before (median, day −22; range, day −174 to day −8) and after (median, day 63; range, day 18 to day 527) allo-HSCT together with BM aspirations. The average number of the BM biopsies performed after allo-HSCT was 1.7 per patient (range, 1 to 6). We found a significant decrease of BM cellularity in samples collected from patients suffering from cytopenias in the peripheral blood (Supplemental Table 1). The characteristics of GVHD, in terms of the duration from its onset to BM biopsy, as well as the percentage of donor chimerism in the samples with cytopenic symptoms are shown in Supplemental Table 2.

BM GVHD and BM Suppression during Acute GVHD

We analyzed a total of 56 BM samples biopsied from day 0 to day 100 after allo-HSCT (Figure 2). Of these 56 samples, 15 were harvested when the patients had acute GVHD. Eight of these 15 samples were harvested from patients suffering from cytopenias and 2 of them displayed partial loss of osteoblasts, identifying 1 sample as an idiopathic cytopenia. We also identified 3 samples presenting the partial loss of osteoblasts; however, none of these 3 samples were collected when patients showed clinical manifestation of acute GVHD symptoms. The causes of cytopenias for these 3 samples included disease relapse and delayed engraftment. Taken together, during the early period after allo-HSCT, we did not observe a strong correlation between loss of osteoblasts and idiopathic cytopenias.

BM GVHD and BM Suppression during Chronic GVHD

We analyzed a total of 33 samples biopsied beyond day 100 (Figure 3A). Of 14 samples harvested from patients when they exhibited symptoms of chronic GVHD and concurrent idiopathic cytopenias, 4 samples displayed partial loss of

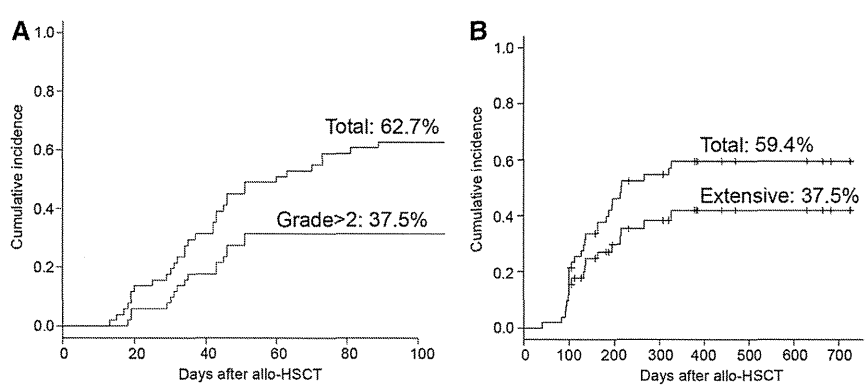

Figure 1. Cumulative incidences of acute (A) and chronic (B) GVHD after allogeneic hematopoietic stem cell transplantation.

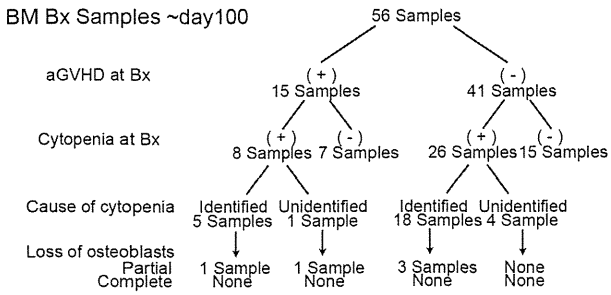

Figure 2. Assessments of bone marrow biopsy samples from patients up to day 100 after allogeneic hematopoietic stem cell transplantation. aGVHD indicates acute graft-versus-host disease; Bx, biopsy.

osteoblasts and another 6 samples displayed complete loss of osteoblasts. We further detailed the types of chronic GVHD affecting these idiopathic cytopenias and found 10 of 19 samples from patients suffering from idiopathic cytopenias displayed extensive chronic GVHD (Figure 3B). The loss of osteoblasts was significantly correlated with the extensive type of chronic GVHD (Table 2, $P = .012$) and also with idiopathic cytopenias in patients with chronic GVHD (Table 3, $P = .0427$). Among samples collected when patients had no cytopenic symptoms, no loss of osteoblasts were observed. We observed a significantly higher frequency of GVHD treatment with steroids in patients with osteoblast loss during chronic GVHD (Table 4). Characteristic pathological analyses of these cases, as well as a control BM sample, are summarized below.

Case 1: A patient with no GVHD and no cytopenias

Figure 4A indicates a pathological sample from a 47-year-old female who had no episodes of GVHD symptoms and no cytopenias when her BM sample was harvested on day 41. In hematoxylin and eosin staining, osteoblasts lining bone trabeculae are well observed before allo-HSCT and on day 41 (arrowheads). The lower panels show CD56 staining from the

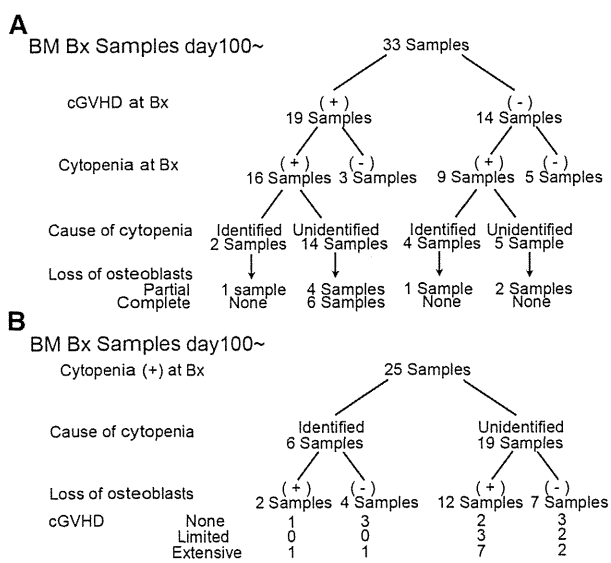

Figure 3. Assessments of bone marrow biopsy samples from patients after day 100 after allogeneic hematopoietic stem cell transplantation. (A) Relations between loss of osteoblasts and idiopathic cytopenias. (B) Grades of chronic GVHD and their relations to idiopathic cytopenias.

Table 2
Loss of Osteoblasts and Its Correlation with Chronic GVHD in BM Samples after Day 100

Chronic GVHD	Loss of Osteoblasts (+) (n = 14 Samples)	Loss of Osteoblasts (−) (n = 19 Samples)	*P* Value
Any chronic GVHD	11	8	**.0324**
Limited chronic GVHD	3	5	.7451
Extensive chronic GVHD	8	3	**.0120**

GVHD indicates graft-versus-host disease; BM, bone marrow.
Bold values indicate 2-tailed chi-square test.

same patient. Previous reports indicate neural cell adhesion molecule (NCAM, CD56) is strongly expressed by human osteoblasts [13]; therefore, we used CD56 staining for our samples to specifically identify osteoblasts.

Case 2: A patient with chronic liver and skin GVHD with cytopenias

A 57-year-old male patient underwent allo-HSCT from an HLA identical sibling. His underlining disease (anaplastic large cell lymphoma) relapsed on day 134 and tacrolimus was tapered off afterwards. Chronic extensive GVHD of the liver manifested on day 168, followed by deterioration of cutaneous and oral chronic GVHD and cytopenias, including platelets and red blood cells (grade 4 in platelets and grade 2 in hemoglobin by Common Terminology Criteria for Adverse Events version 4). BM biopsy on day 176 shows the complete loss of osteoblasts (Figure 4B). Chronic GVHD and cytopenias were then improved by the resumption of low-dose tacrolimus, and BM biopsy on day 521 showed recovery of osteoblasts.

Case 3: A patient with sustained cytopenias with skin GVHD

Figure 4C shows BM from a 37-year-old male patient receiving allo-HSCT from an HLA mismatched unrelated donor. Gradual cytopenias was observed from day 90 after allo-HSCT with stage 1 cutaneous GVHD. BM biopsy on day 127 displays complete loss of osteoblasts. When he recovered from these symptoms, osteoblasts reappeared on the sample taken on day 260.

Case 4: A patient with gradual loss of osteoblasts with worsening GVHD

A 27-year-old male patient underwent allo-HSCT from an unrelated donor. He achieved neutrophil engraftment on day 20; however, he remained dependent on platelet and red blood cell transfusions. He developed stage 1 skin GVHD on day 34. Red blood cell and platelet engraftment were

Table 3
Loss of Osteoblasts and its Correlation with Idiopathic Cytopenias in BM Samples after Day 100

Loss of Osteoblasts*	cGVHD (+), Idiopathic Cytopenia (+) (n = 14 Samples)	cGVHD (+), Idiopathic Cytopenia (−) (n = 5 Samples)†	*P* Value
Any osteoblast loss	10	1	**.0427**
Partial loss	4	1	.7032
Complete loss	6	0	**.0324**

BM indicates bone marrow; cGVHD, chronic graft-versus-host disease.
Bold values indicate 2-tailed chi-square test.

* Loss of osteoblasts is defined as *partial* if loss of osteoblasts is observed in 30% to 90% of the bone trabeculae in the pathological sections and *complete* if more than 90% of the osteoblast are lost.

† These samples include n = 3 samples without cytopenias and n = 2 with cytopenias with identified causes.

Table 4
Steroid Administration and Loss of Osteoblasts in Chronic GVHD BM Samples after Day 100

Steroid Therapy at BM Biopsy	Loss of Osteoblasts (+) cGVHD (+) (n = 11 Samples)	Loss of Osteoblasts (−) cGVHD (+) (n = 8 Samples)	*P* Value
(+)	8	2	.0360
(−)	3	6	

GVHD indicates graft-versus-host disease; BM, bone marrow; cGVHD; chronic graft-versus-host disease.

achieved on day 106 (reticulocytes >1%) and day 30 (>20,000/μL), respectively. Osteoblasts were partially lost on BM samples taken on day 36 (Figure 4D). Cytopenias continued, and he then developed chronic lung GVHD exacerbating from day 97, which was successfully treated with steroid therapy. However, the cytopenias persisted and the complete loss of osteoblasts was observed in BM samples taken on day 147. These results demonstrate the potential correlation between systemic (and supposedly affecting BM) GVHD and loss of BM osteoblasts leading to cytopenias. The patient did not develop bronchiolitis obliterans syndrome.

Suppression of CD19$^+$ B Cells and Increased CD4 to CD8 Ratio in BM Samples with the Loss of Osteoblasts

We next examined the BM aspirates samples taken at the same time points of the BM biopsies. We analyzed samples collected after day 100 by categorizing them into 3 subgroups based on the status of osteoblasts: not affected, partial loss, and complete loss. We observed decreased numbers of CD19$^+$ B cells and CD3$^+$ T cells in parallel with the loss of osteoblasts (Figure 5). Also, we found the ratio of CD4 and CD8 T cells was significantly increased with the loss of osteoblasts ($P = .0002$, not affected versus complete loss). These data indicate the effects of BM GVHD, resulting in disrupted hematopoiesis after allo-HSCT and are consistent with our mouse model data in the setting of BM GVHD [9].

DISCUSSION

In the settings of clinical allo-HSCT, patients frequently suffer from sustained cytopenias that parallel systemic GVHD. Some patients in the outpatient clinics after day 100 develop cytopenias without any signs of infection or GVHD. The causes for these cytopenias include relapse of original disease, viral (or bacterial) infections, and/or side effects of drugs, and it is very important to identify the cause as it directly affects the treatment decision for these patients. By

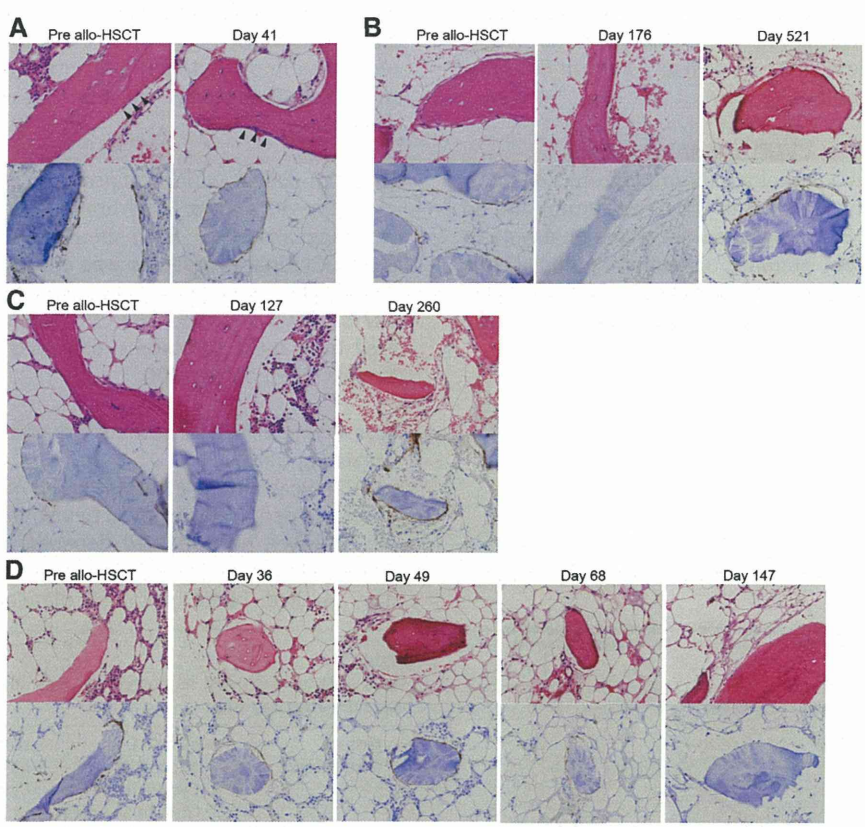

Figure 4. Loss of osteoblasts during GVHD and cytopenias. Biopsied bone marrow (BM) samples were stained with hematoxylin and eosin (upper panels for all pictures), as well as with CD56 (lower panels for all pictures) for assessments of cellularity, morphology, and presence or absence of osteoblasts. Magnification for the images is ×400. (A) BM samples from a 47-year-old patient after unrelated bone marrow transplantation who had no episode of GVHD and cytopenias when BM biopsies were performed. Osteoblasts are well preserved. Arrowheads indicate osteoblasts. (B) A 57-year-old patient who underwent related peripheral blood stem cell transplantation and relapsed on day 134 developed symptoms of chronic GVHD and cytopenias after day 160. Complete loss of osteoblasts is shown on day 176, and when he recovered from those symptoms osteoblasts are back on day 521. (C) A 37-year-old patient after related peripheral blood stem cell transplantation gradually developed cytopenias after day 90 with stage 1 skin GVHD. The BM samples on day 127 show clear loss of osteoblasts. On day 260, when his symptoms subsided, osteoblasts recovered to a normal level. (D) A 27-year-old patient who underwent unrelated bone marrow transplantation attained engraftment of white blood cells on day 20; however, cytopenias had persisted and he developed stage 1 skin GVHD on day 34. Partial loss of osteoblasts was observed from day 36 to day 68 and he had been on high demand of red blood cell and platelet transfusions. The BM samples on day 147 indicate complete loss of osteoblasts.

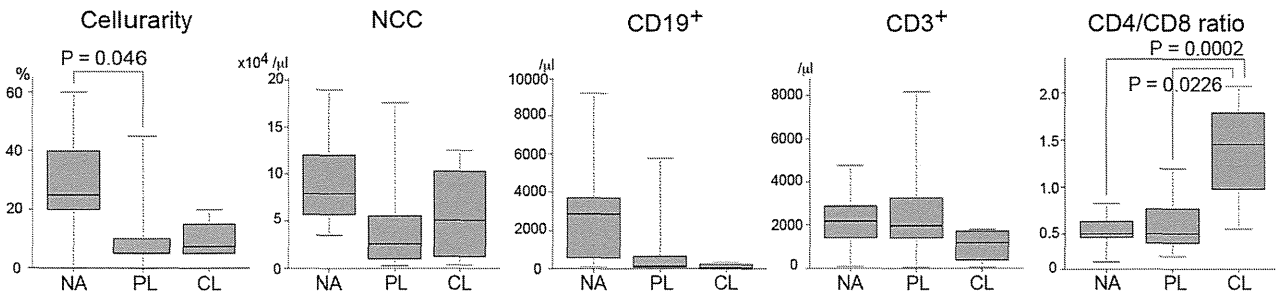

Figure 5. Flow cytometric analyses of BM aspirates in patients after day 100. BM aspiration was performed at the same time of BM biopsy in these patients. Cellularity, nucleated cell count (NCC), $CD19^+$ cells, $CD3^+$ cells, and the ratio of $CD4^+$ to $CD8^+$ cells are shown.

evaluating BM biopsy samples with hematoxylin and eosin, as well as CD56 immunohistochemical staining, we analyzed BM osteoblasts and compared these results with the respective clinical courses. As a result, we confirmed the disappearance of osteoblasts in samples from patients with idiopathic cytopenias and chronic GVHD during the late stage after allo-HSCT, suggesting the correlation between chronic GVHD and BM GVHD, resulting in BM suppression. Bone damage after allo-HSCT [15], especially suppression of B lymphopoiesis during GVHD, has been reported in both clinical and experimental studies [2,6,16-19], and our previous study in murine models unveiled new details of the mechanisms involved in this phenomenon, focusing on the destruction of hematopoietic niches by donor T cells in the course of GVHD [9]. In this article, we also reported the analyses of various clinical factors in BM, including cellularities and B cell analyses, which indicated the correlation with BM GVHD in human chronic GVHD cases. Consistent with our findings in murine GVHD models, we observed a decreased number of $CD19^+$ B cells and an increased CD4 to CD8 ratio in patients with osteoblast destruction; however, these findings were not observed during the early period after allo-HSCT, when in the murine models, donor $CD4^+$ T cells mediated strong BM GVHD. It is possible that in the clinical settings, patients are treated with immunosuppressive therapy and this could have prevented acute BM GVHD [20,21]. In cases of patients treated with steroids, it is quite difficult to separate the effects of chronic GVHD on osteoblasts from those of treatment with steroids, as steroids also decrease osteoblastic proliferation and activity [22]. We observed a higher frequency of GVHD treatment with steroids in patients who had idiopathic cytopenias with osteoblast loss (Table 4), indicating more severe systemic GVHD with BM GVHD that required steroid therapy.

In conclusion, we have shown for the first time, to our knowledge, the direct proof of BM GVHD and the loss of osteoblasts in chronic GVHD patients. Further studies with a large number of patients are warranted; however, our findings explain the cause of idiopathic cytopenias after allo-HSCT and give valuable insights for clinicians to use in treating patients suffering from BM suppression after allo-HSCT.

ACKNOWLEDGMENTS

The authors thank Mr. K. Arita, Mr. A. Yasumoto, Mr. K. Wakasa, Mr. M. Ibata, Mr. H. Goto, Mr. K. Yamaguchi, Mr. M. Onozawa, Ms. Y. Takeda, Ms. J. Iwasaki, Ms. I. Kasahara, Mr. K. Okada, Ms. M. Yamane, Ms. M. Mayanagi, and Ms. Y. Ishimaru for their technical assistance and the Department of Surgical Pathology, Hokkaido University Hospital, for its skillful support for immunohistochemistry.

Financial disclosure: This work was supported by grants from Japan Society for Promotion of Science KAKENHI (25293217 to T.T. and 25860775 to S.S.) and Health and Labor Science Research Grants (to T.T.).

Conflict of interest statement: There are no conflicts of interest to report.

Authorship Statement: Y.S. and S.S. contributed equally to this work.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2013.12.568.

REFERENCES

1. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation.* 1974;18:295-304.
2. Abrahamsen IW, Somme S, Heldal D, et al. Immune reconstitution after allogeneic stem cell transplantation: the impact of stem cell source and graft-versus-host disease. *Haematologica.* 2005;90:86-93.
3. Crooks GM, Weinberg K, Mackall C. Immune reconstitution: from stem cells to lymphocytes. *Biol Blood Marrow Transplant.* 2006;12:42-46.
4. Imamura M. Immunological reconstitution and immunoregulatory cells in hematopoietic stem cell transplantation. *Int J Hematol.* 2002; 76(Suppl 1):191-194.
5. Peggs KS. Immune reconstitution following stem cell transplantation. *Leuk Lymphoma.* 2004;45:1093-1101.
6. Baker MB, Riley RL, Podack ER, Levy RB. Graft-versus-host-disease-associated lymphoid hypoplasia and B cell dysfunction is dependent upon donor T cell-mediated Fas-ligand function, but not perforin function. *Proc Natl Acad Sci U S A.* 1997;94:1366-1371.
7. Iwasaki T, Hamano T, Saheki K, et al. Effect of graft-versus-host disease (GVHD) on host hematopoietic progenitor cells is mediated by Fas-Fas ligand interactions but this does not explain the effect of GVHD on donor cells. *Cell Immunol.* 1999;197:30-38.
8. Mori T, Nishimura T, Ikeda Y, et al. Involvement of Fas-mediated apoptosis in the hematopoietic progenitor cells of graft-versus-host reaction-associated myelosuppression. *Blood.* 1998;92:101-107.
9. Shono Y, Ueha S, Wang Y, et al. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood.* 2010;115:5401-5411.
10. Flowers ME, Kansu E, Sullivan KM. Pathophysiology and treatment of graft-versus-host disease. *Hematol Oncol Clinics North Am.* 1999;13: 1091-1112.
11. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 1995;15:825-828.
12. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant.* 2005;11:945-956.
13. Ely SA, Knowles DM. Expression of CD56/neural cell adhesion molecule correlates with the presence of lytic bone lesions in multiple myeloma and distinguishes myeloma from monoclonal gammopathy of undetermined significance and lymphomas with plasmacytoid differentiation. *Am J Pathol.* 2002;160:1293-1299.
14. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.* 2013;48:452-458.