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 JMDP using frozen samples (Morishima *et al*, 2002; Kawase *et al*, 2007). The extent of HLA testing was exon 2 and 3 for HLA class I and exon 2 for HLA class II, and exon 4 and exon 3 were additionally analysed for class I and class II, respectively, if required. An HLA mismatch in the GVHD was defined as when recipient antigens or alleles were not shared by the donor, and a mismatch in the host-versus-graft direction was defined as when donor antigens or alleles were not shared by the recipient. The direction of mismatch was considered in the analysis of engraftment and GVHD (Morishima *et al*, 2002; Lee *et al*, 2007).

Statistical analyses

The primary endpoint was overall survival after unrelated BMT. Secondary endpoints included the incidences of engraftment, grade III–IV acute GVHD, non-relapse mortality, and relapse. While the follow-up duration differed between patients in the two time periods [early (1993–1999) and late (2000–2009)], for the primary endpoint, we used the data obtained at last contact (Gooley *et al*, 2010). Then, we confirmed that there were no changes in the major findings, when surviving patients were censored at 5 years after BMT.

The chi-square test or Fisher's exact test was used to compare categorical variables and Student's *t*-test or an analysis of variance test was used for continuous variables. Overall survival was estimated according to the Kaplan–Meier method, and compared among groups with the log-rank test. The probabilities of non-relapse mortality, relapse, acute GVHD, and neutrophil engraftment were calculated while treating relapse, death without relapse, relapse or death without GVHD, and death without engraftment, respectively, as competing events, and compared using Gray's test (Gray, 1988).

The impacts of single HLA allele mismatches, the time period when BMT was performed, and the interaction between them were evaluated using multivariate models; Cox proportional hazards model for overall survival and Fine and Gray's proportional hazards model for the other endpoints (Fine & Gray, 1999). 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 total body irradiation (TBI) in the conditioning regimen, cell dose in the bone marrow graft, the use of ciclosporin (CSA) or tacrolimus (TAC) as GVHD prophylaxis, background disease, and disease risk. We divided GVHD prophylaxis regimens into only CSA-based and TAC-based regimens, because more than 95% of the patients received a combination of a calcineurin inhibitor and methotrexate. Acute leukaemia in first or second remission, CML in first or second chronic phase, CML in accelerated phase, and MDS of refractory anaemia or refractory anaemia 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 manner from the model to exclude factors with a *P*-value of 0·05 or higher. Finally, each single HLA allele mismatch and the time periods were added to the model to evaluate the effects of these factors adjusted for the other significant factors with or without interaction terms between the BMT time period and each single HLA allele mismatch. The model without interaction terms evaluated the impact of each single HLA allele mismatch adjusted for the BMT time period and the other significant factors. On the other hand, the model with interaction terms evaluated whether the impact of each single HLA allele mismatch was different between the two time periods, as well as the impact of each single HLA allele mismatch in each time period. Significant interaction means that the impact of the single HLA allele mismatch differs over the two time periods.

All *P*-values were two sided and *P*-values of 0·05 or less were considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Centre, Jichi Medical University; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html; Kanda, 2012), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 2.13.0). More precisely, it is a modified version of R commander (version 1.6-3) that was designed to add statistical functions frequently used in biostatistics.

Results

Patients

The patients characteristics are summarized in Table I. The total number of patients was 3003, and 751 and 2252 BMTs were performed in the early and late time periods, respectively. Of these, 1966 patients received a graft from an HLA-A, -B, -C, and -DRB1 allele matched donor, whereas 187, 31, 524, and 295 patients, respectively, underwent single HLA-A, -B, -C, and DRB1 allele-mismatched BMT. Only the HLA-C mismatch group included HLA mismatch at a serological (antigen) level. Bone marrow was exclusively used as the stem cell source.

Overall survival

To adjust the impact of HLA mismatch for possible confounding factors, we identified the following independently significant factors for overall survival: recipient age, disease, disease risk, and GVHD prophylaxis. After we adjusted for these factors, all single allele mismatches were significantly

Table I. Patient characteristics.

		1 allele mismatch				
	Match	A	B	C	DRB1	
	n = 1966	n = 187	n = 31	n = 524	n = 295	P value
Transplantation time period						
1993–1999	480	74	8	126	63	<0·001
2000–2009	1486	113	23	398	232	
Antigen mismatch						
No	1966 [480/1486]	187 [74/113]	31 [8/23]	38 [7/31]	295 [63/232]	<0·001*
Yes	0	0	0	486 [119/367]	0	
Mismatch in GVH direction						
No	1966 [480/1486]	22 [6/16]	1 [0/1]	38 [9/29]	11 [3/8]	0·0068*
Yes	0	165 [68/97]	30 [8/22]	486 [117/369]	284 [60/224]	
Mismatch in HVG direction						
No	1966 [480/1486]	13 [3/10]	0	43 [10/33]	18 [4/14]	0·29*
Yes	0	174 [71/103]	31 [8/23]	481 [116/365]	277 [59/218]	
Age, years						
Median (range)	37 (16–70) [30/39]	34 (16–56) [30/37]	34 (17–59) [28·5/35]	36 (16–67) [30/38]	37 (16–64) [26/39]	0·21
Age (donor), years						
Median (range)	34 (20–55) [34/34]	35 (20–55) [33/36]	35 (23–49) [29/37]	34 (20–54) [33/34]	34 (20–53) [34/34]	0·90
Sex						
Female	747 [183/564]	76 [31/45]	16 [4/12]	233 [57/176]	117 [30/87]	0·055
Male	1219 [297/922]	111 [43/68]	15 [4/11]	291 [69/222]	178 [33/145]	
Sex (donor)						
Female	651 [159/492]	62 [25/37]	14 [4/10]	218 [45/173]	119 [21/98]	0·016
Male	1307 [317/990]	124 [49/75]	17 [4/13]	303 [81/222]	175 [42/133]	
N.A.	8 [4/4]	1 [0/1]	0	3 [0/3]	1 [0/1]	
Sex mismatch						
Match	1241 [287/954]	101 [36/65]	21 [6/15]	310 [70/240]	159 [28/131]	0·077
Female to Male	311 [83/228]	36 [16/20]	4 [1/3]	99 [22/77]	69 [13/56]	
Male to Female	406 [106/300]	49 [22/27]	6 [1/5]	112 [34/78]	66 [22/44]	
N.A.	8 [4/4]	1 [0/1]	0	3 [0/3]	1 [0/1]	
ABO blood type						
Match	1119 [248/871]	91 [38/53]	13 [6/7]	190 [45/145]	135 [25/110]	<0·001
Minor mismatch	375 [92/283]	44 [14/30]	7 [1/6]	149 [34/115]	69 [17/52]	
Major mismatch	300 [93/207]	23 [8/15]	10 [1/9]	120 [32/88]	56 [13/43]	
Bidirectional mismatch	156 [37/119]	27 [13/14]	1 [0/1]	60 [12/48]	31 [7/24]	
N.A.	16 [10/6]	2 [1/1]	0	5 [3/2]	4 [1/3]	
Disease						
AML	876 [161/715]	64 [15/49]	13 [1/12]	216 [38/178]	136 [22/114]	0·029
ALL	563 [139/424]	58 [21/37]	9 [2/7]	136 [32/104]	81 [20/61]	
CML	321 [142/179]	44 [33/11]	7 [3/4]	94 [41/53]	53 [17/36]	
MDS	206 [38/168]	21 [5/16]	2 [2/0]	78 [15/63]	25 [4/21]	
Disease risk						
Low	1302 [327/975]	120 [51/69]	19 [6/13]	336 [79/257]	180 [36/144]	0·58
High	593 [136/457]	63 [22/41]	10 [1/9]	166 [41/125]	105 [25/80]	
N.A.	71 [17/54]	4 [1/3]	2 [1/1]	22 [6/16]	10 [2/8]	
Cell dose (cells/kg)						
Median	2·80 [3·07/2·70]	2·99 [2·97/2·99]	2·71 [3·10/2·58]	2·79 [3·15/2·60]	2·78 [3·10/2·61]	0·40
GVHD prophylaxis						

Table I. (*Continued*)

		1 allele mismatch				
	Match $n = 1966$	A $n = 187$	B $n = 31$	C $n = 524$	DRB1 $n = 295$	*P* value
CSA-based	918 [377/541]	93 [62/31]	14 [7/7]	243 [100/143]	115 [47/68]	0·17
TAC-based	1017 [93/924]	89 [10/79]	16 [1/15]	267 [24/243]	175 [15/160]	
N.A.	31 [10/21]	5 [2/3]	1 [0/1]	14 [2/12]	5 [1/4]	
Conditioning regimen						
TBI regimen	1634 [467/1167]	168 [74/94]	29 [8/21]	430 [121/309]	249 [63/186]	0·21
Non-TBI regimen	257 [10/247]	14 [0/14]	1 [0/1]	68 [5/63]	37 [0/37]	
N.A.	75 [3/72]	5 [0/5]	1 [0/1]	26 [0/26]	9 [0/9]	

Numbers in the square brackets show the data separated according to the time periods.
HVG, host-versus-graft; GVH (D), graft-versus-host (disease); AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; MDS, myelodysplastic syndrome; N.A., not available; CSA, ciclosporin; TAC, tacrolimus, TBI, total body irradiation.
*Comparison excluding the HLA-matched group.

associated with inferior survival except that the effect of HLA-A allele mismatch was nearly significant [HR 1·22, 95% confidence interval (CI) 1·00–1·51, $P = 0·055$, HR 1·60, 95% CI 1·03–2·49, $P = 0·038$, HR 1·23, 95% CI 1·07–1·41, $P = 0·00037$, and HR 1·26, 95% CI 1·07–1·49, $P = 0·0068$ for HLA-A, -B, -C, and -DRB1 mismatch, respectively]. However, when the effects of single HLA allele mismatches were evaluated separately in the early and late BMT time periods by adding interaction terms between HLA allele mismatches and time periods, only an HLA-B allele mismatch was associated with significantly inferior survival (HR 2·47, 95% CI 1·16–5·24, $P = 0·019$) in the early time period, whereas HLA-A, -C and -DRB1 mismatches did not exhibit a significant effect (HR 1·16, 95% CI 0·84–1·59, $P = 0·37$, HR 0·96, 95% CI 0·73–1·26, $P = 0·77$, and HR 0·83, 95% CI 0·58–1·19, $P = 0·32$, Table II). On the other hand, HLA-C and -DRB1 mismatches were associated with significantly inferior survival in the late time period (HR 1·35, 95% CI 1·15–1·59, $P < 0·001$, and HR 1·45, 95% CI 1·20–1·75, $P < 0·001$). The effects of HLA-A and -B allele mismatches were not statistically significant in the late time period, but the HR values (HR 1·24, 95% CI 0·95–1·62, $P = 0·12$, and HR 1·36, 95% CI 0·78–2·35, $P = 0·28$) were almost equivalent to those of HLA-C and -DRB1 mismatches. In fact, the negative impact of each single HLA allele mismatch was not significantly different among the HLA-A, -B, -C, and -DRB1 mismatches ($P = 0·79$ by the Wald test). Fig 1 shows the survival curves adjusted for other significant factors. In the early time period, the survival curves of the HLA-C and -DRB1 mismatch groups were at least equivalent to that of the HLA matched group, whereas that of the HLA-B mismatch group was separate from those of the other groups (Fig 1A). On the other hand, in the late time period, the survival curves of all of the single HLA allele mismatch groups were close to each other (Fig 1B).

An interaction test between the BMT time period and each single HLA allele mismatch revealed that the effects of single HLA-C and -DRB1 allele mismatches significantly differed over the two time periods ($P = 0·032$ and $P = 0·0072$, Table II). The major reason for these significant interactions was that, while overall survival in the HLA match group significantly improved from the early to the late time periods (HR 0·75, 95% CI 0·64–0·90, $P = 0·0011$), overall survival in the HLA-C and -DRB1 mismatch groups did not improve (HR 1·00, 95% CI 0·73–1·36, $P = 0·98$ and HR 1·20, 95% CI 0·79–1·82, $P = 0·40$, Fig 2). Similarly, overall survival in the HLA-A and -B mismatch groups did not change significantly between the two time periods (HR 0·81, 95% CI 0·49–1·34, $P = 0·41$ and HR 0·55, 95% CI 0·15–2·00, $P = 0·36$).

Engraftment and acute GVHD

The achievement of engraftment was significantly improved over the two time periods (HR 1·13, $P = 0·023$) after adjusting for other significant factors. None of the single HLA allele mismatches in the host-versus-graft direction affected the incidence of engraftment in either the early or late time periods, except for HLA-B allele mismatch in the late time period (HR 0·70, $P = 0·037$, Table III). The HR for engraftment was decreased, from 1·06 to 0·95 in the HLA-A mismatch group and from 1·03 to 0·89 in the HLA-DRB1 mismatch group, but the interaction tests were not significant.

With regard to the incidence of grade III–IV acute GVHD, single HLA-C allele mismatch in the graft-versus-host direction was associated with a significantly higher incidence of severe acute GVHD in the early time period (HR 2·02, $P = 0·0029$). In the late time period, single HLA-A and DRB1 allele mismatches, in addition to the HLA-C allele mismatch, were associated with a significantly higher incidence of grade III–IV acute GVHD (HR 1·72, $P = 0·025$, HR 1·51, $P = 0·0067$, and HR 1·45, $P = 0·045$ for HLA-A, -C, and -DRB1 mismatches, respectively), but the interactions between the time period and HLA-A and DRB1 allele mismatches were not statistically significant (Table III, Fig 3).

Table II. Multivariate analysis to evaluate the impact of single HLA allele mismatches, transplantation time periods, and their interaction on overall survival.

Factor	Hazard ratio	*P* value
Main effects		
Age	1·01 (1·01–1·02)	<0·001
Disease		
AML	1	
ALL	1·16 (1·02–1·32)	0·024
CML	0·90 (0·77–1·07)	0·23
MDS	0·56 (0·47–0·68)	<0·001
Disease risk		
Low	1	
High	2·98 (2·66–3·35)	<0·001
N.A.	2·40 (1·85–3·11)	<0·001
GVHD prophylaxis		
CSA-based	1	
TAC-based	0·94 (0·84–1·06)	0·30
HLA (early years)		
Match	1	
A mismatch	1·16 (0·84–1·59)	0·37
B mismatch	2·47 (1·16–5·24)	0·019
C mismatch	0·96 (0·73–1·26)	0·77
DRB1 mismatch	0·83 (0·58–1·19)	0·32
HLA (late years)		
Match	1	
A mismatch	1·24 (0·95–1·62)	0·12
B mismatch	1·36 (0·78–2·35)	0·28
C mismatch	1·35 (1·15–1·59)	0·0003
DRB1 mismatch	1·45 (1·20–1·75)	0·0001
Transplantation time period		
Early period	1·00	
Late period	0·74 (0·63–0·86)	0·00016
Interactions		
Time period * A mismatch	1·07 (0·70–1·63)	0·75
Time period * B mismatch	0·55 (0·22–1·40)	0·21
Time period * C mismatch	1·41 (1·03–1·93)	0·032
Time period * DRB1 mismatch	1·74 (1·16–2·61)	0·0072

GVHD, graft-versus-host disease; HLA, human leucocyte antigen; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; MDS, myelodysplastic syndrome; N.A., not available; CSA, ciclosporin; TAC, tacrolimus.

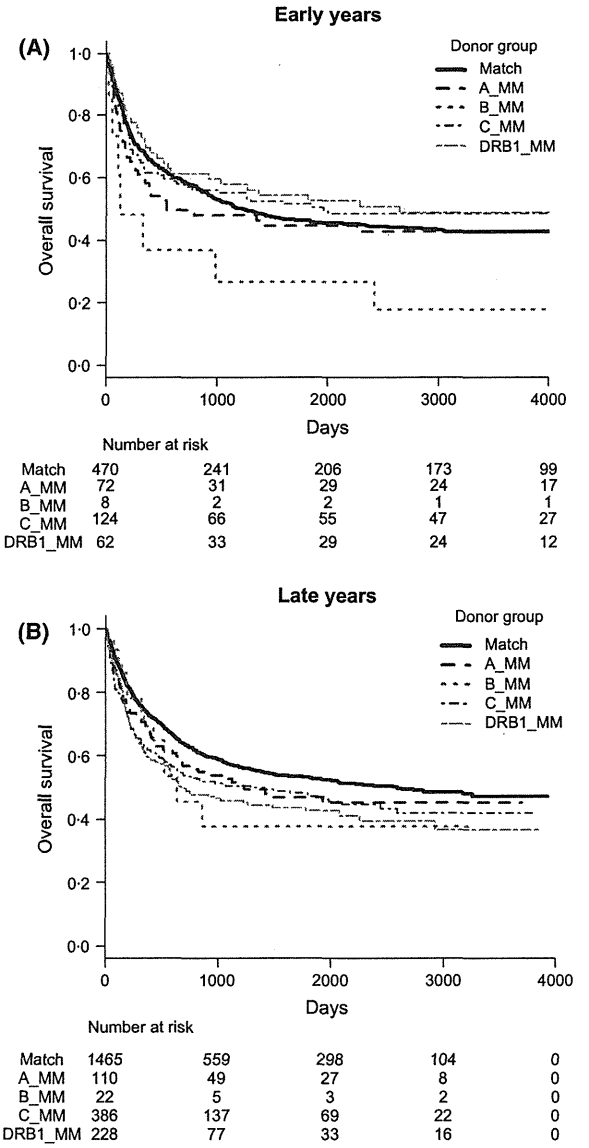

Fig 1. Overall survival grouped according to the human leucocyte antigen mismatch between the donor and recipient in the (A) early (1993–1999) and (B) late (2000–2009) time periods. The survival curves were adjusted for other significant factors by the mean of covariates method, in which average values of covariates are entered into the Cox proportional hazards model.

Non-relapse mortality and relapse

The incidence of non-relapse mortality was higher in the HLA-B allele mismatch group with borderline significance in the early time period (HR 2·48, $P = 0·069$, Table III, Fig 4). In the late time period, single HLA-A and -C allele mismatches were associated with a significantly higher incidence of non-relapse mortality (HR 1·47, $P = 0·027$ and HR 1·33, $P = 0·011$). While the HR for non-relapse mortality was highest in the HLA-B allele mismatch group (HR 1·72, $P = 0·10$), the effect was not statistically significant, probably due to the small sample size.

In the early period, a single HLA-C allele mismatch was associated with a significantly lower incidence of relapse (HR 0·46, $P = 0·0063$, Table III, Fig 5). However, an HLA-C mismatch did not have a significant relationship with the relapse rate in the late time period. There was a significant interaction between the BMT time period and an HLA-C allele mismatch ($P = 0·0094$).

Non-relapse mortality was significantly decreased from the early to late time period (HR 0·69, $P = 0·00078$), whereas the incidence of relapse was not changed (HR 0·96, $P = 0·71$).

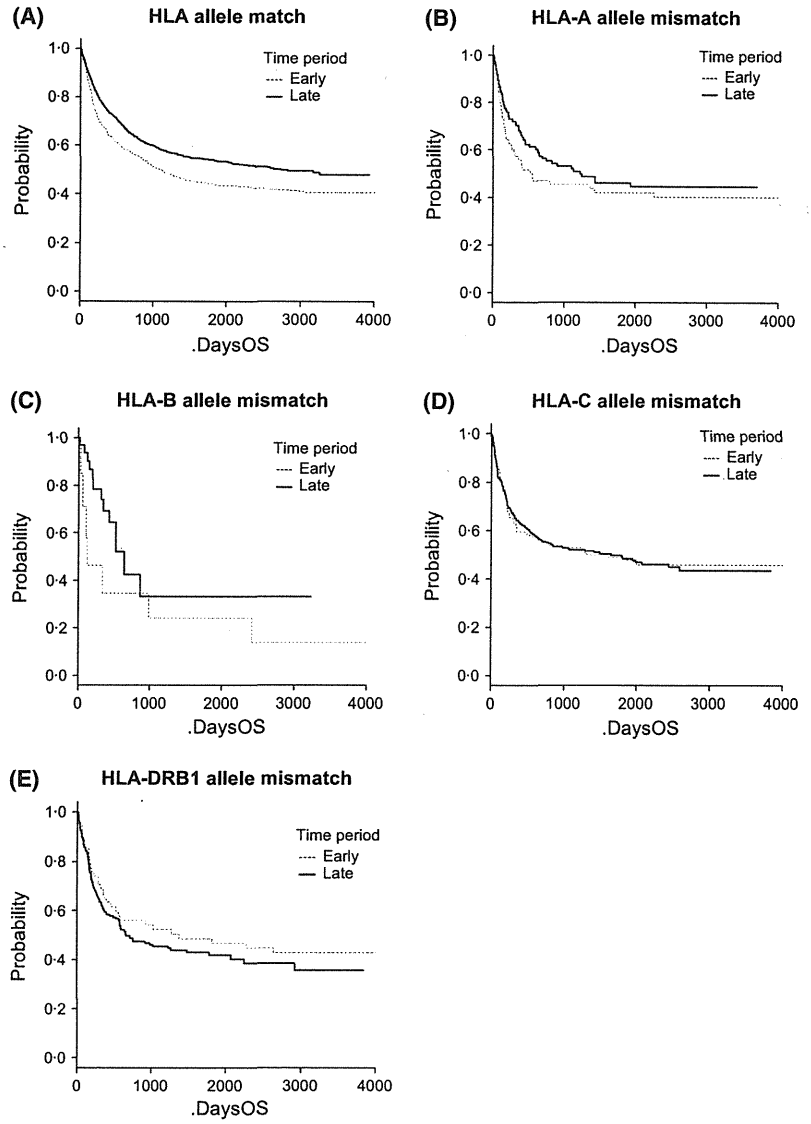

Fig 2. Overall survival grouped according to the transplantation time period in the human leucocyte antigen (HLA) match (A), HLA-A allele mismatch (B), HLA-B allele mismatch (C), HLA-C allele mismatch (D), and HLA-DRB1 allele mismatch (E) groups. The survival curves were adjusted for other significant confounding factors by the mean of covariates method, in which average values of covariates are entered into the Cox proportional hazards model. Early, transplanted between 1993 and 1999; Late, transplanted between 2000 and 2009.

Discussion

This study re-evaluated the effect of a single HLA allele mismatch on the outcome of unrelated BMT in the recent cohort. We chose 2000 as the cutoff of time period, as high-resolution typing for HLA-A and -B became a common practice in Japan after 2000. In contrast to our previous findings (Sasazuki *et al*, 1998), only the effects of single HLA-C and -DRB1 mismatches were statistically significant in the recent time period, but the negative impact of each single HLA allele mismatch was not significantly different among the HLA-A, -B, -C, and -DRB1 mismatches. Previous JMDP studies showed that HLA-A and -B allele mismatches were associated with higher overall mortality, whereas HLA-C or -DRB1 allele mismatches did not affect mortality after unrelated BMT (Sasazuki *et al*, 1998). In contrast, Petersdorf *et al* (2004) reported that a single HLA-A, -B, -C or -DRB1 allele mismatch had no significant relationship with survival in patients with leukaemia other than chronic myeloid leukaemia in chronic phase. The recent NMDP study analysed the effect of a single allele mismatch on survival in 1840 HLA-matched and 985 one-allele mismatched unrelated HSCT and showed that a single mismatch at HLA-B or -C had smaller relationship with survival than single mismatch at HLA-A or -DRB1 (Lee *et al*, 2007). These discrepancies could be explained by the difference in study population or study designs (Bray *et al*, 2008). For example, the distribution of HLA alleles is different between the US and Japanese populations. Several HLA allele mismatch combinations have been shown to have higher risk for severe acute GVHD compared to other mismatch combinations (Kawase *et al*, 2007). The proportion of high-risk mismatch combinations may affect

Table III. Multivariate analysis to evaluate the impact of single human leucocyte antigen (HLA) allele mismatches, transplantation time periods, and their interaction on the incidences of neutrophil engraftment, grade III–IV acute GVHD, non-relapse mortality, and relapse.

Factor	Hazard ratio	*P* value
Engraftment		
Main effects		
HLA (early years)		
Match	1	
A mismatch	1·06 (0·87–1·29)	0·59
B mismatch	0·65 (0·28–1·54)	0·33
C mismatch	0·93 (0·77–1·11)	0·42
DRB1 mismatch	1·03 (0·79–1·36)	0·80
HLA (late years)		
Match	1	
A mismatch	0·95 (0·77–1·18)	0·66
B mismatch	0·70 (0·50–0·98)	0·037
C mismatch	0·95 (0·73–1·08)	0·4
DRB1 mismatch	0·89 (0·77–1·03)	0·12
Transplantation time period		
Early period	1	
Late period	1·13 (1·02–1·25)	0·023
Interactions		
Time period * A mismatch	0·90 (0·68–1·21)	0·49
Time period * B mismatch	1·07 (0·43–2·67)	0·89
Time period * C mismatch	1·02 (0·82–1·27)	0·85
Time period * DRB1 mismatch	0·86 (0·63–1·17)	0·33
Grade III-IV acute GVHD		
Main effects		
HLA (early years)		
Match	1	
A mismatch	1·46 (0·79–2·69)	0·22
B mismatch	1·74 (0·22–13·55)	0·60
C mismatch	2·02 (1·27–3·20)	0·0029
DRB1 mismatch	0·80 (0·37–1·74)	0·58
HLA (late years)		
Match	1	
A mismatch	1·72 (1·07–2·77)	0·025
B mismatch	1·26 (0·42–3·79)	0·68
C mismatch	1·51 (1·12–2·02)	0·0067
DRB1 mismatch	1·45 (1·01–2·09)	0·045
Transplantation time period		
Early period	1	
Late period	1·01 (0·75–1·36)	0·96
Interactions		
Time period * A mismatch	1·18 (0·54–2·55)	0·68
Time period * B mismatch	0·73 (0·07–7·44)	0·79
Time period * C mismatch	0·75 (0·43–1·29)	0·30
Time period * DRB1 mismatch	1·81 (0·77–4·25)	0·17
Non-relapse mortality		
Main effects		
HLA (early years)		
Match	1	
A mismatch	1·41 (0·93–2·12)	0·11
B mismatch	2·48 (0·93–6·57)	0·069
C mismatch	1·20 (0·87–1·67)	0·27
DRB1 mismatch	0·86 (0·52–1·41)	0·55
HLA (late years)		
Match	1	
A mismatch	1·47 (1·05–2·07)	0·027
B mismatch	1·72 (0·90–3·29)	0·1
C mismatch	1·33 (1·07–1·66)	0·011
DRB1 mismatch	1·22 (0·93–1·60)	0·15
Transplantation time period		
Early period	1	
Late period	0·69 (0·56–0·86)	0·00078
Interactions		
Time period * A mismatch	1·05 (0·61–1·79)	0·86
Time period * B mismatch	0·69 (0·21–2·25)	0·54
Time period * C mismatch	1·11 (0·74–1·64)	0·62
Time period * DRB1 mismatch	1·42 (0·81–2·50)	0·23
Relapse		
Main effects		
HLA (early years)		
Match	1	
A mismatch	0·79 (0·45–1·39)	0·42
B mismatch	1·97 (0·57–6·76)	0·28
C mismatch	0·46 (0·27–0·81)	0·0063
DRB1 mismatch	0·90 (0·54–1·51)	0·70
HLA (late years)		
Match	1	
A mismatch	0·71 (0·44–1·14)	0·15
B mismatch	1·10 (0·49–2·49)	0·81
C mismatch	1·04 (0·81–1·33)	0·79
DRB1 mismatch	1·27 (0·95–1·68)	0·10
Transplantation time period		
Early period	1	
Late period	0·96 (0·76–1·20)	0·71
Interactions		
Time period * A mismatch	0·89 (0·43–1·87)	0·77
Time period * B mismatch	0·56 (0·13–2·46)	0·44
Time period * C mismatch	2·23 (1·22–4·08)	0·0094
Time period * DRB1 mismatch	1·40 (0·78–2·52)	0·26

Factors used for adjustment included donor sex, ABO major mismatch, ABO minor mismatch, cell dose, GVHD prophylaxis, and disease risk in analysis for engraftment, donor age, donor sex, female to male transplantation, cell dose, disease, and disease risk in analysis for GVHD, recipient age, donor age, donor sex, female to male transplantation, ABO major mismatch, disease, disease risk, and GVHD prophylaxis in analysis for non-relapse mortality, and donor age, disease, disease risk, and the use of TBI in analysis for relapse.

the effect of each single HLA allele mismatch. With regard to the study designs, the inclusion criteria for disease, phase of disease, and HLA matching were different among studies (Bray *et al*, 2008). Japanese studies included HLA-A, -B, and -DR antigen matched transplantation only, whereas the other studies included one-antigen mismatched transplantation. Earlier studies reported that an allele mismatch and an antigen mismatch had similar effects on mortality, although the risk of graft failure was higher with an antigen mismatch

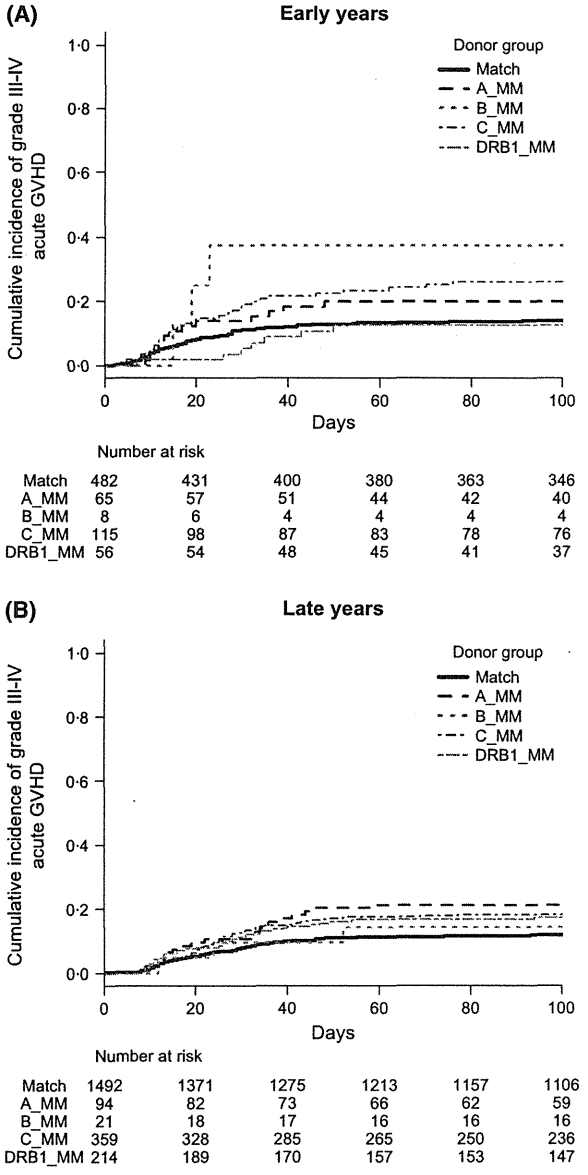

Fig 3. The cumulative incidence of grade III-IV acute graft-versus-host disease (GVHD) grouped according to the human leucocyte antigen mismatch between the donor and recipient in the (A) early (1993–1999) and (B) late (2000–2009) time periods.

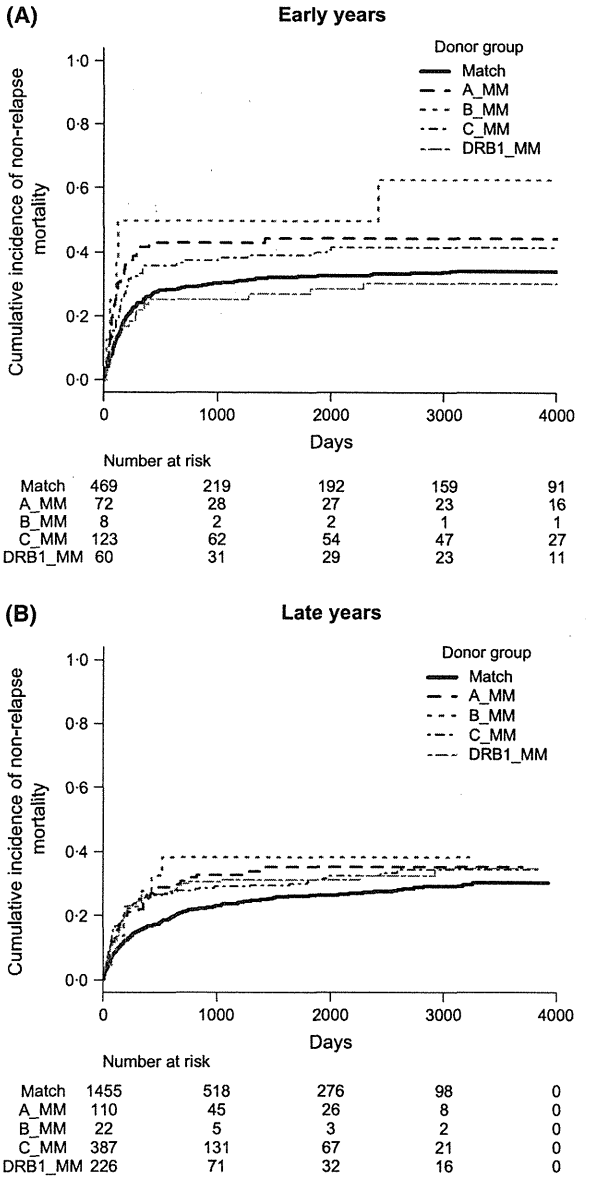

Fig 4. The cumulative incidence of non-relapse mortality grouped according to the human leucocyte antigen mismatch between the donor and recipient in the (A) early (1993–1999) and (B) late (2000–2009) time periods.

(Petersdorf *et al*, 2001, 2004). A subsequent report from NMDP confirmed that there was no significant difference in the effect on survival between a single antigen mismatch and a single allele mismatch (Lee *et al*, 2007). In the current study, only patients who underwent unrelated BMT from an HLA-A, -B, and -DR antigen matched donor were included, as such a donor can be found in more than 90% of patients in Japan. Therefore, only the HLA-C mismatch group included patients with HLA-mismatch at an antigen level. The effect of HLA-C antigen mismatch and HLA-C allele mismatch on survival was equivalent (HR 1·33 vs. 1·28) in the current cohort, although the number of patients with HLA-C allele mismatch was limited.

The second important finding is the positive interaction test that revealed the statistically significant change in the effects of HLA-C and -DRB1 mismatches from the early to the late time period. These significant interactions resulted from the fact that survival after HLA-matched BMT was significantly improved in the late time period, while there was no such improvement after HLA-C or -DRB1 mismatched

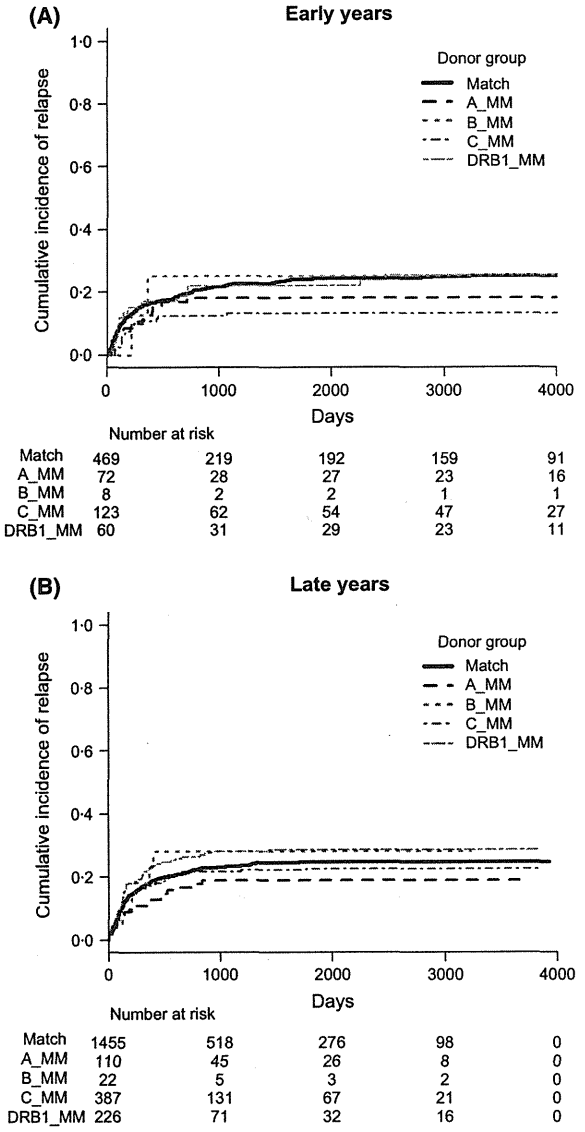

Fig 5. The cumulative incidence of relapse grouped according to the human leucocyte antigen mismatch between the donor and recipient in the (A) early (1993–1999) and (B) late (2000–2009) time periods.

BMT (Fig 2). The improvement in survival in the HLA match group is probably due to the progress in transplantation procedures, including strategies against GVHD and infectious complications. The incidence of grade III–IV acute GVHD in the HLA match group decreased from 13·9% to 11·9% over the two time periods, and furthermore, the incidence of transplant-related mortality among patients who developed grade III–IV acute GVHD decreased, from 25·4% to 15·9%. While such progress should also be reflected in the HLA-C and -DRB1 mismatch groups, other factors may have counterbalanced this benefit. With regard to HLA-DRB1 allele mismatch, significant interaction could be explained by the difference in the availability of information about HLA allele mismatch between the two time periods. In the 1990's, only the presence of an HLA-DRB1 allele mismatch was noted by physicians before BMT, whereas both HLA-A and -B mismatches were also tested before BMT in the 2000's. In addition, the landmark paper from the JMDP was published in 1998 (Sasazuki *et al*, 1998), and the presence of an HLA-A or -B mismatch was recognized as a risk factor for severe acute GVHD. These backgrounds might have induced a trend toward more intensive GVHD prophylaxis in patients with an HLA-DRB1 allele mismatch in the 1990's and in those with an HLA-A or -B mismatch in the 2000's. For example, in the early time period, TAC-based GVHD prophylaxis was most frequently used in the HLA-DRB1 mismatch group (odds ratios for the use of TAC were 0·65, 0·58, 0·97 and 1·29 for the HLA-A, -B, -C, and -DRB1 mismatch groups, respectively, compared to the HLA-matched group). On the other hand, in the late time period, TAC was used almost equally in the HLA-A, -B, and -DRB1 mismatch groups (odds ratios for the use of TAC were 1·49, 1·25, 1·00 and 1·38 for the HLA-A, -B, -C, and -DRB1 mismatch groups, respectively, compared to the HLA-matched group). The statistical interaction was significant even after an adjustment for the use of TAC, and therefore this is not the major reason for the interaction. The target blood concentrations of CSA or TAC and the dose of methotrexate in GVHD prophylaxis may also have been affected by the availability of information about HLA allele mismatch, but such data were not included in the database.

Another bias that may have been caused by the difference in the availability of information about the HLA allele mismatch is the trend to avoid HLA-mismatched BMT for patients with less advanced diseases, because the impact of HLA mismatch is generally more apparent in such diseases (Petersdorf *et al*, 2004; Lee *et al*, 2007; Kanda *et al*, 2003, 2012). In fact, the proportion of patients with low-risk disease in the HLA-DRB1 allele mismatch group was less than that in the other groups (57·1% vs. 62·7–75%) in the early time period, while equivalent proportions were seen in the late time period (62·1% vs. 56·5–65·6%). However, the HR value for HLA-DRB1 allele mismatch increased from 0·79 in the early period to 1·42 in the late period even when we only analysed patients with low-risk disease, although the interaction was not statistically significant ($P = 0·069$). The proportion of patients with a high-risk HLA allele mismatch may also affect the impact of each single HLA-allele mismatch on survival (Kawase *et al*, 2007), but the proportions were similar in the early and late time periods (6·3% and 7·3%). Therefore, this cannot explain the significant interaction between the time period and HLA-DRB1 allele mismatch.

With regard to the interaction between the time period and HLA-C allele mismatch, there was no difference in the availability of information, because HLA-C typing was not routinely performed until 2009. The significant interaction probably resulted from the increased incidence of relapse in the late time period in the HLA-C allele mismatched group.

The proportion of patients with a killer immunoglobulin-like receptor ligand mismatch in the graft-versus-host direction (KIR_L_MM_G) may affect the incidence of relapse (Dupont & Hsu, 2004; Morishima *et al*, 2007). However, the interaction test for relapse was significant even when we excluded patients with a KIR_L_MM_G mismatch ($P = 0{\cdot}022$). Therefore, we could not find a clear explanation for this interaction.

The major limitation of this study is the sample size in the HLA-B mismatch groups, especially in the early time period. Although the major object of this study was to reevaluate the impact of a mismatch in each single allele in the late time period, there were only 23 patients in the HLA-B mismatch group even in the late period, and therefore we could not conclude that the effects of all single HLA mismatches were equivalent, despite that there was no significant difference in the negative impact on survival among the HLA-A, -B, -C, and -DRB1 mismatches. Another limitation of this study was the exclusion of HLA-DQ mismatch in the analyses, as the allele data for HLA-DQ was available only in 493 of the 3003 patients in this study. However, when we included HLA-DQ in the multivariate analysis for overall survival, the effect of HLA-DQ mismatch on survival was not significant (HR 0·93, 95% CI 0·63–1·38, $P = 0{\cdot}73$) and the HRs for HLA-A, -B, -C, and -DRB1 did not obviously change after the addition of HLA-DQ in the model (data not shown).

In conclusion, this retrospective study revealed that the impact of single HLA allele mismatches might have changed after HLA-A and -B mismatch information became available to physicians before BMT. In the recent cohort (BMT between 2000 and 2009), the negative impact of HLA-C and -DRB1 mismatches became apparent. We should reconsider the algorithm for unrelated donor selection in Japan.

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Author contributions

Y.K. and Y.M. designed the study. Y.K., J.K., Y.A., and S.M. analysed the data. Y.M., T.I., K.O., T.F., K.M., H.I., T.M., K.I., T.E., and K.K. gathered the data. Y.K. wrote the first draft of the paper and all other authors contributed to the final version.

Disclosure of conflicts of interest

We declare that we have no conflicts of interest.

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In vivo T-cell depletion with alemtuzumab in allogeneic hematopoietic stem cell transplantation: Combined results of two studies on aplastic anemia and HLA-mismatched haploidentical transplantation

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We evaluated the efficacy of *in vivo* T-cell depletion with alemtuzumab in two prospective studies according to the International Conference on Harmonisation (ICH)—Good Clinical Practice (ICH–GCP) guidelines; one was for patients with aplastic anemia (AA study) and the other was for patients who were undergoing hematopoietic stem cell transplantation (HSCT) from a 2- or 3-antigen-mismatched haploidentical donor (MM study). The final dose of alemtuzumab in these studies was 0.16 mg/kg/day for 6 days. At this dose, all of the 12 and 11 patients in the AA and MM studies, respectively, achieved initial engraftment and the incidences of Grade II–IV acute graft-versus-host disease (GVHD) were 0% and 18%. While cytomegalovirus (CMV) frequently reactivated, none of the patients developed fatal CMV disease. Transplantation-related mortality within 1 year after HSCT was observed in only two and one patients, respectively. The numbers of CD4+ and CD8+ T-cells and T-cell receptor rearrangement excision circles remained low within 1 year after HSCT. These findings suggest that the use of alemtuzumab at this dose in a conditioning regimen enables safe allogeneic HSCT even from a 2- or 3-antigen-mismatched donor. However, the use of a lower dose of alemtuzumab should be explored in future studies to accelerate immune recovery after HSCT. Am. J. Hematol. 88:294–300, 2013. © 2013 Wiley Periodicals, Inc.

Introduction

Graft-versus-host disease (GVHD) is an important complication after allogeneic hematopoietic stem cell transplantation (HSCT), especially in the presence of an HLA-mismatch between the donor and recipient. Alemtuzumab is a humanized monoclonal antibody against CD52, and the use of alemtuzumab in the conditioning regimen before HSCT has been shown to decrease the incidences of acute and chronic GVHD through the *in vivo* depletion of donor T cells [1]. A growing body of evidence supports the efficacy of alemtuzumab at preventing acute GVHD in a variety of HSCT settings [2–9]. In addition, we and others have reported that alemtuzumab enables HLA-mismatched haploidentical HSCT without the *ex vivo* depletion of donor T cells [10,11]. We administered alemtuzumab at 0.2 mg/kg/day for 6 days before allogeneic HSCT from a 2- or 3-anitigen-mismatched related donor in 12 patients [10]. All patients achieved neutrophil engraftment and the cumulative incidence of Grade III to IV acute GVHD was only 9%. Rizzieri et al. used alemtuzumab at a total dose of 100 mg spread over 5 days in reduced-intensity haploidentical HSCT without *ex vivo* T-cell depletion [11]. The incidences of Grade III–IV acute GVHD and transplant-related mortality were 8% and 10.2%, respectively. Therefore, *in vivo* T-cell depletion using alemtuzumab enables haploidentical HSCT without excessive GVHD.

With regard to allogeneic HSCT for aplastic anemia (AA), alemtuzumab has been incorporated into the conditioning regimen to achieve sustained engraftment with minimal toxicity and GVHD [12]. The incidence of graft failure was 9.5% for HSCT from an HLA-matched sibling donor and 14.5% for HSCT from an unrelated donor. Acute GVHD was observed in only 13.5% of the patients and there was no Grade III–IV acute GVHD.

However, the use of alemtuzumab as a conditioning regimen for allogeneic HSCT has not been approved in Japan or in any other countries. Therefore, we performed two pivotal trials according to the International Conference on Harmonisation (ICH)—WHO Good Clinical Practice (GCP) guidelines to obtain approval from the Pharmaceuticals and Medical Devices Agency (PMDA); one was for HSCT for AA study and the other was for HSCT from an HLA-mismatched donor (MM study). These studies were approved by the Institutional Review Board of each participating center and were registered in the UMIN Clinical Trial Registry (C000000356 and C000000357).

Patients and Methods

Patients

The common inclusion criteria of the two studies were age between 20 and 65, ECOG performance status <2, the absence of severe organ dysfunctions (for example, $SaO_2 < 94\%$ or ejection fraction

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Conflict of interest: Nothing to report

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<55%) and the absence of active infection. All patients provided their written informed consent before being enrolled in these studies.

The AA study included patients with very severe, severe, or transfusion-dependent moderate AA. The donor was either an HLA-matched related donor, one-antigen-mismatched related donor, matched unrelated donor, or HLA-DRB1 one-allele-mismatched unrelated donor. Patients who underwent HSCT from a donor other than HLA-matched related donor must have received immunosuppressive treatment and were refractory to or relapsed after such treatment.

The MM study included patients with relapsed or refractory acute leukemia, high-risk acute leukemia such as Philadelphia chromosome-positive acute lymphoblastic leukemia in first complete remission, chronic myelogenous leukemia after blastic transformation, myelodysplastic syndrome with severe cytopenia or a bone marrow blast ratio of at least 20%, and lymphoma that was refractory to chemotherapy or that relapsed after autologous HSCT. Patients who had an available HLA-matched related donor, one-antigen-mismatched related donor, matched unrelated donor, or HLA-DRB1 one-allele-mismatched unrelated donor were excluded.

Conditioning regimens

The conditioning regimen in the AA study (FLU-CY) consisted of fludarabine at 30 mg/kg/day for 4 days (days -6 to -3) and cyclophosphamide at 25 mg/kg/day for 4 days (days -6 to -3). Total body irradiation (TBI) at 2 Gy was added on day-1 in HSCT from a donor other than an HLA-matched related donor. Alemtuzumab was added to this regimen for 6 days (days -10 to -5).

The conditioning regimen in the MM study included myeloablative and reduced-intensity regimens. Patients who were aged at least 55 years or those who had previously undergone autologous HSCT received a reduced-intensity regimen (FLU-BU) that consisted of fludarabine at 30 mg/kg/day for 6 days (days -8 to -3), oral busulfan at 4 mg/kg/day for 2 days (days -5 to -4), and TBI at 4 Gy on day -1. The other patients received a myeloablative conditioning regimen (CY-TBI) that consisted of cyclophosphamide at 60 mg/kg/day for 2 days (days -3 to -2) and TBI at 4 Gy/day for 3 days (days -7 to -5). Alemtuzumab was added to this regimen for 6 days (days -8 to -3).

Dose of alemtuzumab

These studies consisted of two stages. In the first stage, the dose of alemtuzumab was started at 0.2 mg/kg/day, which was used in our previous study [10], and then we planned to change the dose to between 0.16 mg/kg/day and 0.25 mg/kg/day according to the conventional 3 + 3 cohort design. The starting dose of alemtuzumab in the second stage was defined by the results in the first-stage patients, and thereafter, the dose was changed according to the continual reassessment method [13].

To prevent acute infusion-related reactions to alemtuzumab, patients were pretreated with 1 mg/kg of methyl-prednisolone. Alemtuzumab was infused over 4 hr. On the first day of alemtuzumab infusion, 3 mg of alemtuzumab was infused over 2 hr and, after confirming that no severe infusion-related toxicities were observed, we infused the remaining alemtuzumab over the next 2 hr.

Other transplantation procedures

With regard to the stem cell source, bone marrow was exclusively used in the AA study, whereas peripheral blood was used in the MM study. Prophylaxis against GVHD was performed with cyclosporine (CSA) and short-term methotrexate. CSA was started on day -1 at a dose of 3 mg/kg/day by continuous infusion and the dose was adjusted to maintain a blood concentration between 250 and 350 ng/mL. CSA was changed to an oral form when it could be tolerated by the patient. Methotrexate was administered at 10 mg/m^2 on Day 1 and 7 mg/m^2 on days 3 and 6 in the AA study, whereas it was administered at 15 mg/m^2 on Day 1 and 10 mg/m^2 on days 3, 6, and 11 in the MM study. For patients without GVHD, we started to taper CSA from Day 100 by 5% per week in the AA study, whereas we started to taper CSA from Day 30 by 10% per week and discontinued CSA on Day 100 in the MM study.

Prophylaxis against bacterial, fungal and *Pneumocystis jiroveci* infection consisted of fluoroquinolones, azoles, and sulfamethoxazole/trimethoprim. As prophylaxis against herpes simplex virus infection and varicella zoster infection, acyclovir was given from days -7 to 35, followed by long-term low-dose administration [14]. Preemptive therapy with ganciclovir against cytomegalovirus (CMV) was performed by weekly monitoring of an antigenemia assay [15]. EB virus (EBV) reactivation was monitored by polymerase chain reaction (PCR) assay on days 90 and 180 and at optional occasions at the discretion of attending physicians. Patients who developed Grade II–IV acute GVHD were treated with 1 mg/kg of methyl-prednisolone.

Chimerism and immune recovery

Host/donor cell chimerism after transplantation was analyzed by sex-chromosome FISH or the short tandem repeat method using peripheral blood CD3+ cells [16]. Immune reconstitution was evaluated by the quantification of CD3+/CD4+, CD3+/CD8+, CD3-/CD19+, and CD3-/CD56+ cells by flowcytometry. For patients who had HLA-A*0201 or HLA-A*2402, the number of cytotoxic T-cells that were specific for CMV was measured using tetramers loaded with CMV pp65 antigen peptides [17]. T-cell receptor rearrangement excision circles (TRECs) were quantified in purified CD4+ and CD8+ T-cells by real-time quantitative PCR (RQ-PCR) with the 5'-nuclease (TaqMan) assay and an ABI7900 system (Life Technologies, Foster City, CA) as described elsewhere [18]. The mean copy numbers of TRECs in 10^5 CD4+ and CD8+ T-cells from 10 healthy adults were 1440 ± 880 and 1920 ± 1300, respectively [19]. The overall complexity of the T-cell receptor repertoire within a Vβ subfamily was determined by counting the number of discrete peaks and determining their relative sizes on the electropherogram by a complexity scoring system as described elsewhere [19–21]. The mean complexity scores of CD4+ and CD8+ T-cells from 10 healthy adults were 116.1 ± 2.36 and 109.49 ± 6.77, respectively [19].

Pharmacokinetics of alemtuzumab and detection of anti-alemtuzumab antibody

Serum samples were collected before each dose and at various time points afterward and stored frozen at −80°C until analysis. Serum alemtuzumab concentrations were measured using flowcytometry as described elsewhere [22]. The presence of anti-alemtuzumab antibodies was analyzed using YID13.9, a monoclonal anti-idiotype-specific antibody for alemtuzumab, as a standard [22].

Statistical considerations

Treatment was considered successful if all three of the following criteria were met: achievement of neutrophil engraftment, patients alive at Day 60 after HSCT, and the absence of Grade II–IV and Grade III–IV acute GVHD in the AA study and the MM study, respectively. Overall survival was calculated using the Kaplan-Meier method. The serial changes in the alemtuzumab concentrations or the number of lymphocytes were analyzed using a repeated-measures analysis of variance after logarithmic transformation by discarding the patients with missing values. All *P* values were two-sided and *P* values less than 0.05 were considered to indicate statistical significance.

Results

Patients

In the AA study, six patients were enrolled in the first-stage study. The first three patients who received alemtuzumab at 0.20 mg/kg/day for 6 days (CAM20) achieved treatment success and the dose of alemtuzumab was decreased to 0.16 mg/kg/day for 6 days (CAM16). Thereafter, the next three patients in the first stage and all 12 patients in the second stage received alemtuzumab at this decreased dose. Similarly, in the MM study, the first three patients in the first stage received CAM20, whereas the next three patients in the first stage and all 11 patients in the second stage received CAM16. In both studies, no dose modification was required in the second stage according to the continual reassessment method protocol.

In the following analyses, only the 23 patients who received CAM16 were evaluated as predefined in the protocol, unless otherwise specified. The donor was an HLA-matched related donor, a matched unrelated donor, and a one-allele-mismatched unrelated donor in three, four, and five patients, respectively, in the AA study. In the MM study, there was a two- and three-antigen mismatch in the graft-versus-host direction in seven and four patients, respectively.

Engraftment, chimerism, and GVHD

All patients, including 12 in the AA study and 11 in the MM study, achieved engraftment (Table I). However, one patient in the AA study developed secondary graft failure and died on Day 69 after HSCT.

TABLE I. Patient Characteristics

UPN	Age/ Sex	Diagnosis	Stage	Donor	HLA mismatch	Regimen	Alemtuzumab dose	Engraftment (day)	Acute GVHD	Outcome (day 60)	Outcome (day 365)	Cause of death
AA-01	42M	AA		Unrelated	0	FLU-CY-TBI	0.2	15	0	Alive	Died (day 258)	GI bleeding and respiratory failure
AA-02	20F	AA		Unrelated	0	FLU-CY-TBI	0.2	17	0	Alve	Alive	
AA-03	53F	AA		Unrelated	0	FLU-CY-TBI	0.2	17	0	Alive	Alive	
AA-04	46F	AA		Unrelated	0	FLU-CY-TBI	0.16	16	0	Alive	Alive	
AA-05	45F	AA		Related	0	FLU-CY	0.16	15	0	Alive	Alive	
AA-06	25F	AA		Related	0	FLU-CY	0.16	12	0	Alive	Alive	
AA-07	46M	AA		Unrelated	0	FLU-CY-TBI	0.16	15	0	Alive	Alive	
AA-08	33M	AA		Unrelated	1 allele	FLU-CY-TBI	0.16	17	0	Alive	Died (day 69)	Secondary graft faulre
AA-09	44M	AA		Unrelated	0	FLU-CY-TBI	0.16	16	0	Alive	Alive	
AA-10	30F	AA		Unrelated	1 allele	FLU-CY-TBI	0.16	26	1	Alive	Alive	
AA-11	40F	AA		Unrelated	1 allele	FLU-CY-TBI	0.16	15	0	Alive	Alive	
AA-12	35F	AA		Unrelated	1 allele	FLU-CY-TBI	0.16	17	1	Alive	Died (day 321)	Sepsis
AA-13	25F	AA		Unrelated	1 allele	FLU-CY-TBI	0.16	20	0	Alive	Alive	
AA-14	20F	AA		Related	0	FLU-CY	0.16	14	0	Alive	Alive	
AA-15	21F	AA		Unrelated	0	FLU-CY-TBI	0.16	15	0	Alive	Alive	
MM-01	24F	CML	BP	Related	3 antigens	CY-TBI	0.2	15	0	Alive	Alive	
MM-02	57F	AML	CR2	Related	3 antigens	FLU-BU-TBI	0.2	14	0	Alive	Died (day 185)	Relapse
MM-03	61M	AML	CR2	Related	3 antigens	FLU-BU-TBI	0.2	13	0	Alive	Died (day 178)	Renal failure
MM-04	52F	ALL (Ph)	CR1	Related	2 antigens	CY-TBI	0.16	14	1	Alive	Alive	
MM-05	32M	AML	NR	Related	3 antigens	CY-TBI	0.16	20	0	Alive	Died (day 256)	Relapse
MM-06	26M	AML	CR2	Related	3 antigens	CY-TBI	0.16	17	1	Alive	Alive	
MM-07	51M	AML	NR	Related	2 antigens	CY-TBI	0.16	16	0	Alive	Alive	
MM-08	44F	AML-TLD	CR1	Related	3 antigens	CY-TBI	0.16	16	1	Alive	Alive (relapse)	
MM-09	59F	MDS	RA	Related	2 antigens	FLU-BU-TBI	0.16	15	2	Alve	Alive	
MM-10	44M	NHL	NR	Related	3 antigens	FLU-BU-TBI	0.16	13	0	Alive	Died (day 127)	Progression
MM-11	57F	MDS	RAEB	Related	2 antigens	FLU-BU-TBI	0.16	20	0	Alive	Alive	
MM-12	46F	AML	CR2	Related	2 antigens	CY-TBI	0.16	NA	0	Died	Died (day 20)	Sepsis
MM-13	38F	AML	NR	Related	2 antigens	CY-TBI	0.16	14	2	Alive	Alive	
MM-14	23M	AML	CR2	Related	2 antigens	CY-TBI	0.16	16	0	Alive	Alive	

AA = aplastic anemia; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; BP = blast phase; BU = busulfan; CML = chronic myelogenous leukemia; CR = complete remission; CY = cyclophosphamide; FLU = fludarabine; GVHD = graft-versus-host disease; HLA = human leukocyte antigen; MDS = myelodysplastic syndrome; MM = HLA mismatch; NA = not applicable; NHL = non-Hodgkin lymphoma; NR = not in remission; Ph = Philadelphia chromosome; RA = refractory anemia; RAEB = refractory anemia with excess of blasts; TBI = total body irradiation; TLD = tri-lineage dysplasia; UPN = unique patient number.

All but one patient in the MM study showed persistent complete donor chimerism unless the patient had a relapse. On the other hand, mixed chimerism of more than 10% donor cells was observed in six of the 12 patients in the AA study (Fig. 1). With regard to HLA-mismatch in the AA study, only one of the five patients with an HLA-mismatch developed mixed chimerism, whereas this was seen in five of the seven patients without an HLA-mismatch, but this difference was not statistically significant ($P = 0.24$). Two patients received donor lymphocyte infusion of $0.5 - 1.0 \times 10^6$ CD3-positive cells/kg and both achieved complete donor chimerism. At 180 days after HSCT, the mean white blood cell count, hemoglobin level, and platelet count in patients with mixed chimerism were slightly higher than those in patients with complete donor chimerism (3690 vs. 2557/μL, $P = 0.073$, 10.5 vs. 10.2 g/dL, $P = 0.74$, and 117 vs. 71 $\times 10^9$/L, $P = 0.0094$). None of the patients with declining donor chimerism at 1 year after HSCT developed progressive cytopenia in their subsequent follow-up.

Two of the 12 patients in the AA study developed Grade I acute GVHD and two patients each in the MM study developed Grade I and II acute GVHD, respectively (Table I). There were no instances of Grade III–IV acute GVHD or GVHD-related death. Chronic GVHD was observed in two patients each in the AA and MM studies, including extensive chronic GVHD in one and two patients, respectively.

Infectious complications

CMV was frequently reactivated. All 12 patients in the AA study and nine of the 10 patients in the MM study experienced positive CMV antigenemia. CMV disease was observed in one and three patients in the AA and MM studies, respectively, including retinitis in three and gastroenteritis in one. These patients were treated with antiviral agents and none of these cases were fatal.

At 90 and 180 days after HSCT, EBV reactivation of more than 10^3 copies/mL was observed in two patients in the AA study (2.6×10^4 copies and 1.5×10^3 copies, respectively). On the other hand, none of the patients in the MM study developed EBV reactivation. There was no posttransplantation lymphoproliferative disease (PTLD) in either study.

Anti-alemtuzumab antibody and immune reconstitution

Anti-alemtuzumab antibody was not detected at 7, 35, or 70 days after HSCT in any of the patients in the AA and MM studies.

The recovery of CD3 + CD4+ cells was significantly faster in the MM study ($P = 0.029$), but the recovery of CD3 + CD8+ cells was equivalent ($P = 0.90$, Fig. 2). Geometric mean numbers of CD3 + CD4+ and CD3 + CD8+ cells were 11.6/μL and 45.8/μL at 90 days after HSCT and 140.3/μL and 334.9/μL at 180 days after HSCT, respectively, in the AA study. These values were 49.4/μL and 56.7/μL at 90 days after HSCT and 99.7/μL and 244.0/μL at 180 days after HSCT, respectively, in the MM study. There was no significant difference in the recovery of CD3 + CD4+ or CD3 + CD8+ cells between patients who developed mixed chimerism and those who did not in the AA study ($P = 0.23$ and $P = 0.94$).

The median quantities of CMV-specific CD8+ T lymphocytes as measured by the tetramer-based assay were 0.08% (range 0–0.97%), 0.075% (range 0–0.21%), and 0.05% (range 0.01–0.26%) at 90, 180, and 365 days after HSCT in the AA study and 0.05% (range 0–0.76%), 0.01% (range 0–0.02%), and 1.83% (range 0.01–4.05%) in the MM study.

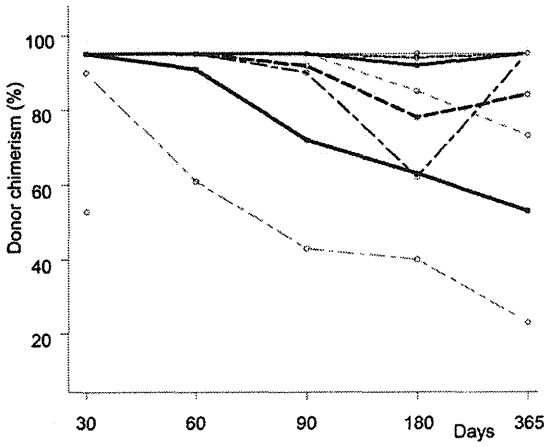

Figure 1. Donor cell chimerism in peripheral blood CD3-positive cell fraction in the AA study.

The results of the serial evaluation of TREC and T-cell repertoire complexity after HSCT are shown in Table II. The TREC levels in CD4+ and CD8+ cells were much lower than those in healthy controls (1440 ± 880 and 1920 ± 1300, respectively) within a year after HSCT [19]. In addition, the TREC level in CD4+ cells in the MM study was lower than that in the AA study ($P = 0.016$ and $P = 0.13$ for CD4+ and CD8+ T-cells, respectively).

On the other hand, the complexity score of T-cell receptor repertoire of CD4+ T-cells became close to that in healthy controls (116.1 ± 2.36) at 180 days after HSCT, while that of CD8+ T-cells remained lower than that in healthy controls (109.49 ± 6.77) within 1 year after HSCT [19]. The complexity scores in the AA study were higher than those in the MM study, but this difference was not statistically significant ($P = 0.13$ and $P = 0.12$ for CD4+ and CD8+ T-cells, respectively).

Transplantation-related mortality, relapse and survival

Overall survival after HSCT among patients who received CAM16 is shown in Fig. 3. The probability of 1-year survival was 83.3% (95% CI: 48.2–95.6%) in the AA study and 72.7% (95% CI: 37.1–90.3%) in the MM study. The causes of death were sepsis and secondary graft failure in one patient each in the AA study, and sepsis in one patient and relapse in two in the MM study. Therefore, transplantation-related mortality was observed in only two and one patients in the AA and MM studies, respectively.

Relapse or progression of underlying malignancy was observed in three of the 11 patients within 1 year after HSCT in the MM study. Another patient developed molecular relapse of acute myeloid leukemia with AML1-MTG8 translocation, but molecular remission was achieved after donor lymphocyte infusion.

Pharmacokinetics

The serial changes in the serum alemtuzumab concentration within 8 days after the first administration of alemtuzumab are shown in Fig. 4. The serum concentration gradually increased and peaked after the last infusion of alemtuzumab. The geometric mean concentrations of alemtuzumab after the last infusion were 10378 ng/mL and 7855 ng/mL in the AA and MM studies, respectively, and the value in the AA study was significantly higher ($P = 0.0063$). On the other hand, there was no significant difference in the peak alemtuzumab concentration between the two conditioning regimens, CY-TBI and FLU-BU, in the MM study ($P = 0.74$). The serum concentrations of alemtuzumab on the day of HSCT in the AA and MM studies were equivalent (geometric mean values 3972 ng/mL and 4070 ng/mL, $P = 0.87$).

The serum alemtuzumab concentration showed a log-linear decrease after HSCT (Fig. 5). There was a significant difference in the serum alemtuzumab concentration after HSCT between patients who received CAM20 and those who received CAM16 in the MM study (Fig. 5A, $P = 0.0088$). On the other hand, a similar difference was not observed in the AA study (Fig. 5B, $P = 0.35$).

There was no significant difference in the alemtuzumab concentration between patients who developed Grade I–IV acute GVHD after HSCT and those who did not among patients who received CAM16 in the MM study (Fig. 5C, $P = 0.84$). Although the alemtuzumab concentration was slightly lower in patients who developed Grade II–IV acute GVHD, this difference was not statistically significant (Fig. 5D, $P = 0.17$). In addition, we did not find a significant correlation between the serum alemtuzumab concentration and the recovery of CD3 + CD4+ cells or CD3 + CD8+ cells (data not shown).

The area under the serum concentration-time curve (AUC) from 0 to infinity was 1,13,609 (S.D. 25631) ng × days /mL in the AA study and 94,521 (S.D. 36538) ng × days /mL in the MM study. The serum half-life was 10.2

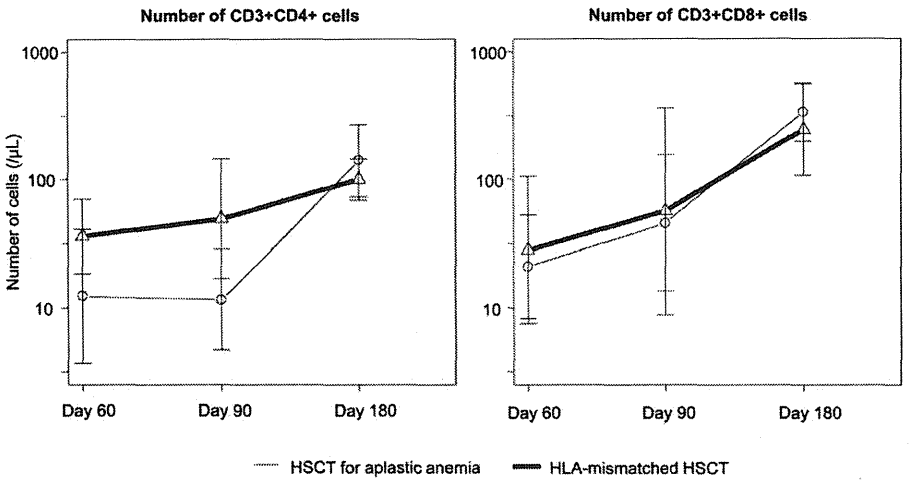

Figure 2. Recovery of T-cell immunity after HSCT.

TABLE II. Immune Reconstitution After Transplantation

	HSCT for aplastic anemia	HLA-mismatched HSCT
CD4 TREC (per 100,000 cells)		
Day 90	5.4	0
Day 180	119.4	15.1
Day 365	177.9	12.8
CD8 TREC (per 100,000 cells)		
Day 90	1.6	0
Day 180	22.6	8.4
Day 365	53.8	6.2
CD4 TCR-CS		
Day 90	76.1	60.7
Day 180	109.3	103.4
Day 365	113.3	104
CD8 TCR-CS		
Day 90	40.6	30.7
Day 180	66.9	65.2
Day 365	76	69.1

HSCT = hematopoietic stem cell transplantation; TREC = T-cell receptor rearrangement excision circle; TCR-CS = T-cell receptor repertoire complexity score.

(S.D. 1.64) days in the AA study and 10.3 (S.D. 1.64) days in the MM study.

Discussion

We evaluated the efficacy of *in vivo* T-cell depletion with alemtuzumab in two prospective studies according to the ICH–GCP guidelines. All of the patients who received alemtuzumab at 0.16 mg/kg/day for 6 days achieved initial engraftment and the incidences of Grade II–IV acute GVHD were 0% and 18% in the AA and MM studies, respectively. Transplantation-related mortality within 1 year after HSCT was observed in only two and one patients, respectively. These findings suggested that the use of alemtuzumab at this dose in the conditioning regimen enables safe allogeneic HSCT in terms of low incidences of acute GVHD and transplantation-related mortality.

The peak alemtuzumab concentrations in our studies were comparable to those in a UK study considering the alemtuzumab dose in each study [23]. Thereafter, the serum alemtuzumab concentration showed a log-linear decrease with a serum half-life of about 10 days. A therapeutic concentration (>100 ng/mL) of alemtuzumab was maintained within 56 days after HSCT [24]. This might have contributed to the low incidence of acute GVHD as well as the high incidence of CMV reactivation.

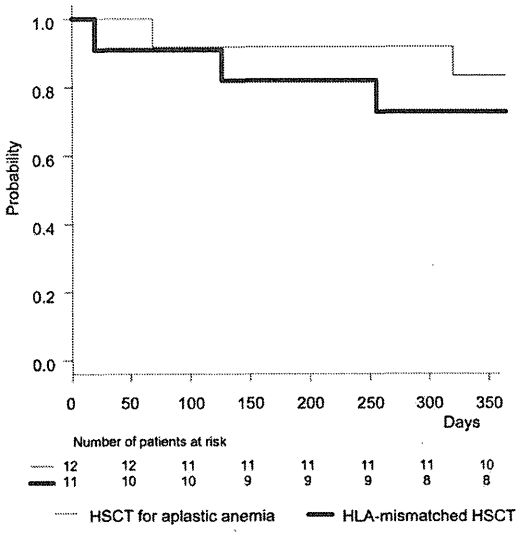

Figure 3. Overall survival after HSCT in the AA study and the HLA-mismatch (MM) study.

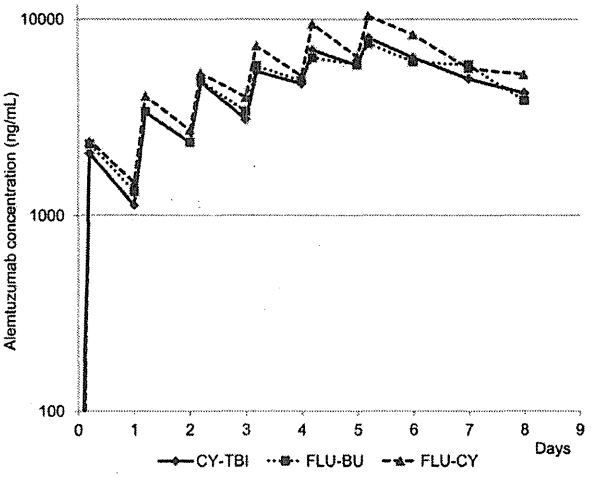

Figure 4. Alemtuzumab concentration within 8 days after the first administration of alemtuzumab grouped according to the conditioning regimen. CY-TBI = cyclophosphamide-total body irradiation, FLU-BU = fludarabine-busulfan.

The dose of alemtuzumab varies widely among previous studies. At first, a total dose of 100 mg of alemtuzumab spread over 5 days was widely used [3,25]. Alemtuzumab at this dose was highly effective for preventing acute and chronic GVHD, but it delayed immune recovery and increased the incidence of viral infection [26,27]. In addition, patients who underwent reduced-intensity HSCT with alemtuzumab and CSA as GVHD prophylaxis required donor lymphocyte infusion for disease control more frequently than those who received GVHD prophylaxis with CSA and methotrexate [28]. Thereafter, several studies have been conducted to decrease the dose of alemtuzumab. Chakraverty et al. showed that the dose of alemtuzumab can be safely reduced to a total of 30 mg in reduced-intensity HSCT from an HLA-identical sibling donor [29]. A Greek group evaluated the efficacy of alemtuzumab at a total dose of 10–20 mg on days -2 and -1 before HSCT [30]. Even with alemtuzumab at these very low doses, there was no Grade II–IV acute GVHD after HSCT from a sibling donor. Grade II and Grade III acute GVHD were each observed in 5% of the patients who underwent HLA-matched unrelated HSCT. In our studies, alemtuzumab was started at a total dose of 1.20 mg/kg and thereafter decreased to a total dose of 0.96 mg/kg. Even in 2- or 3-antigen-mismatched HSCT, the incidence of Grade II–IV acute GVHD was only 18% and there was no Grade III–IV acute GVHD. On the other hand, CMV reactivation was observed in all but one patient. Therefore, it may be possible to further decrease the dose of alemtuzumab to accelerate immune recovery after HSCT.

Mixed chimerism in the CD3+ cell fraction was frequently observed in the AA study, especially in patients who underwent HSCT from an HLA-matched donor. On the other hand, all patients in the MM study achieved sustained complete donor cell chimerism unless they had a relapse. Therefore, the presence of an HLA-mismatch may have enhanced the conversion to complete donor chimerism. The presence of CD3+ cell mixed chimerism was not associated with secondary graft failure or the recurrence of

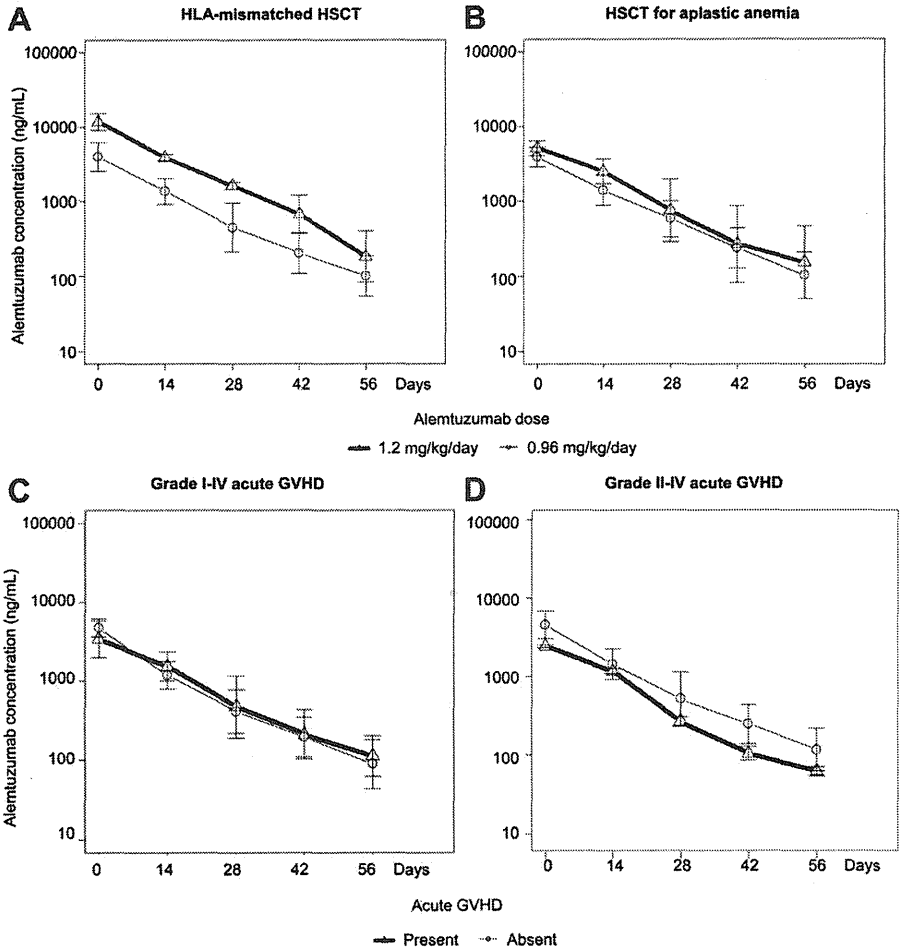

Figure 5. Alemtuzumab concentration after HSCT grouped according to the dose of alemtuzumab (A, B) and grouped according to the development of acute graft-versus-host disease (GVHD) (C, D).

cytopenia, which is consistent with the previous findings by Marsh et al [12].

Immune recovery after HSCT, including the numbers of CD4+ and CD8+ T-cells, copy number of TRECs, and T-cell receptor repertoire complexity, was delayed in both studies. Especially, the TREC levels in the MM study were significantly lower than those in the AA study, and this may have resulted in the slightly lower complexity score in the MM study. The lower TREC levels in the MM study may have been caused by a higher age, the use of TBI in the conditioning regimen, or the presence of an HLA-mismatch. The number of CMV-specific CD8+ T cells in both studies was lower than that in a previous report and this may have contributed to the high incidence of CMV reactivation [17].

In conclusion, the use of alemtuzumab at 0.16 mg/kg/day for 6 days allows donor cell engraftment and strongly prevents acute GVHD even in 2- or 3-antigen-mismatched HSCT. However, frequent CMV reactivation and disease relapse remains a problem, and therefore the optimal dose should be explored in future studies.

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The authors thank Yoko Hamada and Masakane Hayakawa (Bayer Yakuhin, Ltd.) for their tireless efforts in these studies.

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TECHNICAL REPORT

Investigation of the freely available easy-to-use software 'EZR' for medical statistics

Y Kanda

Although there are many commercially available statistical software packages, only a few implement a competing risk analysis or a proportional hazards regression model with time-dependent covariates, which are necessary in studies on hematopoietic SCT. In addition, most packages are not clinician friendly, as they require that commands be written based on statistical languages. This report describes the statistical software 'EZR' (Easy R), which is based on R and R commander. EZR enables the application of statistical functions that are frequently used in clinical studies, such as survival analyses, including competing risk analyses and the use of time-dependent covariates, receiver operating characteristics analyses, meta-analyses, sample size calculation and so on, by point-and-click access. EZR is freely available on our website (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html) and runs on both Windows (Microsoft Corporation, USA) and Mac OS X (Apple, USA). This report provides instructions for the installation and operation of EZR.

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Keywords: statistical software; EZR; competing risk; time-dependent covariate

INTRODUCTION

There are many commercially available statistical software packages, including SAS (SAS Institute Inc., Cary, NC, USA), SPSS (SPSS Inc., Chicago, IL, USA) and Stata (Stata Corporation, College Station, TX, USA). These packages are widely used in the area of medical statistics.[1] However, some of these packages do not implement a competing risk analysis or proportional hazards regression model with time-dependent (TD) covariates, which are necessary in studies on hematopoietic SCT.[2–4] In addition, most packages are not clinician friendly, as they require that commands be written based on statistical languages. R is an open-source freely available software environment for statistical computing and graphics.[5] R supports many functions for statistical analyses, but also requires that the user write commands based on the S statistical language. R commander provides an easy-to-use basic-statistics graphical user interface for R.[6] However, the statistical functions of R commander are limited, especially those in the field of medical statistics. Therefore, I added statistical functions, such as survival analyses, including competing risk analyses and the use of TD covariates, receiver operating characteristics analyses, meta-analyses, sample size calculation and so on, to R commander (version 1.6–3) based on R (version 2.13.0). The result, called 'EZR' (Easy R), is available on our website (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html).[7] EZR runs on both Windows (Microsoft Corporation, Redmond, WA, USA) and Mac OS X (Apple, Cupertino, CA, USA). A complete manual for EZR is currently available only in Japanese.[8] EZR comes with 'absolutely no warranty', just like R itself, and the conditions for redistribution are the same as those for R and R commander (under the GNU General Public License).

INSTALLATION OF EZR

For Windows users, the only required file for installation is EZRsetupENG.exe, which can be downloaded from our website.[7] EZR is installed along with R and R commander just by running this installer on Windows XP, VISTA, 7 or 8 (both 32- and 64-bit versions). The default folder for EZR installation is 'C:\ProgramFiles\EZR', which is different from the default installation folder for R, and therefore the installation of EZR does not interfere with R, which may already be installed. After installation is complete, a shortcut to launch EZR will appear on the desktop. The default data folder is 'C:\EZRDATA', but the data folder can be changed by right-clicking on this shortcut, selecting 'Properties', and replacing the folder name in the 'Start in:' column on the 'Shortcut' tab.

The installation of EZR on Mac OS X is more complicated, but instructions for installation can be found on our website. The following instructions are based on EZR on Windows, but can be applied to EZR on OS X, with some exceptions, such as importing Excel files and creating a new data set on EZR, which are not available in EZR on OS X.

BASIC OPERATIONS IN EZR

EZR can be started by double-clicking on the shortcut on the desktop or selecting EZR from the 'Start menu', which causes two windows to appear on the desktop. The window entitled **'R Console'** on the title bar is the main window for R. This window is not used for usual operation in EZR, but should not be closed, as EZR runs on R. The other window entitled **'EZR on R Commander'** is the main operating window for EZR (Figure 1). Functions of EZR can be selected from the menu bar just below the title bar. This menu bar

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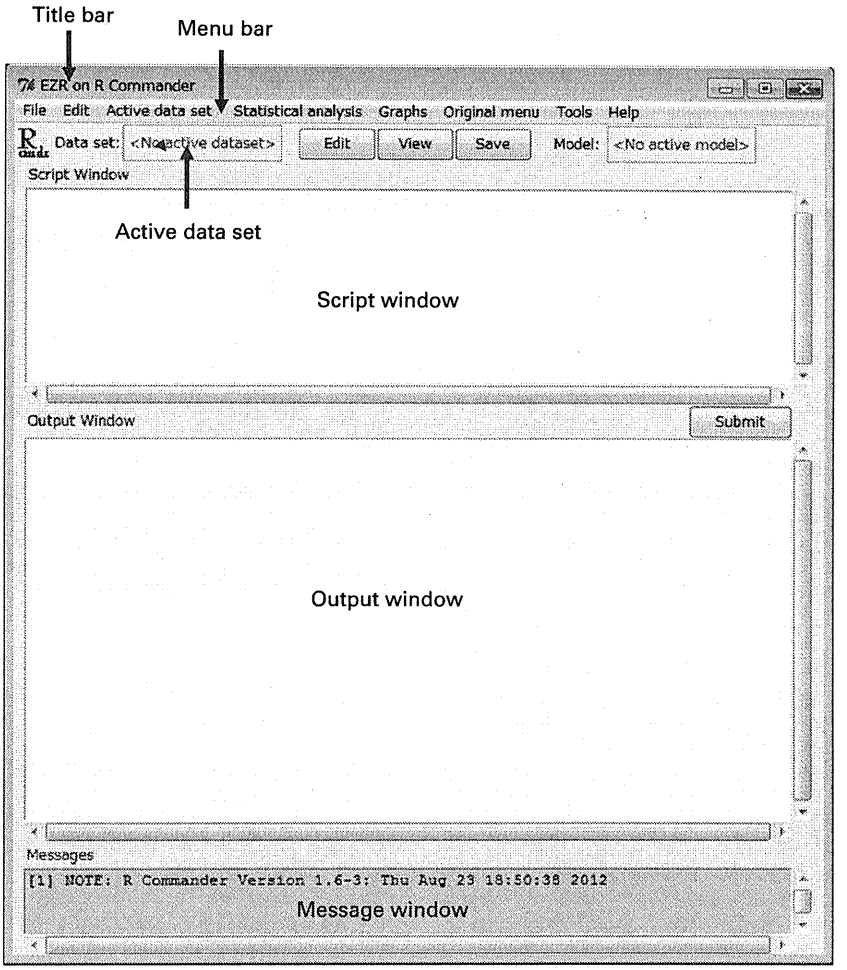

Figure 1. Main window of EZR.

includes the following items: **'File'**, **'Edit'**, **'Active data set'**, **'Statistical analysis'**, **'Graphs'**, **'Original menu'**, **'Tools'** and **'Help'**.

The user can tell EZR what they would like to do by two methods. First, they can type R commands in the **'Script window'** and click on the **'Submit'** button. The alternative method is easier for beginners. EZR functions can be started by point-and-click access using the items on the menu bar. EZR automatically creates and executes corresponding R commands that appear in the **'Script window'**. Results are shown in the **'Output window'**. If any errors or warnings are noted, messages will appear in the **'Message window'**. The created commands can be saved by selecting **'File'** > **'Save script as'** on the menu bar. The output can be saved by selecting **'File'** > **'Save output as'**. By saving the commands, users can reproduce the analyses and can also share the procedure with the other investigators.

CREATING, MODIFYING AND SAVING AN R DATA SET

Windows users can create a new data set directly on EZR by selecting **'File'** > **'New data set'**. However, it is more convenient to create a data set using spreadsheet applications such as Microsoft Excel (Microsoft Corporation). Data sets saved as Excel files (.xls or.xlsx) or comma-separated value (CSV) files (.csv) can be imported to EZR by selecting **'File'** > **'Import data'** > **'From Excel, Access or dBase data set'** or **'File'** > **'Import data'** > **'Read Text Data From File, Clipboard or URL'**, respectively, except that Excel files cannot be imported in EZR on OS X. Alternatively, users can import data by a copy-and-paste approach. Data of interest, copied from a spreadsheet, text file, web site and so on, can be imported into EZR by selecting **'File'** > **'Import data'** > **'Read Text Data From File, Clipboard or URL'**. Authors should choose 'Clipboard' for 'Location of Data file' and 'Tabs' for 'Field Separator' on the dialog to paste from a spreadsheet. EZR can also import SPSS data and Stata data.

In the following instructions, a sample data set that includes 93 fictional patients who received Allo-SCT for acute leukemia will be used. The data set file, 'sample.csv', is available at the http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/sample.csv. Users can directly import the file into EZR by choosing 'Internet URL' for 'Location of Data file' after selecting **'File'** > **'Import data'** > **'Read Text Data From File, Clipboard, or URL'**. Imported data can be viewed by clicking on the **'View'** button and directly edited by clicking on the **'Edit'** button. The list of variables in the data set can be shown by selecting **'Active data set'** > **'Variables'** > **'Show variables in active data set'**. In addition, users can create new variables or modify existing variables using the functions under **'Active data set'** on the menu bar. For example, this sample data set has a continuous variable called 'Age' that represents the patient's age. If a user wants to create a categorical variable, 'Age40', which has a value of 0 for patients less than 40 years old and 1 for those at least 40 years old, they can select **'Active data set'** > **'Variables'** > **'Bin numeric variable with specified**

threshold'. In the dialog, users can select 'Age' from the list of numeric variables, input 'Age40' in the 'New variable name' column and input '40' in the 'Threshold to bin a numeric variable' column. Alternatively, a new variable can be created by selecting **'Active data set'>'Variables'>'Create new variable'**. This function enables more complex computing. For example, if a user wants to create a categorical variable 'ElderlyMale', which would have a value of 1 for male patients aged at least 60 years old and 0 for other patients, they would input 'ifelse(Age>=60 & Sex='Male', 1, 0)' in the 'Expression to compute' column in the dialog.

When a categorical variable with more than two categories is to be analyzed in Fine and Gray regression modeling, 'dummy' variables should be created before analysis, although such 'dummy' variables are automatically created in multiple regression, logistic regression and Cox proportional hazards regression in R. For example, if a user wants to evaluate the effect of the type of stem cell graft, information for which is included in the categorical variable 'Source' as 'BM', peripheral blood 'PB' and cord blood 'CB', they would select **'Active data set'>'Variables'>'Create dummy variables'** to make three categorical variables named 'Source.Dummy.BM', 'Source.Dummy.PB' and 'Source.Dummy.CB'. 'Source.Dummy.BM' has a value of 1 for patients who received BM graft and 0 for others. Users should choose one of the three categories as a reference and input dummy variables for the other two categories into the regression model. The effect size, 95% confidence interval and *P*-value for each category with respect to the reference category will then be shown. If a user directly inputs a categorical variable into multiple regression, logistic regression or Cox proportional hazards regression, the automatically created dummy variables are shown as 'Source [T.CB]' or 'Source [T.PB]', for example. The stepwise selection function of explanatory variables based on Akaike information criterion and Bayesian information criterion only accepts these automatically created dummy variables, whereas stepwise selection based on *P*-value also accepts dummy variables created by a user using EZR. If the option for a 'Wald test for overall *P*-value for factors with >2 levels' is selected in the dialog of the regression analyses, the overall *P*-value for the categorical variable will be calculated.

The modified data set can be saved as an R file (.rda) by clicking the **'Save'** button or selecting **'File'>'Active data set'>'Save active data set'**. Only the active data set, as indicated in the column just to the right of **'Data set:'** is saved. The saved data set can be reloaded to EZR by selecting **'File'>'Load data set'**.

SUMMARIZING DATA

Descriptive statistics enable the user to glance over features of the data, such as the distribution of or outliers among continuous variables. The proportion of categorical variables is shown by selecting **'Statistical analysis'>'Discrete variables'>'Frequency distributions'**. The mean value with the s.d. along with the minimum, median and maximum values of continuous variables are shown by selecting **'Statistical analysis'>'Continuous variables'>'Numerical summaries'**, but plotting a histogram or a dot chart by selecting **'Graphs'>'Histogram'** or **'Dot chart'** may be more useful for evaluating the distribution of continuous variables.

A table that shows patient characteristics can be easily created by selecting **'Statistical analysis'>'Discrete variables'>'Create two-way table and compare two proportions'**. A grouping variable, 'Source' for example, should be specified in the 'Column variable' list and categorical variables that are to be compared among groups should be specified in the 'Row variable' list. More than one variable can be selected by clicking variables while pressing the 'Ctrl' key. A summary table will then be shown in the 'Output window' following the results of statistical tests (Fisher's exact test by default) to compare the proportions of each variable among the groups. A formatted table for presentation can be created by inputting 'w.twoway()' in the **'Script window'** and clicking on the **'Submit'** button. The table will be copied to the clipboard and can be pasted into a spreadsheet.

STATISTICAL ANALYSES FOR CATEGORICAL AND CONTINUOUS VARIABLES

Statistical analysis functions for categorical variables, including Fisher's exact test, χ^2 test, McNemar test, Cochran *Q* test, Cochran–Armitage test and logistic regression, can be accessed in the **'Statistical analysis'>'Discrete variables'** menu. Statistical analysis functions for continuous variables, including the Smirnov–Grubbs test, Kolmogorov–Smirnov test, *t*-test, paired *t*-test, F-test, Bartlett's test, one-way analysis of variance, multi-way analysis of variance, repeated-measures analysis of variance, analysis of covariance, Pearson's correlation test and linear regression, can be accessed in the **'Statistical analysis'>'Continuous variables'** menu. Nonparametric tests, including the Mann–Whitney *U*-test, Wilcoxon's signed rank test, Kruskal–Wallis test, Friedman test, Jonckheere–Terpstra test and Spearman's rank correlation test, are available in the **'Statistical analysis'>'Nonparametric tests'** menu.

SURVIVAL ANALYSIS

A survival analysis, which is often the primary end point of studies on hematopoietic SCT, can be performed by selecting statistical functions in the **'Statistical analysis'>'Survival analysis'** menu. For example, users can plot Kaplan–Meier curves and compare survival curves among groups with a log-rank test by selecting **'Statistical analysis'>'Survival analysis'>'Kaplan-Meier survival curve and logrank test'**. At least two variables are required: a time-to-event variable, which indicates the time to the occurrence of an event (death in survival analysis) or time to the last evaluation for patients without an event, and a status variable, which has a value of 1 for event and 0 for no event. Users can choose many options in the dialog that mainly involve plotting survival curves (Figures 2a and b). In the **'Output window'**, the results of log-rank test can be found following the point estimations with 95% confidence intervals of survival rates (Figures 3a and b). If more than 1 grouping variable is specified, a summary table will be shown, which can be copied to the clipboard by the w.survival() command (Figure 3c).

A Cox proportional hazards regression can be performed by selecting **'Statistical analysis'>'Survival analysis'>'Cox proportional hazard regression'**. Users have to specify a time-to-event variable, a status variable (1 for event and 0 for no event) and explanatory variables (Figure 4a). In addition, users can choose the following options in the dialog; Wald test for overall *P*-value for factors with 2 or more levels, test the proportional hazards assumption, show the baseline survival curve and stepwise selection of explanatory variables based on Akaike information criterion, Bayesian information criterion and *P*-value. In the **'Output window'**, the main result of Cox proportional hazard regression can be found that includes the hazard ratios, their 95% confidence intervals and *P*-values for each explanatory variable, followed by the results of three tests for the global null hypothesis (none of the explanatory variables is associated with the response) (Figure 5a). A summary of proportional hazards regression analysis, the results of Wald test and the results of testing the proportional hazards assumption are shown below the main result (Figure 5b), followed by the results of stepwise selection of explanatory variables (Figure 5c), if requested. The results of a proportional hazards regression analysis can be copied to the clipboard by the w.multi() command. The output of this