## Acknowledgements

We thank all the collaborating institutions and their staff for providing the patient data. This study was funded by the Center for Supporting Hematology-Oncology Trials.

*Conflict of interest:* The authors declare no conflicts of interest.

## References

1. Wisplinghoff H, Seifert H, Wenzel RP, Edmond MB. Current trends in the epidemiology of nosocomial bloodstream infections in patients with hematological malignancies and solid neoplasms in hospitals in the United States. *Clin Infect Dis* 2003;**36**:1103–10.
2. Chatzinikolaou I, Abi-Said D, Bodey GP, Rolston KV, Tarrand JJ, Samonis G. Recent experience with *Pseudomonas aeruginosa* bacteremia in patients with cancer. Retrospective analysis of 245 episodes. *Arch Intern Med* 2000;**160**:501–9.
3. Klastersky J. Management of infection in granulocytopenic patients. *J Antimicrob Chemother* 1983;**12**:102–6.
4. Schimpff S. Empiric antibiotic therapy for granulocytopenic cancer patients. *Am J Med* 1986;**80**:13–33.
5. Ramphal R. Is monotherapy for febrile neutropenia still a viable alternative? *Clin Infect Dis* 1999;**29**:508–14.
6. Pizzo PA, Hathorn JW, Hiemenz J, Browne M, Commers J, Cotton D, et al. A randomized trial comparing ceftazidime alone with combination antibiotic therapy in cancer patients with fever and neutropenia. *N Engl J Med* 1986;**315**:552–8.
7. Tamura K, Imajo K, Akiyama N, Suzuki K, Urabe A, Ohyashiki K, et al. Randomized trial of cefepime monotherapy or cefepime in combination with amikacin as empirical therapy for febrile neutropenia. *Clin Infect Dis* 2004;**39**(Suppl 1):S15–24.
8. Bow EJ, Rotstein C, Noskin GA, Laverdiere M, Schwarer AP, Segal BH, et al. A randomized, open-label, multicenter comparative study of the efficacy and safety of piperacillin–tazobactam and cefepime for the empirical treatment of febrile neutropenic episodes in patients with hematologic malignancies. *Clin Infect Dis* 2006;**43**:447–59.
9. Del Favero A, Menichetti F, Martino P, Bucaneve G, Micozzi A, Gentile G, et al. A multicenter, double-blind, placebo-controlled trial comparing piperacillin–tazobactam with and without amikacin as empiric therapy for febrile neutropenia. *Clin Infect Dis* 2001;**33**:1295–301.
10. Freifeld AG, Bow EJ, Sepkowitz KA, Boeckh MJ, Ito JI, Mullen CA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer—2010 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2011;**52**:e56–93.
11. Senda K, Arakawa Y, Nakashima K, Ito H, Ichiyama S, Shimokata K, et al. Multifocal outbreaks of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum beta-lactams, including carbapenems. *Antimicrob Agents Chemother* 1996;**40**:349–53.
12. Gniadkowski M. Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. *Clin Microbiol Infect* 2001;**7**:597–608.
13. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* 2010;**10**:597–602.
14. Oliphant CM, Green GM. Quinolones: a comprehensive review. *Am Fam Physician* 2002;**65**:455–64.
15. Davis R, Markham A, Balfour JA. Ciprofloxacin. An updated review of its pharmacology, therapeutic efficacy and tolerability. *Drugs* 1996;**51**:1019–74.
16. Peacock JE, Herrington DA, Wade JC, Lazarus HM, Reed MD, Sinclair JW, et al. Ciprofloxacin plus piperacillin compared with tobramycin plus piperacillin as empirical therapy in febrile neutropenic patients. A randomized, double-blind trial. *Ann Intern Med* 2002;**137**:77–87.
17. Bliziotis IA, Michalopoulos A, Kasiakou SK, Samonis G, Christodoulou C, Chrysanthopoulou S, et al. Ciprofloxacin vs an aminoglycoside in combination with a beta-lactam for the treatment of febrile neutropenia: a meta-analysis of randomized controlled trials. *Mayo Clin Proc* 2005;**80**:1146–56.
18. Kern WV, Cometta A, De Bock R, Langenaeken J, Paesmans M, Gaya H. Oral versus intravenous empirical antimicrobial therapy for fever in patients with granulocytopenia who are receiving cancer chemotherapy. International Antimicrobial Therapy Cooperative Group of the European Organization for Research and Treatment of Cancer. *N Engl J Med* 1999;**341**:312–8.
19. Johnson PR, Liu Yin JA, Tooth JA. A randomized trial of high-dose ciprofloxacin versus azlocillin and netilmicin in the empirical therapy of febrile neutropenic patients. *J Antimicrob Chemother* 1992;**30**:203–14.
20. Meunier F, Zinner SH, Gaya H, Calandra T, Viscoli C, Klastersky J, et al. Prospective randomized evaluation of ciprofloxacin versus piperacillin plus amikacin for empiric antibiotic therapy of febrile granulocytopenic cancer patients with lymphomas and solid tumors. The European Organization for Research on Treatment of Cancer International Antimicrobial Therapy Cooperative Group. *Antimicrob Agents Chemother* 1991;**35**:873–8.
21. Bayston KF, Want S, Cohen J. A prospective, randomized comparison of ceftazidime and ciprofloxacin as initial empiric therapy in neutropenic patients with fever. *Am J Med* 1989;**87**:269S–73S.
22. Hoogkamp-Korstanje JA. In-vitro activities of ciprofloxacin, levofloxacin, lomefloxacin, ofloxacin, pefloxacin, sparfloxacin and trovafloxacin against Gram-positive and Gram-negative pathogens from respiratory tract infections. *J Antimicrob Chemother* 1997;**40**:427–31.
23. Kayser FH, Novak J. In vitro activity of ciprofloxacin against Gram-positive bacteria. An overview. *Am J Med* 1987;**82**:33–9.
24. Saengsuwan P, Jaruratanasirikul S, Jullangkoon M, Aeinlang N. Comparative study of pharmacokinetics/pharmacodynamics of ciprofloxacin between 400 mg intravenously every 8 h and 400 mg intravenously every 12 h in patients with Gram negative bacilli bacteremia. *J Med Assoc Thai* 2010;**93**:784–8.
25. Johnson PR, Yin JA, Tooth JA. High dose intravenous ciprofloxacin in febrile neutropenic patients. *J Antimicrob Chemother* 1990;**26**(Suppl F):101–7.
26. Forrest A, Nix DE, Ballow CH, Goss TF, Birmingham MC, Schentag JJ. Pharmacodynamics of intravenous ciprofloxacin in seriously ill patients. *Antimicrob Agents Chemother* 1993;**37**:1073–81.
27. Kelsey SM, Wood ME, Shaw E, Jenkins GC, Newland AC. A comparative study of intravenous ciprofloxacin and benzylpenicillin versus netilmicin and piperacillin for the empirical treatment of fever in neutropenic patients. *J Antimicrob Chemother* 1990;**25**:149–57.
28. Kelsey SM, Collins PW, Delord C, Weinhard B, Newland AC. A randomized study of teicoplanin plus ciprofloxacin versus gentamicin plus piperacillin for the empirical treatment of fever in neutropenic patients. *Br J Haematol* 1990;**76**(Suppl 2):10–3.
29. Philpott-Howard JN, Barker KF, Wade JJ, Kaczmarski RS, Smedley JC, Mufti GJ. Randomized multicentre study of ciprofloxacin and azlocillin versus gentamicin and azlocillin in the treatment of febrile neutropenic patients. *J Antimicrob Chemother* 1990;**26**(Suppl F):89–99.
30. Ball P. Ciprofloxacin: an overview of adverse experiences. *J Antimicrob Chemother* 1986;**18**(Suppl D):187–93.
31. Freifeld A, Marchigiani D, Walsh T, Chanock S, Lewis L, Hiemenz J, et al. A double-blind comparison of empirical oral and intravenous antibiotic therapy for low-risk febrile patients with neutropenia during cancer chemotherapy. *N Engl J Med* 1999;**341**:305–11.

Leukemia (2013) **27**, 286–294

**www.nature.com/leu**

**ORIGINAL ARTICLE**

# Unrelated cord blood transplantation vs related transplantation with HLA 1-antigen mismatch in the graft-versus-host direction

J Kanda[1], T Ichinohe[2], S Kato[3], N Uchida[4], S Terakura[5], T Fukuda[6], M Hidaka[7], Y Ueda[8], T Kondo[9], S Taniguchi[4], S Takahashi[10], T Nagamura-Inoue[11], J Tanaka[12], Y Atsuta[13], K Miyamura[14] and Y Kanda[1] on behalf of the Donor/Source Working Group and HLA Working Group of the Japan Society for Hematopoietic Cell Transplantation

Little information is available regarding whether an unrelated cord blood (UCB) unit or a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the graft-versus-host direction (RD/1AG-MM-GVH) should be selected as an alternative donor for patients without an HLA-matched related/unrelated donor. Therefore, we conducted a retrospective study using national registry data on patients with leukemia or myelodysplastic syndrome who received transplantation using a single UCB ($n = 2288$) unit or an RD/1AG-MM-GVH ($n = 525$). We found that the survival rate in the UCB group was comparable to that in the RD/1AG-MM-GVH group, although the RD/1AG-MM-GVH group with an HLA-B mismatch showed significantly higher overall and non-relapse mortality. Neutrophil and platelet engraftment were significantly faster, whereas the incidence of acute or chronic graft-versus-host disease (GVHD) was significantly higher in the RD/1AG-MM-GVH group. The incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group with *in vivo* T-cell depletion was comparable to that in the UCB group, which translated into a trend toward better overall survival, regardless of the presence of an HLA-B mismatch. In conclusion, UCB and RD/1AG-MM-GVH are comparable for use as an alternative donor, except for RD/1AG-MM-GVH involving an HLA-B mismatch.

*Leukemia* (2013) **27,** 286–294; doi:10.1038/leu.2012.203

**Keywords:** cord blood transplantation; related transplantation; HLA mismatch; alternative donor

## INTRODUCTION

For patients who lack an HLA-identical sibling, an HLA-matched unrelated donor (MUD) is considered to be the preferred alternative donor in allogeneic hematopoietic cell transplantation (HCT).[1–5] However, it is difficult to find an MUD for patients with rare HLA haplotypes. Furthermore, it takes at least a few months from the start of an unrelated donor search to actually receive a graft. Therefore, there is a large demand for an alternative source to an HLA-identical sibling or MUD, particularly for patients who have a rare haplotype or who need immediate transplantation.

Unrelated cord blood (UCB) has emerged as a promising alternative source for pediatric and adult patients.[6–17] In UCB transplantation, up to two antigen/allele mismatches between a recipient and cord blood unit are acceptable without an increased risk of acute graft-versus-host disease (GVHD). The clinical outcome in UCB transplantation is improving, and is almost comparable to that in HLA 8/8 allele MUD transplantation, although a high risk of graft failure and early treatment-related complications are still major issues.[15–17]

Another alternative source is an HLA-mismatched related donor, particularly when a related donor with a 1-antigen mismatch at the HLA-A, HLA-B, or HLA-DR locus in the graft-versus-host (GVH) direction (RD/1AG-MM-GVH) is available. HCT from an RD/1AG-MM-GVH results in a higher but acceptable incidence of acute GVHD.[18–20] In previous studies, HLA mismatches in the host-versus-graft (HVG) direction were associated with a higher incidence of graft failure and lower overall survival (OS).[18,19,21] However, the risk of graft failure might have been improved by the use of conditioning regimens that strongly suppress the recipient's immune system.[22] Therefore, in current clinical practice in Japan, stem cell transplantation from an RD/1AG-MM-GVH is being performed while accepting multiple antigen mismatches in the HVG direction without specific *ex vivo* stem cell manipulation.[18,19,23] We have recently reported that OS in transplantation from an RD/1AG-MM-GVH involving an HLA-B antigen mismatch was inferior, whereas that from an RD/1AG-MM-GVH involving an HLA-A or -DR antigen mismatch was comparable to that from an 8/8-MUD in standard-risk diseases.[23]

Unlike transplantation from an MUD, transplantation using a UCB unit or an RD/1AG-MM-GVH can be performed immediately when necessary. However, little information is available regarding the priority in selecting these alternative donors. Therefore, we conducted a retrospective study using national registry data on 2813 patients with leukemia or myelodysplastic syndrome (MDS)

[1]Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama, Japan; [2]Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, Japan; [3]Department of Cell Transplantation & Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan; [4]Department of Hematology, Toranomon Hospital, Tokyo, Japan; [5]Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan; [6]Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo, Japan; [7]Department of Internal Medicine, National Hospital Organization, Kumamoto Medical Center, Kumamoto, Japan; [8]Department of Haematology/Oncology, Kurashiki Central Hospital, Kurashiki, Japan; [9]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [10]Department of Molecular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [11]Department of Cell Processing & Transfusion, Research Hospital, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [12]Hematology and Oncology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [13]Department of Hematopoietic Stem Cell Transplantation Data Management/Biostatistics, Nagoya University School of Medicine, Nagoya, Japan and [14]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan. Correspondence: Dr J Kanda, Division of Hematology, Saitama Medical Center, Jichi Medical University, 1-847 Amanuma-cho, Omiya-ku, Saitama city, Saitama, Japan.
E-mail: jkandajp@gmail.com
Received 15 June 2012; revised 5 July 2012; accepted 11 July 2012; accepted article preview online 18 July 2012; advance online publication, 10 August 2012

who received transplantation using a single UCB or an RD/1AG-MM-GVH.

## MATERIALS AND METHODS

### Data collection

Data for patients (age: ⩾16 years) with acute myeloid leukemia, acute lymphoblastic leukemia, MDS and chronic myelogenous leukemia who received a first HCT using a single HLA 0–2 antigen-mismatched UCB unit or an RD/1AG-MM-GVH between 1 January 1998 and 31 December 2009 were obtained from the Transplant Registry Unified Management Program (TRUMP),[24] which includes data from the Japan Cord Blood Bank Network (JCBBN) and the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Our analysis included 2306 patients who received a single UCB graft (UCB group) and 541 patients who received a graft from an RD/1AG-MM-GVH (RD/1AG-MM-GVH group). As of January 2012, double UCB grafts for HCT are not available in Japan. The following patients were excluded: 26 patients who lacked data on survival status, survival date, sex of recipient, or GVHD prophylaxis and 8 patients who received stem cells that had been manipulated by *ex vivo* T-cell depletion or CD34 selection. Overall, 2288 patients who received a UCB unit and 525 who received a graft from an RD/1AG-MM-GVH fulfilled the criteria. The study was approved by the data management committees of TRUMP and by the institutional review boards of Japanese Red Cross Nagoya First Hospital and Saitama Medical Center, Jichi Medical University, where this study was organized.

### Histocompatibility

Histocompatibility data for the HLA-A, HLA-B and HLA-DR loci were obtained from reports from the institution where the transplantation was performed or from cord blood banks. To reflect current practice in Japan, HLA matching in UCB or RD/1AG-MM-GVH transplantation was assessed by serological data for HLA-A, HLA-B, and HLA-DR loci. An HLA mismatch in the GVH direction was defined as when the recipient's antigens or alleles were not shared by the donor, whereas a mismatch in the HVG direction was defined as when the donor's antigens or alleles were not shared by the recipient.

### End points

The primary end point of the study was to compare OS rates between the UCB and RD/1AG-MM-GVH groups. Other end points were the cumulative incidences of neutrophil and platelet engraftment, acute and chronic GVHD, relapse, and non-relapse mortality (NRM). Neutrophil recovery was considered to have occurred when the absolute neutrophil count exceeded $0.5 \times 10^9$/l for 3 consecutive days following transplantation. Platelet recovery was considered to have occurred when the absolute platelet count exceeded $50 \times 10^9$/l without platelet transfusion. The physicians who performed transplantation at each center diagnosed and graded acute and chronic GVHD according to the traditional criteria.[25,26] The incidence of chronic GVHD was evaluated in patients who survived for at least 100 days.

### Statistical analysis

Descriptive statistics were used to summarize variables related to the patient characteristics. Comparisons between groups were performed with the $\chi^2$-test or extended Fisher's exact test as appropriate for categorical variables and the Mann–Whitney *U*-test for continuous variables. The probability of OS was estimated according to the Kaplan–Meier method, and the groups were compared with the log-rank test. The adjusted probability of OS was estimated according to the Cox proportional-hazards model, with other significant variables considered in the final multivariate model. The probabilities of neutrophil and platelet engraftment, acute and chronic GVHD, NRM, and relapse were estimated on the basis of cumulative incidence methods, and the groups were compared with the Gray test;[27,28] competing events were death without engraftment for neutrophil and platelet engraftment, death or relapse without GVHD for acute and chronic GVHD, death without relapse for relapse, and relapse for NRM. The Cox proportional-hazards model was used to evaluate variables that may affect OS, whereas the Fine and Gray proportional-hazards model was used to evaluate variables that may affect engraftment, GVHD, NRM and relapse.[29] We classified the conditioning regimen as myeloablative if either total body irradiation >8 Gy, oral busulfan ⩾9 mg/kg, intravenous busulfan ⩾7.2 mg/kg, or melphalan >140 mg/m$^2$ was used in the conditioning regimen, and otherwise classified it as reduced intensity, based on the report by the Center for International Blood and Marrow Transplant Research.[30] For patients for whom the doses of agents used in the conditioning regimen were not available, we used the information on conditioning intensity (myeloablative or reduced intensity) reported by the treating clinicians. Acute leukemia in the first or second remission, chronic myelogenous leukemia in the first or second chronic phase or accelerated phase, and MDS with refractory anemia or refractory anemia with ringed sideroblasts were defined as standard-risk diseases, and other conditions were defined as high-risk diseases. The following variables were considered when comparing the UCB and RD/1AG-MM-GVH groups: the recipient's age group (⩽50 years or >50 years at transplantation), sex of recipient, disease (acute myeloid leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia or MDS), disease status before transplantation (standard- or high-risk), type of conditioning regimen (myeloablative or reduced intensity), type of GVHD prophylaxis (calcineurin inhibitor and methotrexate, calcineurin inhibitor only, or other), year of transplantation (1998–2004, 2005–2009), and the time from diagnosis to transplantation (<6 months or ⩾6 months). In the analysis within the RD/1AG-MM-GVH group, the use of *in vivo* T cell depletion (no vs yes), stem cell source (peripheral blood (PB) stem cells vs bone marrow (BM)), and the number of HLA mismatches in the HVG direction (0–1 vs 2–3) were also considered. Factors without a variable of main interest were selected in a stepwise manner from the model with a variable retention criterion of $P<0.05$. We then added a variable of main interest to the final model. All tests were two-sided, and $P<0.05$ was considered to indicate statistical significance. All statistical analyses were performed with Stata version 12 (Stata Corp., College Station, TX, USA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan).[31] EZR is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0, Vienna, Austria). More precisely, it is a modified version of R commander (version 1.6–3) that was designed to add statistical functions that are frequently used in biostatistics.

## RESULTS

### Characteristics of patients and transplants

Table 1 shows the patient and transplant characteristics. Recipients of an RD/1AG-MM-GVH were younger than recipients of a UCB unit. Approximately half of the recipients in the RD/1AG-MM-GVH group received PB. The number of HLA mismatches in the GVH direction between a UCB unit and recipient was 0 in 10%, 1 in 33% and 2 in 57%. In the RD/1AG-MM-GVH group, the number of antigen mismatches in the HVG direction was 0 in 12%, 1 in 68%, 2 in 18% and 3 in 3%. Most of the recipients of an RD/1AG-MM-GVH received a calcineurin inhibitor with methotrexate for GVHD prophylaxis, whereas 25% of UCB recipients received only calcineurin inhibitor. *In vivo* T-cell depletion including antithymocyte globulin (ATG) or alemtuzumab was used in 10% of the RD/1AG-MM-GVH group, but in only 1% of the UCB group. Alemtuzumab was used in only one patient, who received transplantation from an RD/1AG-MM-GVH. Information regarding the dose and type of ATG was missing in two-third of the patients who received ATG. Available data showed that the median dose of thymoglobulin was 2.5 (range 2.5–9.0, $n=9$) and 2.5 (range 1.25–5.0, $n=10$) mg/kg and the median dose of ATG-Fresenius was 8.0 (range 5.0–10.0, $n=3$) and 8.0 (range 5.0–10.0, $n=7$) mg/kg, in the UCB and RD/1AG-MM-GVH groups, respectively. Two-third of UCB transplantations were performed between 2005 and 2009. The median duration of follow-up for survivors was 2 and 4 years in the UCB and RD/1AG-MM-GVH groups, respectively.

### Neutrophil and platelet engraftment

The incidence of neutrophil engraftment at day 50 in the RD/1AG-MM-GVH group was higher than that in the UCB group (UCB group, 73%, 95% confidence interval (CI), 71–75%; RD/1AG-MM-GVH group, 93%, 95% CI, 91–95%; Gray test, $P<0.001$; Figure 1a). The incidence of platelet engraftment at day 150 in the

**Table 1.** Patient characteristics

| *Variable* | *UCB (n = 2288)* | *RD/1AG-MM-GVH (n = 525)* | P |
|---|---|---|---|
| Age at transplant, median (range) | 49 (16–82) | 43 (16–74) | <0.001 |
| *Recipient sex* | | | |
| Female | 1004 (44%) | 239 (46%) | 0.494 |
| Male | 1284 (56%) | 286 (54%) | |
| *Disease* | | | |
| Acute myelogenous leukemia | 1365 (60%) | 269 (51%) | 0.003 |
| Acute lymphoblastic leukemia | 498 (22%) | 137 (26%) | |
| Chronic myelogenous leukemia | 124 (5%) | 42 (8%) | |
| Myelodysplastic syndrome | 301 (13%) | 77 (15%) | |
| *Duration from diagnosis to transplant* | | | |
| Median time (range), months | 7.9 (0.2–768.5) | 7.6 (0–251.7) | 0.233 |
| *Disease risk* | | | |
| Standard | 959 (42%) | 249 (47%) | 0.050 |
| High | 1217 (53%) | 257 (49%) | |
| Unknown | 112 (5%) | 19 (4%) | |
| *Source of stem cells* | | | |
| Bone marrow | — | 251 (48%) | — |
| Peripheral blood | — | 274 (52%) | |
| Cord blood | 2288 (100%) | — | |
| *HLA compatibility in the graft-versus-host direction* | | | |
| Matched | 225 (10%) | — | <0.001 |
| One-antigen mismatch | 753 (33%) | 525 (100%) | |
| Two-antigen mismatch | 1310 (57%) | — | |
| *HLA compatibility in the host-versus-graft direction* | | | |
| Matched | 233 (10%) | 62 (12%) | <0.001 |
| One-antigen mismatch | 716 (31%) | 355 (68%) | |
| Two-antigen mismatch | 1339 (59%) | 94 (18%) | |
| Three-antigen mismatch | — | 14 (3%) | |
| *Conditioning regimen* | | | |
| Myeloablative | 1390 (61%) | 253 (48%) | <0.001 |
| CY + TBI ± | 1062 | 164 | |
| Other TBI regimen | 130 | 20 | |
| BU + CY ± | 88 | 45 | |
| Other non-TBI regimen | 110 | 24 | |
| Reduced intensity | 894 (39%) | 162 (31%) | |
| FLU ± TBI ± | 840 | 138 | |
| Other regimen | 54 | 24 | |
| Unclassifiable | 4 (0.2%) | 110 (21%) | |
| *GVHD prophylaxis* | | | |
| CSA/TAC + MTX | 1410 (62%) | 448 (85%) | <0.001 |
| CSA/TAC + MMF | 246 (11%) | 12 (2%) | |
| CSA/TAC + Steroid | 28 (1%) | 13 (2%) | |
| CSA/TAC only | 571 (25%) | 45 (9%) | |
| Unknown | 33 (1%) | 7 (1%) | |
| *Use of* in vivo *T-cell depletion* | | | |
| No | 2258 (99%) | 472 (90%) | <0.001 |
| Yes | 30 (1%) | 53 (10%) | |
| *Year at transplant* | | | |
| 1998–2004 | 760 (33%) | 260 (50%) | <0.001 |
| 2005–2009 | 1528 (67%) | 265 (50%) | |
| *Follow-up of survivors* | | | |
| Median time (range), years | 2.1 (0.0–10.0) | 4.0 (0.1–12.2) | <0.001 |

Abbreviations: BU, busulfan; CSA, cyclosporine; CY, cyclophosphamide; FLU, fludarabine; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus; TBI, total body irradiation; UCB, unrelated cord blood.

RD/1AG-MM-GVH group was also higher than that in the UCB group (UCB group, 53%, 95% CI, 51–55%; RD/1AG-MM-GVH group, 70%, 95% CI, 66–74%; Gray test, $P<0.001$; Figure 1b). The use of RD/1AG-MM-GVH was significantly associated with a higher incidence of neutrophil and platelet engraftment in the multivariate analysis (neutrophil engraftment, hazard ratio (HR), 3.46,

95% CI, 3.00–3.98, $P<0.001$; platelet engraftment, HR 2.20, 95% CI, 1.89–2.57, $P<0.001$; Supplementary Table 1). As our previous study revealed that an HLA-B mismatch had an adverse effect on OS in transplantation from an RD/1AG-MM-GVH, patients in the RD/1AG-MM-GVH group with an HLA-A, -B, or -DR mismatch were separately compared with the UCB group. We consistently observed superior neutrophil and platelet engraftment in each RD/1AG-MM-GVH group as compared with the UCB group (Supplementary Table 1).

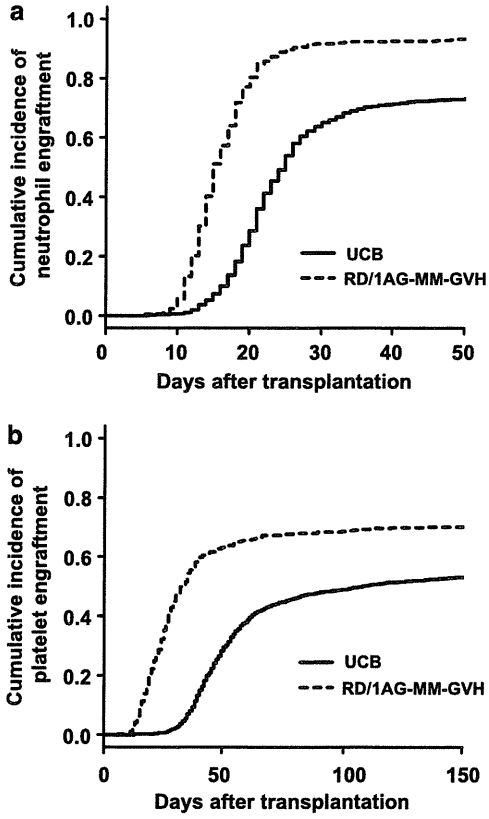


**Figure 1.** Neutrophil (**a**) and platelet engraftment (**b**).

## Acute and chronic GVHD

The incidence of grade II–IV or grade III–IV acute GVHD in the RD/1AG-MM-GVH group was significantly higher than that in the UCB group (grade II–IV acute GVHD at day 100: UCB group, 34%, 95% CI, 32–36%; RD/1AG-MM-GVH group, 50%, 95% CI, 45–54%; Gray test, $P<0.001$; grade III–IV acute GVHD at day 100: UCB group, 11%, 95% CI, 10–13%; RD/1AG-MM-GVH group, 21%, 95% CI, 17–24%; Gray test, $P<0.001$; Figures 2a and b). The incidence of chronic GVHD or extensive type of chronic GVHD in the RD/1AG-MM-GVH group was also significantly higher than that in the UCB group (chronic GVHD at 3 years: UCB group, 25%, 95% CI, 23–27%; RD/1AG-MM-GVH group, 42%, 95% CI, 38–47%; Gray test, $P<0.001$; extensive chronic GVHD at 3 years: UCB group, 11%, 95% CI, 10–13%; RD/1AG-MM-GVH group, 29%, 95% CI, 25–34%; Gray test, $P<0.001$; Figures 2c and d). A multivariate analysis confirmed a higher risk of grade II–IV or grade III–IV acute GHVD, chronic or extensive chronic GVHD in the RD/1AG-MM-GVH group than in the UCB group (grade II–IV acute GVHD; HR 1.64, 95% CI, 1.43–1.90, grade III–IV acute GVHD; HR 2.28, 95% CI, 1.80–2.88, chronic GVHD; HR 1.47, 95% CI, 1.24–1.73, extensive chronic GVHD; HR 2.35, 95% CI, 1.90–2.91, Supplementary Table 2).

## OS

The 3-year unadjusted OS rates in the UCB and RD/1AG-MM-GVH groups were 38% (36–41%) and 39% (34–43%), respectively ($P=0.115$). The use of either UCB or RD/1AG-MM-GVH was not associated with OS rates in the multivariate analysis (UCB vs RD/1AG-MM-GVH, HR, 0.99, 95% CI, 0.87–1.12, $P=0.833$) in all-risk patients, or either standard-risk ($P=0.588$) or high-risk patients ($P=0.639$; Table 2), after adjusting for the following significant risk factors: age >50 years, male recipient, acute myeloid leukemia vs MDS, high-risk disease, GVHD prophylaxis using only calcineurin inhibitor vs calcineurin inhibitor + methotrexate, and earlier year

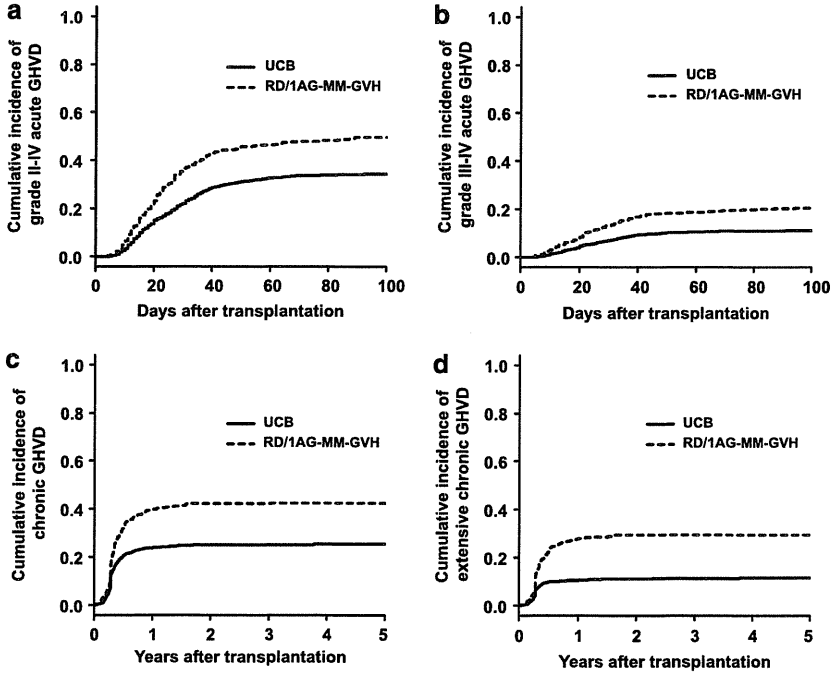


**Figure 2.** Acute and chronic GVHD. Cumulative incidences of grade II–IV (**a**) and grade III–IV acute GVHD (**b**) and chronic (**c**) and extensive chronic GVHD (**d**) are shown.

**Table 2.** Multivariate analysis of overall mortality

| *Variable* | *Total*[a] | | *Standard risk*[b] | | *High risk*[c] | |
|---|---|---|---|---|---|---|
| | *HR (95% CI)* | P *value* | *HR (95% CI)* | P *value* | *HR (95% CI)* | P *value* |
| *(A)* | | | | | | |
| UCB | 1.00 | reference | 1.00 | reference | 1.00 | reference |
| RD/1AG-MM-GVH | 0.99 (0.87–1.12) | 0.833 | 1.06 (0.86–1.31) | 0.588 | 0.96 (0.81–1.13) | 0.639 |
| *(B)* | | | | | | |
| UCB | 1.00 | reference | 1.00 | reference | 1.00 | reference |
| RD/HLA-A-MM-GVH | 0.92 (0.72–1.18) | 0.519 | 0.99 (0.66–1.48) | 0.959 | 0.90 (0.64–1.26) | 0.551 |
| RD/HLA-B-MM-GVH | 1.20 (1.01–1.44) | 0.043 | 1.44 (1.05–1.96) | 0.023 | 1.12 (0.89–1.41) | 0.326 |
| RD/HLA-DR-MM-GVH | 0.85 (0.70–1.02) | 0.084 | 0.88 (0.66–1.19) | 0.411 | 0.84 (0.65–1.08) | 0.170 |

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CI, confidence interval; CML, chronic myelogenous leukemia; CSA, cyclosporine; HR, hazard ratio; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus. [a]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.50, 95% CI, 1.35–1.66, $P<0.001$); sex of recipient, female (reference, 1.00), male (HR, 1.12; 95% CI, 1.02–1.24; $P=0.023$); diagnosis, AML (reference, 1.00),ALL (HR, 1.11, 95% CI, 0.98–1.26, $P=0.112$), CML (HR, 0.90, 95% CI, 0.72–1.13, $P=0.374$), MDS (HR, 0.81, 95% CI, 0.68–0.95, $P=0.001$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.24; 95% CI, 2.00–2.50; $P<0.001$), status not known, (HR, 1.59; 95% CI, 1.21–2.09; $P=0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00),CSA/TAC only (HR, 1.23; 95% CI, 1.09–1.39; $P=0.001$), CSA/TAC + steroid/MMF (HR, 1.02; 95% CI, 0.86–1.21; $P=0.820$), other/missing (HR, 1.21; 95% CI, 0.82–1.78; $P=0.342$); year of transplantation, 1998–2004 (reference, 1.00), 2005–2009 (HR, 0.89; 95% CI, 0.80–0.99; $P=0.038$). [b]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.72, 95% CI, 1.42–2.07, $P<0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 1.43; 95% CI, 1.14–1.78; $P=0.002$), CSA/TAC + steroid/MMF (HR, 1.00; 95% CI, 0.73–1.37; $P=0.995$), other/missing (HR, 1.51; 95% CI, 0.67–3.39; $P=0.319$). [c]Other significant variables were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.41, 95% CI, 1.23–1.61, $P<0.001$); diagnosis, AML (reference, 1.00), ALL (HR, 1.13, 95% CI, 0.95–1.34, $P=0.183$), CML (HR, 0.94, 95% CI, 0.70–1.27, $P=0.704$), MDS (HR, 0.73, 95% CI, 0.60–0.89, $P=0.002$).

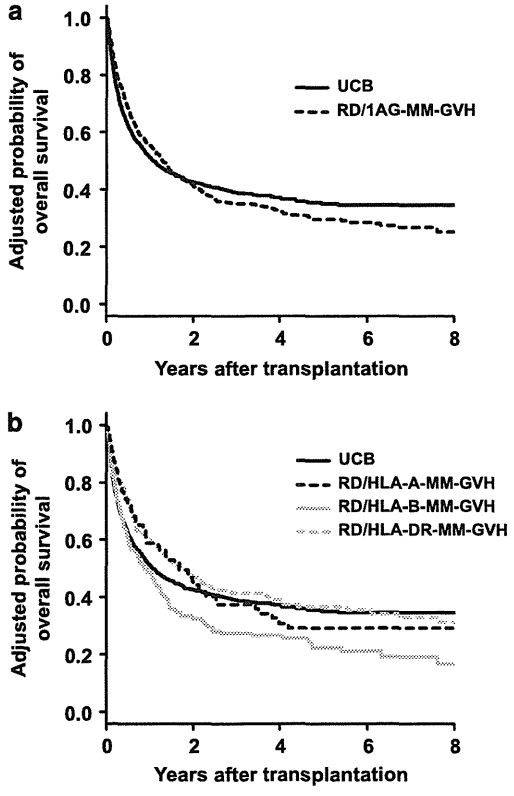


**Figure 3.** Overall survival. Overall survival rates in the transplantation using an unrelated cord blood vs a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the GVH direction (**a**) or with an HLA-A, -B, or -DR antigen mismatch in the GVH direction (**b**) are shown.

of transplantation (1998–2004). Figure 3a shows the adjusted survival curves of the two groups. Next, the HLA-A, HLA-B and HLA-DR mismatched groups in transplantation from an RD/1AG-MM-GVH were compared with the UCB group. The OS rate of patients who received transplantation from an RD/1AG-MM-GVH involving an HLA-B mismatch was significantly lower than that in the UCB group ($P=0.043$; Figure 3b and Table 2), and a subgroup analysis revealed that the adverse effect of an HLA-B mismatch was significant only in standard-risk patients (standard-risk, $P=0.023$; high-risk, $P=0.326$; Table 2).

### Relapse and NRM

The 3-year relapse rates in the UCB and RD/1AG-MM-GVH groups were 35% (95%CI, 33–37%) and 32% (95% CI, 28–36%), respectively (Gray test; $P=0.041$; Figure 4a), and a significant decrease in the incidence of relapse was found in the RD/1AG-MM-GVH group in the multivariate analysis (RD/1AG-MM-GVH vs UCB, HR, 0.78, 95%CI, 0.64–0.95, $P=0.012$; Table 3). The impact of reducing the incidence of relapse did not differ according to the HLA mismatch antigen in the RD/1AG-MM-GVH group (Table 3 and Figure 4b). The 3-year NRM rates in the UCB and RD/1AG-MM-GVH groups were 30% (95% CI, 28–32%) and 32% (95% CI, 28–36%), respectively (Gray test; $P=0.474$; Figure 4c), and a significant increase in the NRM rate was observed in the RD/1AG-MM-GVH group in the multivariate analysis (RD/1AG-MM-GVH vs UCB, HR, 1.24, 95% CI, 1.04–1.47, $P=0.016$; Table 3). In particular, the NRM rate of patients who received transplantation from an RD/1AG-MM-GVH with an HLA-B mismatch was significantly higher than that in the UCB group (RD/1AG-MM-GVH vs UCB, HR, 1.50, 95% CI, 1.17–1.92, $P=0.001$; Figure 4d and Table 3).

The causes of death in patients who died without relapse are shown in Supplementary Table 3. The rates of GVHD and organ failure in the RD/1AG-MM-GVH group were higher than those in the UCB group (GVHD, 18 vs 10%, organ failure, 28 vs 19%), whereas the rates of graft failure and infection were lower in the RD/1AG-MM-GVH group (graft failure, 1 vs 5%; infection, 26 vs 38%).

### The impact of the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group

Based on the fact that the leading causes of death in the RD/1AG-MM-GVH group were GVHD and organ failure, we analyzed the risk factors for the development of acute GVHD in this group.

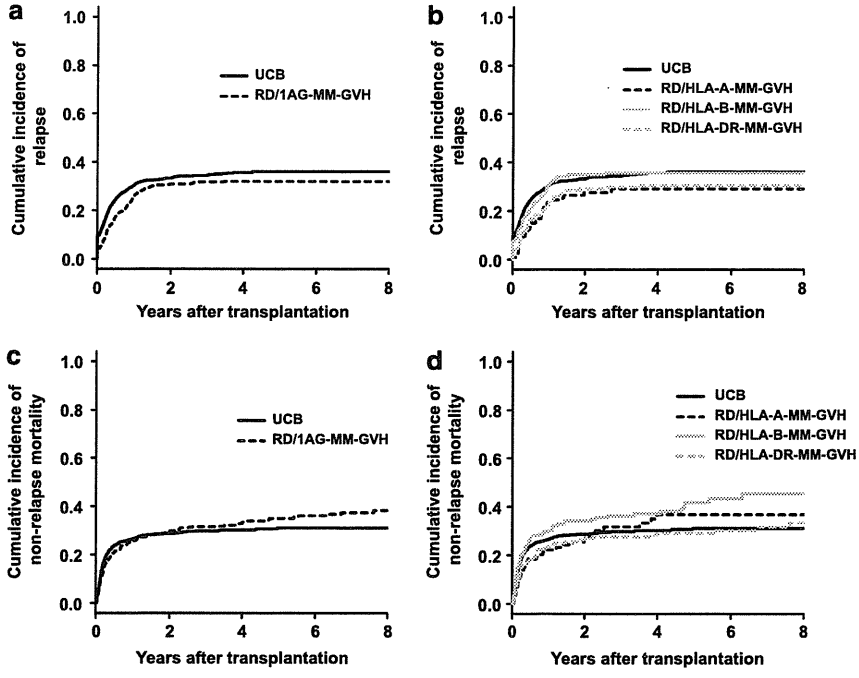


**Figure 4.** Relapse and non-relapse mortality. Cumulative incidence of relapse and non-relapse mortality after transplantation using an unrelated cord blood vs a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the GVH direction (**a**, **c**) or with an HLA-A, -B, or -DR antigen mismatch in the GVH direction (**b**, **d**) are shown.

**Table 3.** Multivariate analysis of relapse and non-relapse mortality

| *Variable* | *Relapse*[a] | | *Non-relapse mortality*[b] | |
|---|---|---|---|---|
| | *HR (95% CI)* | P *value* | *HR (95% CI)* | P *value* |
| *(A)* | | | | |
| UCB | 1.00 | reference | 1.00 | reference |
| RD/1AG-MM-GVH | 0.78 (0.64–0.95) | 0.012 | 1.24 (1.04–1.47) | 0.016 |
| *(B)* | | | | |
| UCB | 1.00 | reference | 1.00 | reference |
| RD/HLA-A-MM-GVH | 0.70 (0.49–1.00) | 0.050 | 1.28 (0.93–1.76) | 0.130 |
| RD/HLA-B-MM-GVH | 0.81 (0.62–1.07) | 0.134 | 1.50 (1.17–1.92) | 0.001 |
| RD/HLA-DR-MM-GVH | 0.80 (0.61–1.04) | 0.096 | 1.02 (0.78–1.32) | 0.901 |

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CI, confidence interval; CML, chronic myelogenous leukemia; CSA, cyclosporine; HR, hazard ratio; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus. [a]Other significant variables in model A were; diagnosis, AML (reference, 1.00), ALL (HR, 1.09, 95% CI, 0.92–1.29, $P=0.336$), CML (HR, 1.39, 95% CI, 1.05–1.82, $P=0.019$), MDS (HR, 0.59, 95% CI, 0.46–0.76, $P<0.001$); time from diagnosis to transplantation, <6 months (reference, 1.00), ⩾6 months (HR, 0.80; 95% CI, 0.70–0.92; $P=0.002$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.81; 95% CI, 2.41–3.27; $P<0.001$), status not known, (HR, 2.17; 95% CI, 1.45–3.23; $P<0.001$); conditioning intensity, myeloablative (reference, 1.00), reduced intensity (HR, 1.22; 95% CI, 1.04–1.44; $P=0.014$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 0.65; 95% CI, 0.53–0.78; $P<0.001$), CSA/TAC + steroid/MMF (HR, 0.75; 95% CI, 0.59–0.96; $P=0.024$), other/missing (HR, 0.94; 95% CI, 0.55–1.61; $P=0.825$). [b]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.70, 95% CI, 1.47–1.98, $P<0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00),CSA/TAC only (HR, 1.70; 95% CI, 1.44–2.01; $P<0.001$), CSA/TAC + steroid/MMF (HR, 1.18; 95% CI, 0.94–1.49; $P=0.158$), other/missing (HR, 1.47; 95% CI, 0.86–2.51; $P=0.154$); year of transplantation, 1998–2004 (reference, 1.00), 2005–2009 (HR, 0.76; 95% CI, 0.66–0.88; $P<0.001$).

In multivariate analysis, two factors were found to be significantly associated with the risk of developing grade II–IV acute GVHD in the RD/1AG-MM-GVH group: the use of *in vivo* T-cell depletion and source of stem cells (use of *in vivo* T-cell depletion, yes vs no, HR 0.40, $P=0.002$, PB vs BM, HR 1.61, $P<0.001$).

Because the use of *in vivo* T-cell depletion significantly lowered the risk of acute GVHD, we re-compared the RD/1AG-MM-GVH group and the UCB group while focusing on the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group. The incidence of grade II–IV or grade III–IV acute GVHD or chronic or extensive chronic GVHD in the RD/1AG-MM-GVH group using *in vivo* T-cell depletion was comparable to that in the UCB group (Supplementary Figure 1 and Supplementary Table 4), whereas the incidences of neutrophil and platelet engraftment were significantly higher in the RD/1AG-MM-GVH group using *in vivo* T-cell depletion than in the UCB group (neutrophil engraftment, HR, 5.52, 95% CI, 3.36–9.05, $P<0.001$; platelet engraftment, HR 2.01, 95% CI, 1.26–3.21, $P<0.001$). Compared to the UCB group, the RD/1AG-MM-GVH group with T-cell depletion showed lower overall and NRM, albeit these differences were not significant, which suggests that the use of *in vivo* T-cell depletion may improve the outcome of transplantation from an RD/1AG-MM-GVH (Figure 5, Supplementary Table 5). It is interesting to note that the adverse impact of an HLA-B mismatch vs HLA-A or -DR

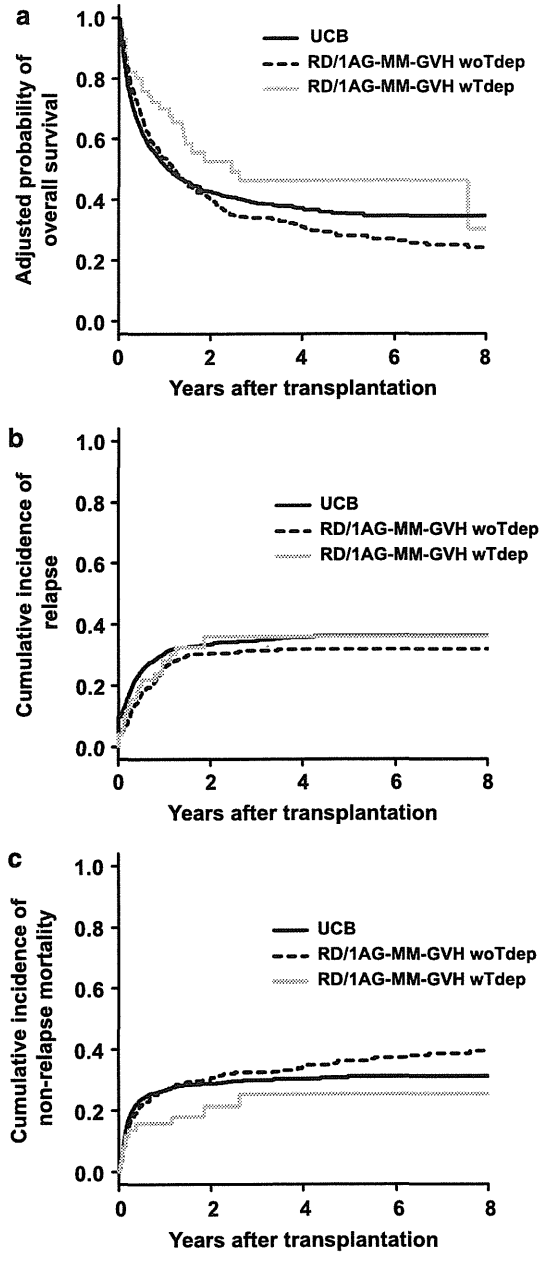


**Figure 5.** OS (**a**), relapse (**b**) and NRM (**c**) according to the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group.

mismatch in the RD/1AG-MM-GVH group disappeared with the use of *in vivo* T-cell depletion (with *in vivo* T-cell depletion; HLA-B vs HLA-A/DR mismatch; HR 1.08, 95% CI, 0.45–2.62, $P=0.864$, without *in vivo* T-cell depletion; HLA-B vs HLA-A/DR mismatch; HR 1.59, 95% CI, 1.25–2.01, $P<0.001$).

With regard to the effect of stem cell source, the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group using BM was lower than that with PB but higher than that with UCB (Supplementary Figure 2). The use of PB or BM did not affect OS, relapse, or NRM (Supplementary Table 5).

## DISCUSSION

In this nationwide retrospective study, we found that the survival rate in the UCB group was comparable to that in the RD/1AG-MM-GVH group regardless of the disease risk. The RD/1AG-MM-GVH group with an HLA-B mismatch showed significantly higher overall and NRM, whereas the RD/1AG-MM-GVH group with an HLA-A or HLA-DR mismatch showed an OS comparable to that in the UCB group. Neutrophil and platelet engraftment in the RD/1AG-MM-GVH group were significantly faster than those in the UCB group, whereas the incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group was significantly higher. However, the incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group with *in vivo* T-cell depletion was comparable to that in the UCB group, which translated into a better, but not significantly better, OS than that in the UCB group.

In Japan, unrelated BM donor coordination (from donor search to transplantation) takes a median of 4 months, whereas much less time is required for UCB or RD/1AG-MM-GVH transplantation if there is a candidate. This was reflected in the longer duration from diagnosis to transplantation in unrelated BM transplantation.[32] In contrast, UCB and RD/1AG-MM-GVH transplantation show a similar and shorter duration (Table 1 ; 7.9 months vs 7.6 months). Therefore, in cases where both UCB and RD/1AG-MM-GVH are available, donors should be chosen based on their advantages and disadvantages. Compared with UCB, the use of RD/1AG-MM-GVH has a great advantage in neutrophil and platelet engraftment, which is not inconsistent with a previous finding that engraftment in the UCB group was significantly delayed comparing with that in MUD.[33] This translated into a lower rate of death from graft failure or infection in the RD/1AG-MM-GVH group. However, these advantages were offset by a substantial increase in the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group. The risk of grade III–IV acute GVHD and extensive chronic GVHD in the RD/1AG-MM-GVH group was twice that in the UCB group. If UCB units containing adequate total nucleated cell doses (ex. $>2.5\times10^7$/kg) are available,[34] the selection of UCB would be appropriate to avoid the risk of chronic GVHD. In contrast, RD/1AG-MM-GVH would be more appropriate when early neutrophil engraftment should be prioritized, such as for a patient with an active infectious disease at transplantation.

The high incidences of GVHD and GVHD-related death in the RD/1AG-MM-GVH group indicate the need for stronger immunosuppression to improve the clinical outcome. The use of T-cell depletion, mostly by ATG, was significantly associated with a lower incidence of grade III–IV acute GVHD and extensive chronic GVHD in the RD/1AG-MM-GVH group. Although this effect was not statistically significant, the RD/1AG-MM-GVH group with *in vivo* T-cell depletion showed lower overall and treatment-related mortality, which would outweigh a possible increased risk of relapse. These findings in our cohort suggest that ATG may be effective, and the addition of ATG in the RD/1AG-MM-GVH group should be assessed in a prospective study.

As shown in our previous study,[23] overall mortality in the RD/1AG-MM-GVH group involving an HLA-B mismatch was significantly higher than that in the RD/1AG-MM-GVH group with an HLA-A or -DR mismatch, probably because of an additional HLA-C antigen mismatch as expected from linkage disequilibrium between HLA-B and HLA-C and available data on HLA-C antigen.[23,35] The incidence of grade III–IV acute GVHD in the HLA-B mismatch group was higher than that in the HLA-DR mismatch group, but was comparable to that in the HLA-A mismatch group. In addition, the incidence of death from GVHD was similar in the HLA-B and HLA-A/DR mismatch groups (data not shown). Therefore, the reason for the lower overall morality in the RD/1AG-MM-GVH group with an HLA-B mismatch remains unclear. However, the adverse effect of an HLA-B mismatch disappeared when *in vivo* T-cell depletion was used, which suggests that an immunological effect is involved in this mechanism.

This study has several limitations. First, in clinical practice in Japan, matching of HLA-DR is counted at a low resolution, as with HLA-A and HLA-B, whereas it is counted at a high resolution in the

United States and Europe. To evaluate the impact of this difference, we divided patients in the UCB group with two antigen mismatches into two groups by using available HLA-DRB1 allele information: a group with two antigen mismatches with one additional HLA-DRB1 allele mismatch ($n = 609$) and another group with two antigen mismatches without an additional HLA-DRB1 mismatch ($n = 295$). We did not find a significant difference in OS between these two groups ($P = 0.758$), which suggests that HLA-matching using HLA-DR antigen or allele information will not affect OS in the present study. Second, the findings in the present study are based on Asian cohort who received a 'single' UCB or RD/1AG-MM-GVH transplantation. Lighter body weight in Asian population than Caucasian population may make it easy to find a suitable single UCB unit that contains adequate total nucleated cell doses. In addition, as suggested by Oh *et al.*,[36] limited heterogeneity of Japanese population may affect the outcomes of transplantation. Therefore, the findings should be externally validated in the non-Asian cohort or transplantation using double UCB units. Third, information on the dose and type of ATG was missing in two-third of the patients who received ATG. However, the available data showed that the median dose of thymoglobulin (2.5 mg/kg) or ATG-F (8 mg/kg) was equivalent to the dose that is widely used in our daily practice. Lastly, heterogeneous backgrounds may have resulted in a bias, although we tried to adjust for possible confounders by multivariate analyses. Lastly, the effect of multiple testing should be taken into account for the interpretation of secondary end points.

In conclusion, our findings suggest that both UCB and RD/1AG-MM-GVH are suitable as alternative donors for patients without an HLA-matched sibling or unrelated donor. However, the presence of an HLA-B-antigen mismatch in the GVH direction has an adverse effect on OS because of treatment-related complications. Neutrophil and platelet engraftment in the RD/1AG-MM-GVH group were significantly faster than those in the UCB group, whereas the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group was significantly higher, which translated into a high incidence of death from GVHD. Donor selection between UCB and RD/1AG-MM-GVH should be determined based on the presence of an HLA-B mismatch in RD/1AG-MM-GVH and from the risks and benefits derived from the risk of graft failure and infection in the UCB group and acute or chronic GVHD in the RD/1AG-MM-GVH group. Additional immune suppression using *in vivo* T-cell depletion may improve the clinical outcome in the RD/1AG-MM-GVH group by decreasing the incidences of GVHD and NRM and may also overcome the adverse effect of an HLA-B mismatch. This approach should be assessed in a prospective study.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We are indebted to all of the physicians and data managers who contributed valuable data on transplantation to the Japan Society for Hematopoietic Cell Transplantation and the Japan Cord Blood Bank Network. We also thank the members of the data management committees of the Japan Society for Hematopoietic Cell Transplantation and the Japan Cord Blood Bank Network for managing data. JK is a research fellow of the Japan Society for the Promotion of Science. This work was supported in part by Grant-in-Aid for JSPS Fellows (JK).

## AUTHOR CONTRIBUTIONS

JK and YK designed the research, organized the project and wrote the manuscript; JK, YA, and YK performed the statistical analysis and analyzed the data; KK and TN-I collected data from JCBBN; and all of the authors interpreted the data and reviewed and approved the final manuscript.

## REFERENCES

1 Szydlo R, Goldman JM, Klein JP, Gale RP, Ash RC, Bach FH *et al.* Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J Clin Oncol* 1997; **15**: 1767–1777.
2 Petersdorf EW, Gooley TA, Anasetti C, Martin PJ, Smith AG, Mickelson EM *et al.* Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. *Blood* 1998; **92**: 3515–3520.
3 Hansen JA, Gooley TA, Martin PJ, Appelbaum F, Chauncey TR, Clift RA *et al.* Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. *N Engl J Med* 1998; **338**: 962–968.
4 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.
5 Yakoub-Agha I, Mesnil F, Kuentz M, Boiron JM, Ifrah N, Milpied N *et al.* Allogeneic marrow stem-cell transplantation from human leukocyte antigen-identical siblings versus human leukocyte antigen-allelic-matched unrelated donors (10/10) in patients with standard-risk hematologic malignancy: a prospective study from the French Society of Bone Marrow Transplantation and Cell Therapy. *J Clin Oncol* 2006; **24**: 5695–5702.
6 Wagner JE, Rosenthal J, Sweetman R, Shu XO, Davies SM, Ramsay NK *et al.* Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. *Blood* 1996; **88**: 795–802.
7 Kurtzberg J, Laughlin M, Graham ML, Smith C, Olson JF, Halperin EC *et al.* Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N Engl J Med* 1996; **335**: 157–166.
8 Gluckman E, Rocha V, Boyer-Chammard A, Locatelli F, Arcese W, Pasquini R *et al.* Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. *N Engl J Med* 1997; **337**: 373–381.
9 Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J, Migliaccio AR *et al.* Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med* 1998; **339**: 1565–1577.
10 Rocha V, Wagner Jr. JE , Sobocinski KA, Klein JP, Zhang MJ, Horowitz MM *et al.* Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. *N Engl J Med* 2000; **342**: 1846–1854.
11 Rocha V, Cornish J, Sievers EL, Filipovich A, Locatelli F, Peters C *et al.* Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. *Blood* 2001; **97**: 2962–2971.
12 Laughlin MJ, Eapen M, Rubinstein P, Wagner JE, Zhang MJ, Champlin RE *et al.* Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med* 2004; **351**: 2265–2275.
13 Rocha V, Labopin M, Sanz G, Arcese W, Schwerdtfeger R, Bosi A *et al.* Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med* 2004; **351**: 2276–2285.
14 Takahashi S, Iseki T, Ooi J, Tomonari A, Takasugi K, Shimohakamada Y *et al.* Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. *Blood* 2004; **104**: 3813–3820.
15 Eapen M, Rubinstein P, Zhang MJ, Stevens C, Kurtzberg J, Scaradavou A *et al.* Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet* 2007; **369**: 1947–1954.
16 Atsuta Y, Suzuki R, Nagamura-Inoue T, Taniguchi S, Takahashi S, Kai S *et al.* Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood* 2009; **113**: 1631–1638.
17 Rocha V, Gluckman E. Improving outcomes of cord blood transplantation: HLA matching, cell dose and other graft- and transplantation-related factors. *Br J Haematol* 2009; **147**: 262–274.
18 Kanda Y, Chiba S, Hirai H, Sakamaki H, Iseki T, Kodera Y *et al.* Allogeneic hematopoietic stem cell transplantation from family members other than HLA-identical siblings over the last decade (1991-2000). *Blood* 2003; **102**: 1541–1547.
19 Teshima T, Matsuo K, Matsue K, Kawano F, Taniguchi S, Hara M *et al.* Impact of human leucocyte antigen mismatch on graft-versus-host disease and graft failure after reduced intensity conditioning allogeneic haematopoietic stem cell transplantation from related donors. *Br J Haematol* 2005; **130**: 575–587.
20 Anasetti C, Beatty PG, Storb R, Martin PJ, Mori M, Sanders JE *et al.* Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. *Hum Immunol* 1990; **29**: 79–91.

21 Anasetti C, Amos D, Beatty PG, Appelbaum FR, Bensinger W, Buckner CD *et al.* Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med* 1989; **320**: 197–204.
22 Lu DP, Dong L, Wu T, Huang XJ, Zhang MJ, Han W *et al.* Conditioning including antithymocyte globulin followed by unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation can achieve comparable outcomes with HLA-identical sibling transplantation. *Blood* 2006; **107**: 3065–3073.
23 Kanda J, Saji H, Fukuda T, Kobayashi T, Miyamura K, Eto T *et al.* Related transplantation with HLA-1 Ag mismatch in the GVH direction and HLA-8/8 allele-matched unrelated transplantation: a nationwide retrospective study. *Blood* 2012; **119**: 2409–2416.
24 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.
25 Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J *et al.* 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant* 1995; **15**: 825–828.
26 Sullivan KM, Agura E, Anasetti C, Appelbaum F, Badger C, Bearman S *et al.* Chronic graft-versus-host disease and other late complications of bone marrow transplantation. *Semin Hematol* 1991; **28**: 250–259.
27 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.
28 Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. *Ann Stat* 1988; **16**: 1141–1154.
29 Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. *J Am Stat Assoc* 1999; **94**: 456–509.
30 Giralt S, Ballen K, Rizzo D, Bacigalupo A, Horowitz M, Pasquini M *et al.* Reduced-intensity conditioning regimen workshop: defining the dose spectrum. Report of a workshop convened by the center for international blood and marrow transplant research. *Biol Blood Marrow Transplant* 2009; **15**: 367–369.
31 Kanda Y. Free statistical software: EZR (Easy R) on R commander. Available from http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html (Accessed on 1 February 2012).
32 Kanda J, Hishizawa M, Utsunomiya A, Taniguchi S, Eto T, Moriuchi Y *et al.* Impact of graft-versus-host disease on outcomes after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study. *Blood* 2012; **119**: 2141–2148.
33 Eapen M, Rocha V, Sanz G, Scaradavou A, Zhang MJ, Arcese W *et al.* Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol* 2010; **11**: 653–660.
34 Cohen YC, Scaradavou A, Stevens CE, Rubinstein P, Gluckman E, Rocha V *et al.* Factors affecting mortality following myeloablative cord blood transplantation in adults: a pooled analysis of three international registries. *Bone Marrow Transplant* 2011; **46**: 70–76.
35 Prasad VK, Heller G, Kernan NA, O'Reilly RJ, Yang SY. The probability of HLA-C matching between patient and unrelated donor at the molecular level: estimations based on the linkage disequilibrium between DNA typed HLA-B and HLA-C alleles. *Transplantation* 1999; **68**: 1044–1050.
36 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.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Cancer Science
The official journal of the Japanese Cancer Association JCA

# Phase II study of dose-modified busulfan by real-time targeting in allogeneic hematopoietic stem cell transplantation for myeloid malignancy

Yachiyo Kuwatsuka,[1,2,11] Akio Kohno,[3] Seitaro Terakura,[2] Shigeki Saito,[2,3,12] Kazuyuki Shimada,[2,3] Takahiko Yasuda,[1,2,12] Yoshihiro Inamoto,[1,2,13] Koichi Miyamura,[1] Masashi Sawa,[4] Makoto Murata,[2] Takahiro Karasuno[5,14] Shuichi Taniguchi,[6] Koji Nagafuji,[7,15] Yoshiko Atsuta,[8] Ritsuro Suzuki,[8] Mariko Fukumoto,[9] Tomoki Naoe,[2] Yoshihisa Morishita[3,10] and the Nagoya Blood and Marrow Transplantation Group

[1]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya; [2]Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya; [3]Department of Hematology and Oncology, JA Aichi Konan Kosei Hospital, Konan; [4]Department of Hematology and Oncology, Anjo Kosei Hospital, Anjo; [5]Department of Hematology and Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; [6]Department of Hematology, Toranomon Hospital, Tokyo; [7]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka; [8]Department of Hematopoietic Stem Cell Transplantation Data Management, Nagoya University School of Medicine, Nagoya; [9]Division of Toxicology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan

(Received January 4, 2012/Revised April 6, 2012/Accepted May 19, 2012/Accepted manuscript online May 28, 2012/Article first published online July 16, 2012)

**We aimed to evaluate the efficacy and safety of allogeneic hematopoietic stem cell transplantation with targeted oral busulfan (BU) and cyclophosphamide (CY) in a phase II study. Busulfan (1.0 mg/kg) was given initially in six doses. Based on the estimated concentration at steady state after the first dose of BU, subsequent (7th–16th) doses were adjusted to obtain a targeted overall concentration at steady state of 700–900 ng/mL. The primary endpoint was 1-year overall survival (OS). Fifty patients were registered and 46 (median age, 53 years; range, 18–62 years) received planned transplant, including 24 with AML, 16 with myelodysplastic syndrome, and six with CML. Fourteen patients were categorized as standard risk. Nineteen patients received transplant from human leukocyte antigen-identical siblings, 27 from unrelated donors. The BU dose required reduction in 32 patients and escalation in six patients. One-year OS was 65% (95% confidence interval, 50–77%). Cumulative incidence of hepatic sinusoidal obstruction syndrome was 11%. One-year transplant-related mortality was 18%. Both OS and transplant-related mortality were favorable in this study, including patients of older age and with high risk diseases. Individual dose adjustment based on BU pharmacokinetics was feasible and effective in the current phase II study. This trial is registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). (*Cancer Sci* 2012; 103: 1688–1694)**

Busulfan is an alkylating agent widely used in high-dose chemotherapy regimens for HSCT.[1,2] The BU level in serum has been shown to be an important factor for graft rejection and regimen-related toxicity such as SOS.[3–5] Unfavorable profiles of oral BU include delayed and variable absorptive characteristics and high variability in drug metabolism.[6] Individualized dose adjustment of BU using the LSM, and its transplantation results, have been investigated widely in Caucasian patients and pediatric populations, but few prospective studies have investigated results in Asian patients.[7] Prior to the current study, we carried out a prospective PK study to analyze BU concentration using gas chromatography–mass spectrometry.[8] Nine patients were enrolled in the study, and received preparative regimen containing oral BU 1 mg/kg every 6 h for eight or 16 doses. Out of nine patients, only three met the average steady-state plasma concentration levels in the safety range of 650–1000 ng/mL[4,9] after the first and 13th dose. From the results, we developed LSM to estimate the AUC using two different formulas in order to fit even delayed clearance. Subsequently, we carried out a pilot study that used the same targeting method as the current study, and six patients with myeloid malignancy received tBU+CY conditioning with a targeting AUC of Css 700–900 ng/mL. Four patients received dose reduction after the seventh dose of BU, and overall Css of three patients met the safety range of 786–905 ng/mL (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003).

From these results, we carried out a prospective phase II trial in Japanese patients with myeloid malignancies to evaluate the clinical results of allogeneic HSCT undergoing individualized high-dose oral BU+CY conditioning.

## Materials and Methods

**Eligibility criteria.** Patients from 16 to 65 years old were eligible if they had a diagnosis of AML, CML, or MDS, with an Eastern Cooperative Oncology Group performance status of 0–2, and no previous history of HSCT. Standard risk was defined as AML in first complete remission, MDS in refractory anemia or refractory anemia with ringed sideroblasts, and CML in chronic phase. High risk was defined as the remaining disease type. Patients receiving T cell depletion, or those with clinically significant infection or severe abnormalities of cardiac, pulmonary, and hepatic functions were excluded. Included patient/donor pairs were either related HLA matched by serological typing of A, B, and DR locus, unrelated HLA matched, or HLA DRB1 one locus mismatched by genotypical typing of A, B, and DRB1 locus. Unrelated donors were chosen by coordination with the Japan Marrow Donor Program. Written informed consent was obtained from each patient according to the Declaration of Helsinki. The study

[10]To whom correspondence should be addressed.
E-mail: m-59@mb.ccnw.ne.jp
[11]Present address: Medical College of Wisconsin, Milwaukee, WI, USA.
[12]Present address: Nagoya University Graduate School of Medicine, Nagoya, Japan.
[13]Present address: Fred Hutchinson Cancer Research Center, Seattle, WA, USA.
[14]Present address: Yao Municipal Hospital, Yao, Japan.
[15]Present address: Kurume University School of Medicine, Kurume, Japan.


doi: 10.1111/j.1349-7006.2012.02342.x

protocol was approved by the Institutional Review Board of each center.

**Conditioning regimen, GVHD prophylaxis, and supportive care.** Patients received a conditioning regimen consisting of BU 1.0 mg/kg given orally four times a day for six doses on two consecutive days (dose 1–6). Six hours after dose 6, patients received an adjusted dose of BU four times a day for 10 doses (dose 7–16) on three consecutive days (Fig. S1). Cyclophosphamide 60 mg/kg was given i.v. on two successive days. Both BU and CY were dosed based on actual body weight if it was <120% of ideal body weight, and adjusted body weight for those exceeding 120%. Sodium valproate was given as seizure prophylaxis before and during BU treatment. Fluconazole was used as fungal prophylaxis.

Either cyclosporine or tacrolimus in combination with methotrexate was used for GVHD prophylaxis. Cyclosporine was given i.v. at a dose of 3 mg/kg per day in two divided doses starting on day −1. Tacrolimus was given i.v. at a dose of 0.025 mg/kg continuously starting on day −1. Methotrexate was given at a dose of 10 mg/m$^2$ on day 1 and 7 mg/m$^2$ on days 3 and 6. Oral cyclosporine or tacrolimus was substituted for i.v. administration when tolerated. In the absence of GVHD, the cyclosporine and tacrolimus doses were tapered after day 50. Acute GVHD of grade 2 or more was treated with methylprednisolone 1–2 mg/kg. Chronic GVHD was treated by the protocols of each institute.

Supportive care measures were used according to institutional guidelines. Daily granulocyte colony stimulating factor was started on day 6 and continued until absolute neutrophil count exceeded 500/μL for two consecutive days.

**Pharmacokinetic studies of BU.** For PK studies of BU, blood samples were obtained 0, 30, 60, 120, 300, and 360 min after the first oral dose. Frozen plasma samples were sent to the laboratory at Kitasato University, and plasma BU concentrations were assayed by gas chromatography–mass spectrometry.[8] The AUC was calculated by LSM using the formulas shown in Table 1.

Average Css levels of BU were determined by the ratio of the BU $AUC_{LSM}$ over the dosing interval to the time between doses. The BU dose after the sixth dose was adjusted when Css after the first dose was not within 700–900 ng/mL. Dose adjustment was not carried out for patients whose Css after the first dose was 700–900 ng/mL. A targeted dose was calculated to achieve an average Css after all doses of 800 ng/mL. The optimal dose of BU was calculated as follows: optimal single dose of BU (mg/kg) = 800 (ng/mL) × first dose (mg/kg)/Css of first dose (ng/mL).

The dose of the 7th to 16th BU was calculated as follows: revised dose (mg/kg) = [optimal single dose (mg/kg) × 16 (times) − first dose (mg/kg) × 6 (times)]/10 (times).

**Definitions of outcomes.** The study was designed as a phase II prospective trial. The primary endpoint of the study was 1-year OS after transplantation. The secondary endpoint was DFS, PK of BU, aGVHD, and cGVHD, and the frequency and severity of SOS, regimen-related toxicity up to day 28, mortality at day 100, hematological recovery, and DFS and OS of each disease category.

All patients were prospectively monitored for engraftment,[10] post-transplant toxicities, GVHD, hepatic SOS, and infection. Failure to reach an absolute neutrophil count of 0.5 × 10$^9$ cells/L by day 28 after transplantation was defined as graft failure, and the patient was withdrawn from the study. The aGVHD was evaluated daily until day 28 and weekly from day 29 to 100 and graded by established criteria.[11] The cGVHD was evaluated up to day 365. Treatment and the outcome of aGVHD and cGVHD were also evaluated. Sinusoidal obstruction syndrome was clinically evaluated before day 28, and diagnosed,[12–14] then graded clinically[12] according to the published criteria. Liver toxicity that occurred after day 21 and fulfilled the above criteria of SOS was defined as late-onset SOS. Clinical data after day 29 until day 100 was additionally surveyed to evaluate late-onset SOS retrospectively.

Disease monitoring was carried out by bone marrow aspiration within 1 week before or after days 30, 60, and 90 after transplantation. Relapse was defined by hematological recurrence for AML,[15,16] and by hematological or cytogenetic relapse for CML. Deaths in the absence of persistent relapse were categorized as non-relapse mortality. Additional surveillance was carried out and the onset of SOS and regimen-related toxicities from days 29 to 100 were collected retrospectively. Long-term survival data and data of relapse after day 365 were also collected retrospectively.

**Statistical analysis.** The primary endpoint of the study was 1-year OS after transplantation. The expected 1-year OS was estimated to be 60%, and its threshold was estimated to be 40%. With a statistical power of 90% and a one-sided, type I error of 5%, the number of eligible patients required for this study was calculated to be 46 using a binominal analysis method. The projected sample size was 50 patients, with the expectation that 10% of patients would be deemed ineligible.

Disease-free survival was calculated from the date of transplantation until the date of relapse or the date of death in complete remission. This trial has been registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). Data were analyzed with Stata 9.2 statistical software (Stata, College Station, TX, USA).

**Table 1. Formulas for limited sample model (LSM) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide**

| | |
|---|---|
| i | In cases $C_6/C_2$ = or <0.5 |
| | $AUC_{LSM} = 0.5C_{0.5} + 0.75C_1 + 2.5C_2 + 2.0C_6 + 4C_6/(LnC_2 - LnC_6)$ |
| ii | In cases $C_6/C_2 > 0.5$ |
| | $AUC_{LSM} = 0.5C_{0.5} + 0.75C_1 + 2.5C_2 + 2.0C_6 + 2C_6/(LnC_2 - LnC_6)$ |

In the previous pilot study, formula (i) bore a strong approximation to actual area under the blood concentration time curve (AUC), but not in patients with an elongated absorption or a delayed elimination of busulfan. The formula of the LSM was modified in the case of $C_6/C_2 > 0.5$ and formula (ii) was used for those patients. $C_x$, serum busulfan level obtained at x hours after the first dose.

## Results

**Patient characteristics.** Patients were registered from October 2003 through March 2007. Fifty patients were registered. One patient who developed severe hemorrhagic ulcer of the ileum after registration was considered to be ineligible. One patient developed metastatic breast cancer before receiving the conditioning regimen and was withdrawn. Forty-eight patients received tBU+CY conditioning. One patient developed systemic convulsion on day −6 before transplantation, and the study was discontinued. Another patient received cord blood transplantation due to unexpected emergent unavailability of the unrelated bone marrow and was included only in the PK analysis. The remaining 46 patients who completed tBU+CY conditioning and received the planned transplantation were analyzed in the subsequent outcome study. Characteristics and a transplantation summary of these 46 patients at the time of registration are shown in Tables 2 and 3, respectively.

**Treatment-related toxicity and hepatic veno-occlusive disease.** Forty-five of 46 patients undergoing tBU+CY conditioning (98%) experienced grade II or higher regimen-related toxicity, and 38 of 48 patients (79%) experienced grade III or more toxicity within 28 days post-transplantation (Table S1). Infection (70%), oral mucositis (52%), nausea and vomiting (30%), and

**Table 2. Characteristics of patients receiving allogeneic hematopoietic stem cell transplantation (*n* = 46)**

| Characteristics | |
|---|---|
| Median age of patients (range), years | 53 (18–62) |
| Sex of recipient (%) | |
| Male | 29 (63) |
| Female | 17 (37) |
| Sex, donor versus recipient (%) | |
| Match | 25 (54) |
| Male to female | 11 (24) |
| Female to male | 10 (22) |
| Disease type (%) | |
| AML | 24 (52) |
| 1st CR | 5 |
| 2nd CR | 10 |
| 1st relapse | 5 |
| No treatment† | 4 |
| MDS | 16 (35) |
| RA | 4 |
| RAEB | 9 |
| CMML | 1 |
| RAEB-t | 2 |
| CML | 6 (13) |
| CP | 5 |
| AP | 1 |
| Disease risk‡ (%) | |
| Standard | 14 (30) |
| High | 32 (70) |
| Performance status§ (%) | |
| 0 | 40 (86) |
| 1 | 6 (13) |
| 2 | 0 (0) |
| Donor (%) | |
| Related | 19 (41) |
| Unrelated | 27 (59) |
| HLA (%) | |
| HLA identical sibling | 19 (41) |
| HLA 6/6 matched, unrelated | 23 (50) |
| HLA mismatched, unrelated | 4 (9) |

†Two patients with overt leukemia from myelodysplastic syndrome (MDS) and another two patients with hypoplastic AML did not receive induction chemotherapy before transplantation. ‡Standard risk was defined as AML in 1st complete remission (CR), MDS in refractory anemia (RA) or RA with ringed sideroblasts, and CML in chronic phase (CP). §According to Eastern Cooperative Oncology Group criteria. AP, accelerated phase; CMML, chronic myelomonocytic leukemia; HLA, human leukocyte antigen; RAEB, refractory anemia with excess of blasts; RAEB-t, RAEB in transformation.

**Table 3. Summary of transplantation in patients with AML (*n* = 24), myelodysplastic syndrome (*n* = 16), or CML (*n* = 6)**

| | |
|---|---|
| Stem cell source | |
| G-PBMC | 7 |
| Bone marrow | 39 |
| GVHD prophylaxis† | |
| sMTX+CyA | 22 |
| sMTX+FK | 21 |
| aGVHD, grade (%) | |
| None | 26 (56) |
| I | 4 (9) |
| II | 11 (24) |
| III | 4 (9) |
| IV | 1 (2) |
| cGVHD, type (%) | |
| None | 16 (43) |
| Lmt | 9 (24) |
| Ext | 12 (32) |

†One patient received short-term methotrexate + tacrolimus prophylaxis and subsequently received short-term methotrexate + cyclosporine. aGVHD, acute graft versus host disease; chronic GVHD, chronic graft versus host disease; GVHD, graft versus host disease.

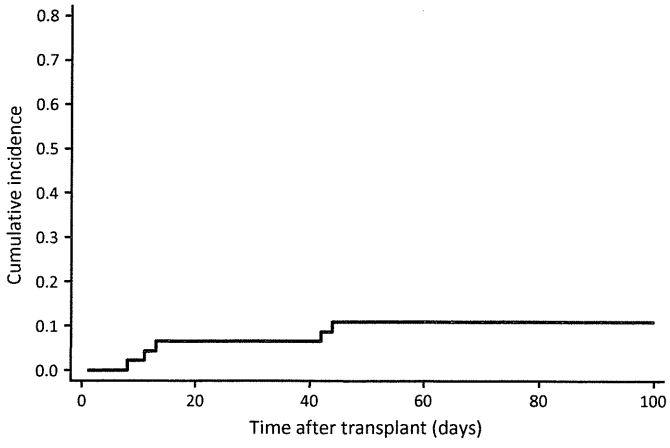


**Fig. 1.** Cumulative incidence of sinusoidal obstruction syndrome in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. The cumulative incidence of overall sinusoidal obstruction syndrome was 11% (95% confidence interval, 4–22%) at day 100 after transplantation.

diarrhea (30%) were frequent grade III or more adverse reactions. Severe neurological toxicity of grade III or more was observed in five patients (11%). One patient developed subarachnoid hemorrhage and died on day 1 after transplantation. Another patient developed tacrolimus encephalopathy on day 23 after transplantation. This patient died of acute bleeding from gastric ulcer on day 57. Another patient developed neurological toxicity during the course of septic shock and died on day 15. One patient who received dose reduction had delayed engraftment, but subsequently engrafted on day 31.

Among 46 patients undergoing planned transplantation, four patients experienced grade III or IV liver toxicity before day 28 (Table S1). Grade III or more long-term liver toxicity between days 29 and 100 was observed in nine patients (Table S2). Three patients were reported to have SOS before day 20, and two were reported to have late-onset SOS from days 21 to 100. Cumulative incidence of overall SOS was 11% (95% CI, 4–22%) at day 100 after transplantation (Fig. 1). Two patients had mild SOS on day 8 and 11 after transplantation, and both improved. One of these patients died of an unrelated cause (acute renal failure and infection). The third patient was reported to have moderate SOS on day 13. This patient died of an unrelated cause (septic shock) on day 15 after transplantation. Two patients developed severe SOS on days 42 and 44. These patients died of hepatic failure on day 64 and 81, respectively.

**Graft versus host disease.** The cumulative incidence of grade II–IV and III/IV aGVHD at day 100 were 35% and 11%, respectively. The cumulative incidence of grades II–IV aGVHD in the recipients who underwent transplant from an HLA-identical related donor or unrelated donor was 26% and 41%, respectively, and those of grades III/IV aGVHD was 11% and 11%, respectively. The cumulative incidence of cGVHD at 1 year after transplantation was 52%. Of the 21 patients who developed cGVHD, 12 had extensive disease and nine had limited disease.

**Survival outcome.** Twenty-six patients were alive with a median follow-up of 43 months (range, 11.9–65 months) after



doi: 10.1111/j.1349-7006.2012.02342.x

transplant. Overall survival was 65% (95% CI, 50–77%) at 1 year after transplantation, 66% (95% CI, 47–79%) for high risk and 64% (95% CI, 34–83%) for standard risk patients (Fig. 2a). Overall survival of AML was 71% (95% CI, 48–85%; $n = 24$) 1 year after transplantation, 50% (95% CI, 25–71%; $n = 16$) for MDS, and 83% (95% CI, 27–97%; $n = 6$) for CML patients. Two patients died before day 28 as described above. From days 28 to 100, seven patients died due to treatment-related mortality (four patients), infection (two patients), and relapse (one patient). Of the four patients who died of TRM, two died from hepatic toxicity, one from gastrointestinal bleeding, and one from thrombotic microangiopathy.

Disease-free survival was 57% (95% CI, 41–69%) 1 year after transplantation, 56% (95% CI, 38–71%) for high risk and 57% (95% CI, 28–78%) for standard risk patients (Fig. 2b). Disease-free survival of AML was 58% (95% CI, 36–75%) at 1 year, 44% (95% CI, 20–66%) for MDS, and 83% (95% CI, 27–97%) for CML patients.

**Relapse and TRM.** Thirteen patients (28%) experienced disease recurrence. Cumulative incidence of relapse was 24% at 1 year after transplantation. Cumulative incidence of relapse was 22% among patients with high risk disease, and 14% among patients with standard risk disease (Fig. 3a). Cumulative incidence of TRM was 18% at 1 year after transplantation (Fig. 3b).

**Pharmacokinetic studies and dose modification.** Among the 47 patients who completed the 16 BU doses, Css of the first dose was 1090 ± 318 ng/mL (range, 593–1673). The mean $AUC_{inf}$ estimated after the first dose of BU was 6760 μg·h/L (range, 3656–13058 μg·h/L). The mean values of oral clearance, distribution volume, and elimination half-life were 0.159 L/h/kg (0.079–0.263 L/h/kg), 0.55 L/kg (0.178–0.989 L/kg), and 2.54 h (0.98–5.49 h), respectively. Six patients received dose escalation of BU, and 32 received dose reduction (Fig. 4a). Median decreasing dose of BU was 4.5 mg/kg (28% of 16 mg/kg). Mean actual dose of BU was 12.7 ± 3.7 mg/kg (range, 7.6–21.3 mg/kg).

One patient was excluded from the analysis due to systemic convulsions on day −6, as described above. The Css of the first dose was 683.1 ng/mL in this patient. Although dose escalation was carried out to receive 18.7 mg/kg, the conditioning regimen was not completed.

**Busulfan targeting and transplant outcome.** Overall survival was not different between patients who received dose reduction, no modification, or escalation of BU (68%, 67%, and 50% at 1 year, respectively). Significantly more grade III–IV toxicities from days 29 to 100 were observed in patients who received dose escalation (Fisher's exact test, $P = 0.023$) (Table S2). No difference in TRM was observed among these three groups.

All three patients who developed early-onset SOS within 20 days after transplant had received dose reduction of BU. Two developed grade II liver toxicity, and another developed grade IV liver toxicity before day 28 (Fig. 4b). Two patients who had late-onset SOS and died had received dose escalation (Fig. 4c).

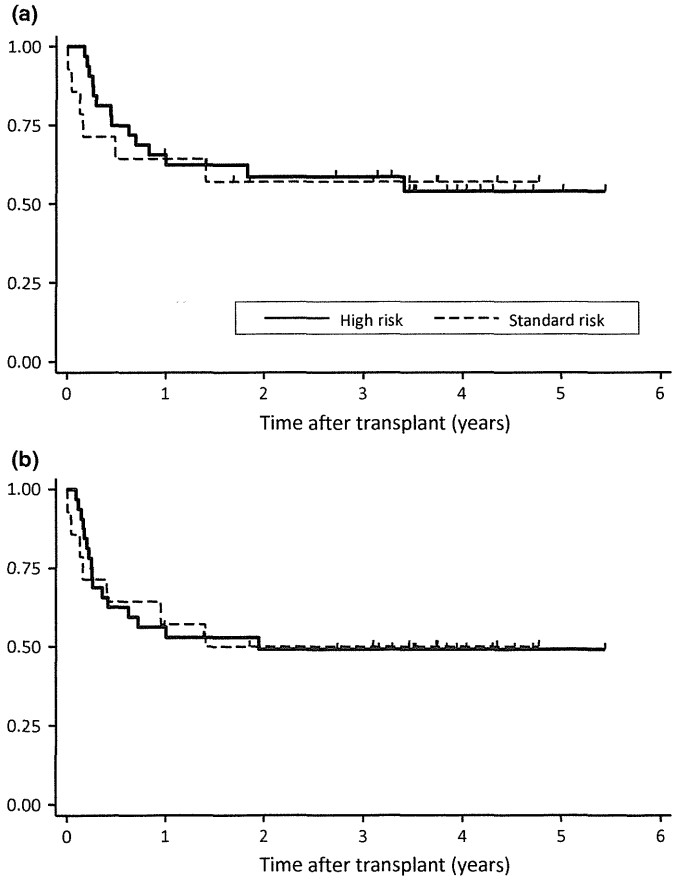


**Fig. 2.** Overall survival and disease-free survival curves according to disease risk in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. Overall survival (a) and disease-free survival (b), each stratified according to disease risk. Data were analyzed with the Kaplan–Meier method.

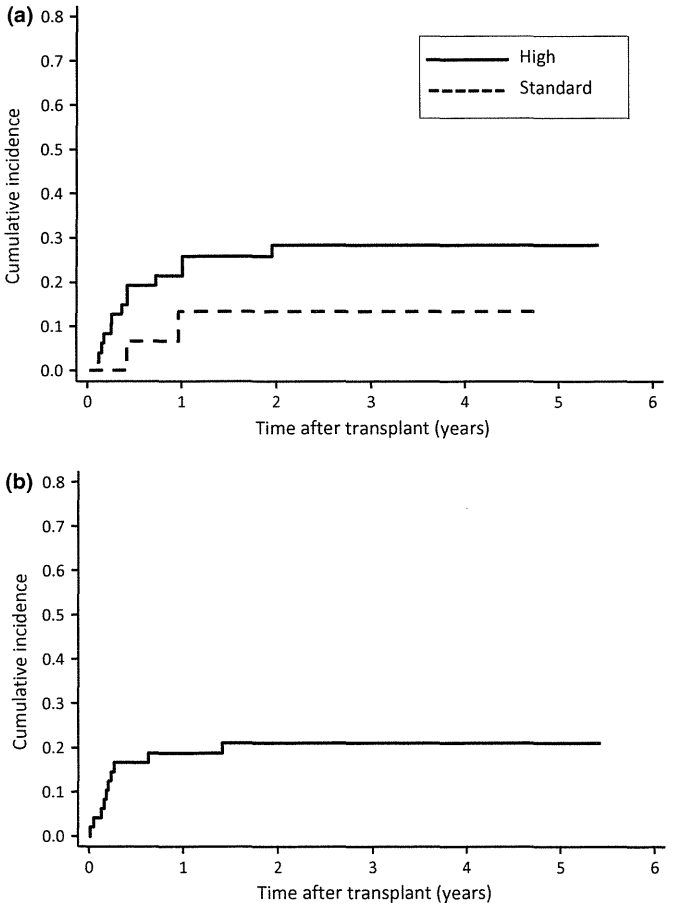


**Fig. 3.** Cumulative incidence of relapse and transplant-related mortality in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. Cumulative incidence of relapse with (a) high risk disease (22% at 1 year), standard risk disease (14%), and (b) cumulative incidence of treatment-related mortality (18%).

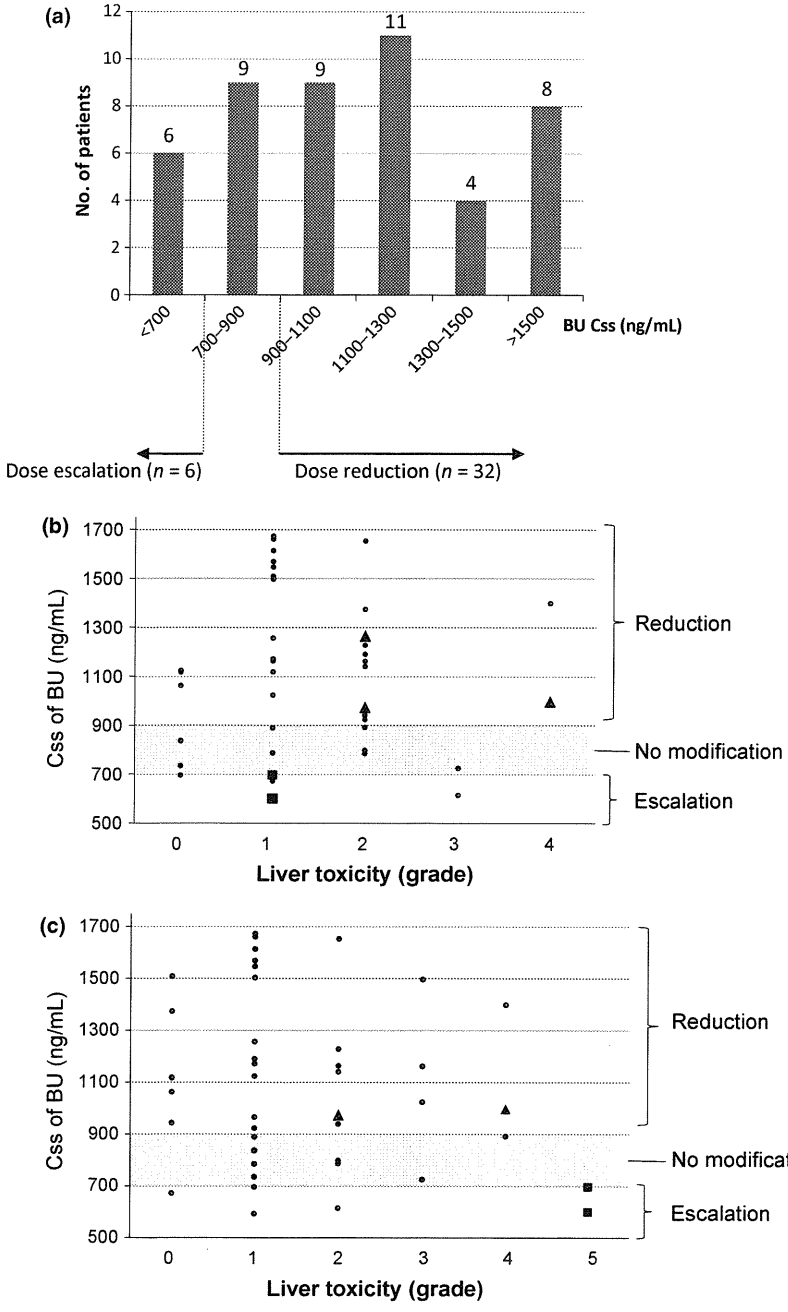


**Fig. 4.** Pharmacokinetic studies of busulfin (BU) in patients receiving allogeneic hematopoietic stem cell transplantation. (a) Number of the patients reaching concentration at steady state (Css) with the first BU dose. Six patients received dose escalation, nine patients received no modification, and 32 patients received dose reduction. (b) Liver toxicity in the first 28 days, sinusoidal obstruction syndrome (SOS), and BU dose modification. Triangles (▲) indicate patients diagnosed with SOS before day 20. Two of these patients developed grade II liver toxicity, and another developed grade IV liver toxicity. Two of these patients with early onset SOS died of an unrelated cause. Squares (■) indicate two patients who developed grade I liver toxicity in the first 28 days and were later diagnosed with severe late-onset SOS. (c) Liver toxicity from days 29 to 100, SOS and BU dose modification. Triangles (▲) and squares (■) indicate the same patients as (b). Two patients who had late-onset SOS and died had received dose escalation.

## Discussion

We carried out a phase II study of individualizing the oral BU and CY conditioning regimen for adult allogeneic HSCT for myeloid malignancies. In the current study, 1-year OS (65%; 95% CI, 50–77%) clearly exceeded the threshold level of 40%.

Oral administration of BU had been associated with erratic gastrointestinal absorption and resulted in unpredictable systemic drug exposure.[3–5,17] Pharmacokinetic studies of BU and subsequent dose adjustment strategies for the BU and CY conditioning regimen have been reported, mainly among pediatric patients.[18–22] Although no essential difference in PK analysis has been reported between data from Japan and North America,[23] survival data and information on the benefit of the tBU+CY regimen for Asian adult populations are limited.[7] In this phase II study to target the BU Css range of 700–900 ng/mL, 32 patients received BU dose reduction and the median dose of total BU was reduced. Nevertheless, no increase in relapse was observed and the incidence of TRM was comparable to the BU+CY regimen using the i.v. form.[23] Notably, the incidence of SOS (11% at day 100) was relatively lower than in the previous report of an adult population receiving the CY+total body irradiation regimen[24] or oral non-targeted BU+CY.[6] Severe SOS was not observed within 20 days after transplantation, and this targeting strategy may contribute to reduce the severity of early-onset SOS. Our positive results could be a consequence of adjusting the BU dose, considering that 38 of 47 patients (81%) actually had not achieved optimal Css after the first dose. That is, the fixed dose of BU was not optimal in 81% of these Japanese patients.

In our previous study, SOS was not observed among patients whose Css range was within the target dose or when the BU dose


doi: 10.1111/j.1349-7006.2012.02342.x

was reduced (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003). In the current study, three patients in the BU reduction group developed early-onset SOS, although the estimated cumulative Css remained within the targeted range. Liver toxicity in these patients might also be related to increased exposure to toxic metabolites of CY.[24] A dose-escalation study using test dose PK also showed that patients who showed a high level of AUC in the first dose developed severe toxicity, including hepatic SOS.[25]

Two of the six patients who received dose escalation experienced late-onset severe SOS. We may need to be cautious of possible late-onset severe SOS after dose escalation of BU. However, the causal relationship between dose escalation of BU based on low initial Css and SOS needs to be further evaluated, because individual oral BU PK are influenced by many factors. Glutathione S-transferase-mediated conjugation with GSH is the main mechanism to detoxify BU. Accumulation of the active metabolite of CY through depletion of the cellular GSH pool may contribute hepatic toxicity.[26] Hepatic GST activity and GST gene polymorphisms have been shown to be associated with BU clearance as well as transplant outcome. Polymorphism of GSTM1 is reported as a risk factor of SOS.[27] The heterozygous variant of GSTA1 (GSTA1*A/*B), which is observed in 26% of the Japanese population, resulted in slower elimination of BU than the wild-type.[28] Analysis using the Japan Marrow Donor Program showed a higher risk of TRM among recipients with the GSTM1-positive genotype, which was different from the Caucasian population.[29] We are currently investigating gene polymorphisms reported to be related with the risk factors of transplantation, such as GST genes and the UDP glucosyltransferase gene family[30] in a prospective trial.

Dose targeting possibly improves the OS by alleviating the variable absorptive characteristics among individuals. However, our results also suggest that dose modification might increase the chance of toxicity after day 28, especially in the case of dose escalation, although we should be careful of this interpretation. Dose reduction could generally lead to rejection of the graft. However, in this study, only one patient had 3 days' delay of engraftment in spite of the large number of patients in the study who received a dose reduction of BU. Busulfan in i.v. form has enabled us to accomplish narrow-ranged dose adjustment.[31] Careful validation of the clinical efficacy of PK-based targeting using i.v. BU is warranted.

In conclusion, individual dose adjustment based on BU PK was feasible and effective in the current phase II study.

## Acknowledgments

We are grateful to Mio Kurata, Seiko Amano, and Kaori Sugiura for clinical data management and data retrieval. We wish to thank all of the staff of the participating institutions.

## Disclosure Statement

The authors have no conflicts of interest.

## Abbreviations

| | |
|---|---|
| aGVHD | acute graft versus host disease |
| AUC | area under the blood concentration time curve |
| BU | busulfan |
| cGVHD | chronic graft versus host disease |
| CI | cumulative incidence |
| Css | concentration at steady state |
| CY | cyclophosphamide |
| DFS | disease-free survival |
| GSH | glutathione |
| GVHD | graft versus host disease |
| HLA | human leukocyte antigen |
| HSCT | hematopoietic stem cell transplantation |
| LSM | limited sample model |
| MDS | myelodysplastic syndrome |
| OS | overall survival |
| PK | pharmacokinetic |
| SOS | sinusoidal obstruction syndrome |
| tBU+CY | targeting BU+CY |
| TRM | transplant-related mortality |

## References

1 Tutschka PJ, Copelan EA, Klein JP. Bone marrow transplantation for leukemia following a new busulfan and cyclophosphamide regimen. *Blood* 1987; **70**: 1382–8.
2 Socie G, Clift RA, Blaise D *et al.* Busulfan plus cyclophosphamide compared with total-body irradiation plus cyclophosphamide before marrow transplantation for myeloid leukemia: long-term follow-up of 4 randomized studies. *Blood* 2001; **98**: 3569–74.
3 Slattery JT, Clift RA, Buckner CD *et al.* Marrow transplantation for chronic myeloid leukemia: the influence of plasma busulfan levels on the outcome of transplantation. *Blood* 1997; **89**: 3055–60.
4 Dix SP, Wingard JR, Mullins RE *et al.* Association of busulfan area under the curve with veno-occlusive disease following BMT. *Bone Marrow Transplant* 1996; **17**: 225–30.
5 Vassal G, Deroussent A, Hartmann O *et al.* Dose-dependent neurotoxicity of high-dose busulfan in children: a clinical and pharmacological study. *Cancer Res* 1990; **50**: 6203–7.
6 Kashyap A, Wingard J, Cagnoni P *et al.* Intravenous versus oral busulfan as part of a busulfan/cyclophosphamide preparative regimen for allogeneic hematopoietic stem cell transplantation: decreased incidence of hepatic venoocclusive disease (HVOD), HVOD-related mortality, and overall 100-day mortality. *Biol Blood Marrow Transplant* 2002; **8**: 493–500.
7 Takamatsu Y, Sasaki N, Eto T *et al.* Individual dose adjustment of oral busulfan using a test dose in hematopoietic stem cell transplantation. *Int J Hematol* 2007; **86**: 261–8.
8 Fukumoto M, Kubo H, Ogamo A. Quantitative determination of busulfan in serum using gas chromatography - Mass spectrometry in negative-ion chemical ionization mode. *Anal Lett* 2001; **34**: 761–71.
9 Grochow LB, Jones RJ, Brundrett RB *et al.* Pharmacokinetics of busulfan: correlation with veno-occlusive disease in patients undergoing bone marrow transplantation. *Cancer Chemother Pharmacol* 1989; **25**: 55–61.
10 Terakura S, Atsuta Y, Sawa M *et al.* A prospective dose-finding trial using a modified continual reassessment method for optimization of fludarabine plus melphalan conditioning for marrow transplantation from unrelated donors in patients with hematopoietic malignancies. *Ann Oncol* 2011; **22**: 1865–71.
11 Deeg HJ, Lin D, Leisenring W *et al.* Cyclosporine or cyclosporine plus methylprednisolone for prophylaxis of graft-versus-host disease: a prospective, randomized trial. *Blood* 1997; **89**: 3880–7.
12 McDonald GB, Hinds MS, Fisher LD *et al.* Veno-occlusive disease of the liver and multiorgan failure after bone marrow transplantation: a cohort study of 355 patients. *Ann Intern Med* 1993; **118**: 255–67.
13 Jones RJ, Lee KS, Beschorner WE *et al.* Venoocclusive disease of the liver following bone marrow transplantation. *Transplantation* 1987; **44**: 778–83.
14 Shulman HM, Hinterberger W. Hepatic veno-occlusive disease–liver toxicity syndrome after bone marrow transplantation. *Bone Marrow Transplant* 1992; **10**: 197–214.
15 Cheson BD, Cassileth PA, Head DR *et al.* Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. *J Clin Oncol* 1990; **8**: 813–9.
16 Kolb HJ. Management of relapse after hematopoietic cell transplantation. In: Thomas EDBK, Forman SJ, eds. *Hematopoietic Cell Transplantation*, 2nd edn. Boston, MA: Blackwell Science, 1999; 929–36.
17 Grochow LB. Busulfan disposition: the role of therapeutic monitoring in bone marrow transplantation induction regimens. *Semin Oncol* 1993; **20**: 18–25; quiz 26.
18 Bolinger AM, Zangwill AB, Slattery JT *et al.* Target dose adjustment of busulfan in pediatric patients undergoing bone marrow transplantation. *Bone Marrow Transplant* 2001; **28**: 1013–8.

19 Brice K, Valerie B, Claire G *et al.* Risk-adjusted monitoring of veno-occlusive disease following Bayesian individualization of busulfan dosage for bone marrow transplantation in paediatrics. *Pharmacoepidemiol Drug Saf* 2008; **17**: 135–43.
20 Tran H, Petropoulos D, Worth L *et al.* Pharmacokinetics and individualized dose adjustment of intravenous busulfan in children with advanced hematologic malignancies undergoing allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2004; **10**: 805–12.
21 Dupuis LL, Sibbald C, Schechter T *et al.* IV busulfan dose individualization in children undergoing hematopoietic stem cell transplant: limited sampling strategies. *Biol Blood Marrow Transplant* 2008; **14**: 576–82.
22 Zwaveling J, Bredius RG, Cremers SC *et al.* Intravenous busulfan in children prior to stem cell transplantation: study of pharmacokinetics in association with early clinical outcome and toxicity. *Bone Marrow Transplant* 2005; **35**: 17–23.
23 Kim SW, Mori SI, Tanosaki R *et al.* Busulfex (i.v. BU) and CY regimen before SCT: Japanese-targeted phase II pharmacokinetics combined study. *Bone Marrow Transplant* 2009; **43**: 611–7.
24 McDonald GB, Slattery JT, Bouvier ME *et al.* Cyclophosphamide metabolism, liver toxicity, and mortality following hematopoietic stem cell transplantation. *Blood* 2003; **101**: 2043–8.
25 O'Donnell PH, Artz AS, Undevia SD *et al.* Phase I study of dose-escalated busulfan with fludarabine and alemtuzumab as conditioning for allogeneic hematopoietic stem cell transplant: reduced clearance at high doses and occurrence of late sinusoidal obstruction syndrome/veno-occlusive disease. *Leuk Lymphoma* 2010; **51**: 2240–9.
26 Hassan M, Ljungman P, Ringden O *et al.* The effect of busulphan on the pharmacokinetics of cyclophosphamide and its 4-hydroxy metabolite: time interval influence on therapeutic efficacy and therapy-related toxicity. *Bone Marrow Transplant* 2000; **25**: 915–24.
27 Srivastava A, Poonkuzhali B, Shaji RV *et al.* Glutathione S-transferase M1 polymorphism: a risk factor for hepatic venoocclusive disease in bone marrow transplantation. *Blood* 2004; **104**: 1574–7.
28 Kusama M, Kubota T, Matsukura Y *et al.* Influence of glutathione S-transferase A1 polymorphism on the pharmacokinetics of busulfan. *Clin Chim Acta* 2006; **368**: 93–8.
29 Terakura S, Murata M, Nishida T *et al.* Increased risk for treatment-related mortality after bone marrow transplantation in GSTM1-positive recipients. *Bone Marrow Transplant* 2006; **37**: 381–6.
30 Terakura S, Murata M, Nishida T *et al.* A UGT2B17-positive donor is a risk factor for higher transplant-related mortality and lower survival after bone marrow transplantation. *Br J Haematol* 2005; **129**: 221–8.
31 Pidala J, Kim J, Anasetti C *et al.* Targeted i.v. BU and fludarabine (t-i.v. BU/Flu) provides effective control of AML in adults with reduced toxicity. *Bone Marrow Transplant* 2011; **46**: 641–9.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Study scheme.

**Table S1.** Regimen-related toxicity before day 28 in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.

**Table S2.** Regimen-related toxicity (day 29–100) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.



doi: 10.1111/j.1349-7006.2012.02342.x

Cellular Immunology 276 (2012) 75–82

Contents lists available at SciVerse ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

# Escape of leukemia blasts from HLA-specific CTL pressure in a recipient of HLA one locus-mismatched bone marrow transplantation

Tomonori Kato [1], Seitaro Terakura [1], Makoto Murata *, Kyoko Sugimoto, Miho Murase, Chisako Iriyama, Akihiro Tomita, Akihiro Abe, Momoko Suzuki, Tetsuya Nishida, Tomoki Naoe

*Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan*

ARTICLE INFO

*Article history:*
Received 26 January 2012
Accepted 7 March 2012
Available online 11 April 2012

*Keywords:*
Cytotoxic T lymphocyte
HLA
Immune escape

ABSTRACT

A case of leukemia escape from an HLA-specific cytotoxic T lymphocyte (CTL) response in a recipient of bone marrow transplantation is presented. Only the expression of HLA-B51, which was a mismatched HLA locus in the graft-versus-host direction, was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts. All CTL clones, that were isolated from the recipient's blood when acute graft-versus-host disease developed, recognized the mismatched B*51:01 molecule in a peptide-dependent manner. The pre-transplant leukemia blasts were lysed by CTL clones, whereas the post-transplant leukemia blasts were not lysed by any CTL clones. The IFN-γ ELISPOT assay revealed that B*51:01-reactive T lymphocytes accounted for the majority of the total alloreactive T lymphocytes in the blood just before leukemia relapse. These data suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA molecule can lead to clinical relapse after bone marrow transplantation.



## 1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative for leukemia by virtue of the immune reaction mediated by donor T lymphocytes, termed the graft-versus-leukemia (GVL) effect [1]. For HSCT recipients from HLA-matched donors, the GVL effect can be triggered by minor histocompatibility antigens [2–4], and several studies using sequential flow cytometric analysis with tetramers have clearly demonstrated that minor histocompatibility antigen-specific T lymphocytes increase in frequency in the recipient's blood before and during clinical regression of leukemia [5–10]. On the other hand, for HLA-mismatched HSCT recipients, extremely limited biological studies have demonstrated that the GVL effect can be mediated by mismatched HLA-specific donor T lymphocytes [11].

Allogeneic HSCT is a well-established immunotherapy for leukemia, but, unfortunately, some recipients relapse after transplantation. It is difficult to evaluate the role of individual factors in relapse. Nevertheless, it is reasonable to assume that the selective pressure exerted by donor T lymphocytes can lead to the outgrowth of pre-existing leukemia variants that have lost expression of gene products such as HLA molecules. Some studies have demonstrated loss of the mismatched HLA haplotype in the leukemia blasts of HSCT recipients as a consequence of loss of heterozygosity in chromosome 6 [12–14]. However, the mechanisms involved in leukemia relapse after HLA locus-mismatched HSCT remain largely uninvestigated.

This paper presents a case of selective HLA down-regulation in post-transplant leukemia blasts but not in pre-transplant blasts of a recipient who received bone marrow transplantation from an HLA one locus-mismatched donor. All cytotoxic T lymphocyte (CTL) clones that were isolated from the recipient's blood during acute graft-versus-host disease (GVHD) demonstrated cytotoxicity specific for the mismatched HLA-B molecule, lysed pre-transplant blasts but not post-transplant blasts, and persisted in the patient's blood until leukemia relapse. These results suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA allele can lead to clinical relapse.

## 2. Patient, materials and methods

### 2.1. Patient

A 24-year-old man with primary refractory T lymphoblastic leukemia/lymphoma received allogeneic bone marrow transplantation without ex vivo T lymphocyte depletion from his mother. Because the patient had neither a sibling nor an HLA-matched unrelated donor, his mother was chosen as an alternative donor. PCR sequencing-based typing for HLA alleles of the patient and mother revealed one HLA-B allele mismatch in

* Corresponding author. Fax: +81 52 744 2157.
*E-mail address:* mmurata@med.nagoya-u.ac.jp (M. Murata).
[1] These authors contributed equally.

0008-8749/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellimm.2012.03.011

**Table 1**
HLA types of the patient and donor.

| | A | B | C | DRB1 | DQB1 | DPB1 |
|---|---|---|---|---|---|---|
| Patient | 1101/2402 | 5401/5101 | 0102/– | 0901/– | 0303/– | 0501/– |
| Donor | 1101/2402 | 5401/5201 | 0102/1202 | 0901/1502 | 0303/0601 | 0501/– |

The mismatched HLA allele in the graft-versus-host direction is underlined.

the graft-versus-host direction (Table 1). The preparative regimen consisted of 180 mg/m$^2$ melphalan and 12 Gy total body irradiation. GVHD prophylaxis consisted of 0.03 mg/kg tacrolimus and short-term methotrexate. Neutrophil engraftment (neutrophil count $\geqslant 0.5 \times 10^9$/l) was achieved 14 days after transplantation with full donor-type chimera. The patient developed severe acute GVHD involving the skin, gut, and liver on day 46 (maximum stage: skin 3, gut 2, and liver 1; maximum grade: III on day 53), evaluated according to previously published criteria [15]. Acute GVHD was temporarily controlled by additional immunosuppressants, but it was incurable and transitioned to chronic GVHD. On day 261, the patient relapsed with ascites, a hydrocele, and a subpapillary tumor. Leukemia blasts in the ascites fluid were confirmed by cytological examination. Immunosuppressant therapy was required to control GVHD until his death on day 279.

### 2.2. Cell culture

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the recipient on day 56, when severe acute GVHD developed, were stimulated in vitro with aliquots of γ-irradiated PBMCs that had been obtained from the recipient pre-transplant and cryopreserved. After three weekly stimulations, the CTL clones were isolated from the polyclonal T lymphocyte culture by limiting dilution. The CTLs were expanded by stimulation every 14 days with 30 ng/ml OKT3 monoclonal antibody (Janssen Pharmaceutical), using unrelated allogeneic γ-irradiated (25 Gy) PBMCs and γ-irradiated (75 Gy) EB virus-transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture medium consisted of RPMI-1640-HEPES (Sigma–Aldrich) containing 10% pooled, heat-inactivated human serum, and recombinant human IL-2 (R&D Systems). The T lymphocytes were used in assays 14 days after stimulation or 1 day after thawing of a frozen aliquot. All samples were collected after written informed consent had been obtained. B-LCLs were maintained in RPMI-1640-HEPES with 10% FBS. COS cells were maintained in DMEM (Sigma–Aldrich) with 10% FBS.

### 2.3. Flow cytometric analysis

Leukemia blasts were incubated at 37 °C for 30 min with anti-HLA-A24/A23 (One lambda), anti-HLA-A11/A1/A26 (One lambda), and anti-HLA-B51/B52/B49/B56 (One lambda) antibodies to detect A24, A11, and B51, respectively, of patient cells followed by incubation at 37 °C for 15 min with fluorescein isothiocyanate-conjugated antimouse IgM (Beckman Coulter). To detect HLA-DR9 of patient cells, leukemia blasts were incubated at 37 °C for 30 min with fluorescein isothiocyanate-conjugated anti-HLA-DR antibody (BD Pharmingen). Antibody to detect HLA-B54 without cross-reaction to B51 was not available. After washing, the cells were analyzed by a BD FACSAria (BD Biosciences). Leukemia blasts were sorted by BD FACSAria with anti-CD7 (BD Biosciences) and anti-CD10 (eBiosciences) antibodies from pre-transplant bone marrow and post-transplant ascites fluid samples. The purities of pre-transplant and post-transplant blasts were ~62% and ~99%, respectively. CTL clones were analyzed using three-color flow cytometry for expression of CD3, CD4, and CD8 using phycoerythrin-cyanin 5.1-conjugated anti-CD3 (Beckman Coulter), phycoerythrin-conjugated anti-CD4 (BD Biosciences), and fluorescein isothiocyanate-conjugated anti-CD8 (BD Biosciences) antibodies.

### 2.4. Chromium release assay

Leukemia blasts and B-LCLs were used as target cells in a cytotoxicity assay. Leukemia blasts and B-LCLs were labeled for 2 h with $^{51}$Cr. After washing, the cells were dispensed at $2 \times 10^3$ cells/well into triplicate cultures in 96-well plates and incubated for 4 h at 37 °C with CTL clones at various E:T ratios. Percent-specific lysis was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] ×100.

### 2.5. Determination of T cell receptor (TCR)-Vβ gene usage and nucleotide sequences

TCR Vβ usage was assessed by RT-PCR using primers covering the entire families of functional TCR Vβ chains [17–19]. Briefly, total RNA was extracted from individual CTL clones, and cDNA was synthesized using SuperScript III RT (Invitrogen). RT-PCR reactions were carried out with the appropriate Vβ sense primers specific for different Vβ families and a primer specific for the constant region of TCR-β. Subsequently, the complementarity determining region 3(CDR3) of each positive PCR product was sequenced with corresponding antisense primer. TCR Vβ gene usage was determined by the international ImMunoGeneTics information system (IMGT) software, IMGT/V-QUEST (http://www.imgt.org/).

### 2.6. HLA-B cDNA constructs

Total RNA was extracted from the patient and donor B-LCLs and converted into cDNA. Constructs containing the full-length *HLA-B*51:01*, *B*52:01*, and *B*54:01* cDNA were generated from the cDNA by PCR and cloned into the pEAK10 expression vector (Edge BioSystems). Two mutated *HLA-B*51:01* cDNA constructs, in which amino acid at position 63 or 67 was substituted with the corresponding amino acid in *B*52:01*, and two more mutated *HLA-B*51:01* cDNA constructs, in which the amino acid at position 194 or 199 was substituted with the corresponding amino acid in *B*44:03*, were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

### 2.7. Transfection of B-LCLs and COS cells with HLA cDNA

B-LCL ($5 \times 10^6$) were transfected by electroporation (200 V, 500 μFD) in 200 μl of potassium-PBS with the 15 μg of pEAK10 plasmid encoding *HLA-B*51:01* cDNA and selected with puromycin (Edge BioSystems), beginning 48 h after transfection. Three days after selection, they were used as targets in a chromium release assay. COS cells ($5 \times 10^3$) were plated in individual wells of 96-well flat-bottom plates and transfected with 100 ng of the pEAK10 plasmid encoding *HLA-B*51:01*, *HLA-B*52:01*, *HLA-B*54:01*, or mutated *HLA-B*51:01* cDNA using the FuGENE 6 Transfection Reagent (Roche).

### 2.8. CTL stimulation assay

COS transfectants ($5 \times 10^3$) were cocultured with CTL clones ($2 \times 10^4$) in individual wells of 96-well flat-bottom plates for 24 h at 37 °C, and IFN-γ production was measured in the supernatant using ELISA (Endogen).

### 2.9. Enzyme-linked immunospot (ELISPOT) assay

T lymphocytes were isolated from recipient's PBMCs by negative depletion using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and used as responder T cells. Responder T cells at a concentration of $2 \times 10^5$ per well were plated in individual wells of the 96-well MultiScreen-IP filter plates (Millipore) coated with anti-human interferon (IFN)-γ antibody (5 μg/ml; Mabtech) and tested in triplicate against a total of $2 \times 10^5$ stimulator cells: patient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. The plates were incubated for 24 h at 37°C, washed, and incubated with biotinylated anti-human IFN-γ antibody (1 μg/ml; Mabtech) for 2 h at room temperature. After addition of streptavidin (Fitzgerald Industries International) to the wells, the plates were developed with a 3-amino-9-ethylcarbazol substrate kit (Vector Laboratories). Spots were counted using a microscope, and mean numbers were calculated from triplicate wells after subtraction of the number of spots obtained with medium alone.

## 3. Results

### 3.1. Selective down-regulation of HLA-B locus in post-transplant leukemia blasts

To determine whether expressions of some HLA loci in post-transplant relapsed leukemia blasts were down-regulated or lost, flow cytometric analysis was performed for HLA-A*24:02, A*11:01, B*51:01, and DR*09:01 using anti-HLA-A24/A23, -HLA-A11/A1/A26, -HLA-B51/B52/B49/B56, and -pan HLA-DR antibodies, respectively. The expression of B*51:01 was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts, whereas expressions of A*24:02, A*11:01, and DR*09:01 were the same or higher in post-transplant blasts than in pre-transplant blasts (Fig. 1). These data led us to question whether B*51:01-selective pressure mediated by donor T lymphocytes was present in the patient post-transplant.

### 3.2. Isolation of alloreactive CTL clones

Ten CTL clones, termed TK1 to TK10, were isolated from the peripheral blood of the recipient during acute GVHD. In a cytotoxicity assay, all isolated clones lysed recipient B-LCL but failed to lyse donor B-LCL (Fig. 2), demonstrating that all clones were alloreactive. Flow cytometric analysis revealed that all CTL clones

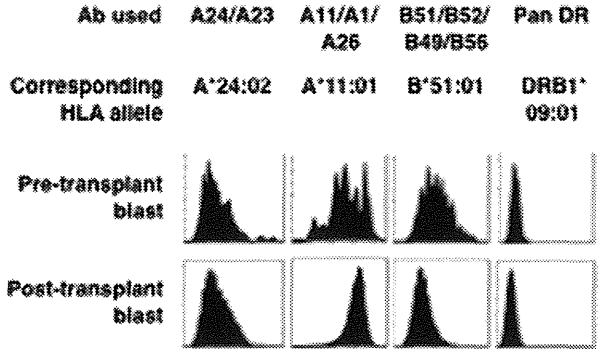


**Fig. 1.** HLA expression on leukemia blasts. Pre-transplant and post-transplant leukemia blasts were stained with anti-HLA-A24/A23, anti-HLA-A11/A1/A26, anti-HLA-B51/B52/B49/B56, and anti-HLA-pan DR antibodies to detect A*24:02, A*11:01, B*51:01, and DRB1*09:01, respectively. Data are representative of four experiments.

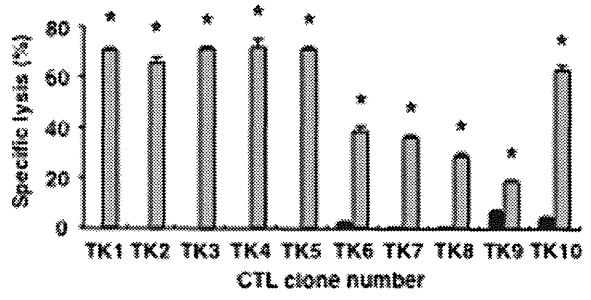


**Fig. 2.** Cytotoxicities of CTL clones against B-LCLs. B-LCLs that originated from the recipient (gray) and the donor (black) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at an E:T ratio of 10:1. *Significant difference ($p < 0.0001$: Student's *t*-test) in the lysis of recipient B-LCL compared with donor B-LCL. Data are representative of three experiments.

**Table 2**
Clonotypes of isolated CTL clones.

| CTL | TCR Vβ | Nucleotide and deduced amino acid sequences of complementarity determining region 3 | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TK1 | Vβ6.5 | GCC | AGC | AGT | CCC | GGG | ACT | AGC | GGA | ACC | TAC | GAG | CAG | TAC | TTC | | | | |
| | | A | S | S | P | G | T | S | G | T | Y | E | Q | Y | F | | | | |
| TK2 | Vβ20 | AGT | CAG | GGG | CCG | GCG | GTT | ACC | GGG | GAG | CTG | TTT | TTT | | | | | | |
| | | S | Q | G | P | A | V | T | G | E | L | F | F | | | | | | |
| TK3 | Vβ20 | AGT | CAG | GGG | CCG | GCG | GTT | ACC | GGG | GAG | CTG | TTT | TTT | | | | | | |
| | | S | Q | G | P | A | V | T | G | E | L | F | F | | | | | | |
| TK4 | Vβ19*1 | GCC | AGT | ACT | TGG | GGT | TAC | CCA | CAG | GGG | CCC | GGT | GCG | GAT | ACC | GGG | GAG | CTG | TTT | TTT |
| | | A | S | T | W | G | Y | P | Q | G | P | G | A | D | T | G | E | L | F | F |
| TK5 | Vβ19*1 | GCC | AGT | ACT | TGG | GGT | TAC | CCA | CAG | GGG | CCC | GGT | GCG | GAT | ACC | GGG | GAG | CTG | TTT | TTT |
| | | A | S | T | W | G | Y | P | Q | G | P | G | A | D | T | G | E | L | F | F |
| TK6 | Vβ12 | GCC | AGC | AGT | TTA | GCT | AGC | GGG | AGG | GCC | TCC | CAT | GAG | CAG | TTC | TTC | | | | |
| | | A | S | S | L | A | S | G | R | A | S | H | E | Q | F | F | | | | |
| TK7 | Vβ12 | GCC | AGC | AGT | TTA | GCT | AGC | GGG | AGG | GCC | TCC | CAT | GAG | CAG | TTC | TTC | | | | |
| | | A | S | S | L | A | S | G | R | A | S | H | E | Q | F | F | | | | |
| TK8 | ND | | | | | | | | | | | | | | | | | | | |
| TK9 | Vβ12 | GCC | AGC | AGT | TTA | GCT | AGC | GGG | AGG | GCC | TCC | CAT | GAG | CAG | TTC | TTC | | | | |
| | | A | S | S | L | A | S | G | R | A | S | H | E | Q | F | F | | | | |
| TK10 | Vβ2 | GCC | AGC | AGT | GAC | TCT | ATC | GCG | GAT | GAG | CAG | TTC | TTC | | | | | | | |
| | | A | S | S | D | S | I | A | D | E | Q | F | F | | | | | | | |

ND, not detected.