With regard to specific amino acid substitutions of HLA-DPB1, we found no significant association among these with a decreased risk of relapse. Shaw et al[27] reported that mismatches at position 57 and 65 in the HLA-DPB1 molecule were associated with transplant complications, but not with GVHD or relapse, which is consistent with our present data. We speculate that, compared with MHC class I, the conformational diversity of MHC class II and peptide complex hampers the identification of strict rules of association between specific amino acid substitutions in MHC class II molecules and the occurrence of alloreaction such as GVHD and GVL. In HLA class I, binding peptides are held by their ends, whereas peptides bind to HLA class II by attachment in the middle, allowing them to vary greatly in length.[28]

Given that this analysis was conducted using a Japanese cohort of patients who received transplants through the Japan Marrow Donor Program, the applicability of our data to other ethnic groups warrants discussion. We speculate that the effect of alloreaction is a reflection and summation of HLA allele mismatch combinations. Discrepancies in the effect of HLA locus on alloreactions between ethnically diverse transplantation might be explained by the proportions of each HLA mismatch combination in each HLA locus. In HLA-DPB1, on the other hand, the allele variations between white and Japanese populations are relatively close, hence our findings in HLA-DPB1 might also be useful for white populations. Regarding HLA-Cw and killer immunoglobulin-like receptor (KIR) incompatibility, we previously reported adverse effects in unrelated T cell–replete HSCT through the Japan Marrow Donor Program,[18] although Ruggieri et al[29] demonstrated that beneficial effects were shown in T-cell depleted haploidentical transplantation. We speculated that in vivo and/or in vitro T-cell depletion could account for this discrepancy.[30] Therefore, results for mismatch combinations in HLA-Cw obtained in other populations treated in other settings may differ from our results. Nevertheless, clarification of these questions would require the same study in other ethnic populations.

Given the general acceptance that GVL is more closely correlated with chronic GVHD than acute GVHD,[3] separating GVL from chronic GVHD may be more difficult than separating it from acute GVHD. On this basis, our results suggest that GVL could be separated from acute GVHD in HSCT from a specific HLA partially mismatched donor. Clarification of whether GVL can also be separated from chronic GVHD requires further study.

In conclusion, we identified 4 HLA-C and 6 HLA-DPB1 mismatch combinations that decrease the risk of relapse in patients after HSCT. Eight of 10 GVL combinations were different from those responsible for severe acute GVHD. In particular, all 6 GVL combinations in HLA-DPB1 were different. Further, pairs with these GVL combinations of HLA-DPB1 were associated with significantly better OS than completely matched pairs. These findings suggest that donor selection according to these results could separate the occurrence of GVL from acute GVHD, especially in HLA-DPB1. Further, amino acid substitutions on specific positions responsible for this decreased risk of relapse were also elucidated in HLA-C, but not in HLA-DPB1. Our finding that specific amino acid substitutions decrease the risk of relapse might be key to revealing the mechanism of the decreased risk of relapse due to GVL with regard to the HLA molecule.

Acknowledgments

We thank the staff members of the transplant centers, donor centers, and the Japan Marrow Donor Program office for their generous cooperation; Ms Ryoko Yamauchi for the data management; and Drs Toshitada Takahashi and Setsuko Kawase for their expert technical assistance.

This study was supported in part by Health and Labor Science Research Grant (Research on Allergic disease and Immunology) from the Ministry of Health, Labor, and Welfare (Tokyo, Japan) and the Grant-in-Aid for Cancer Research (19-1) from the Ministry of Health, Labor, and Welfare (Tokyo, Japan).

Authorship

Contribution: T.K., Y.M., T.S., S.O., and Y.K. participated in the conception of this study; K.K., H.I., and H.S. participated in the assessment of histocompatibility; Y.M. and S.K. participated in the execution of transplantation; T.K. and K.M. participated in the statistical data analysis; T.K. and Y.M. wrote the paper; and all authors checked the final version of the manuscript.

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

Correspondence: Yasuo Morishima, Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya, 464-8681, Japan; e-mail: ymorisim@aichi-cc.jp.

References

1. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med. 1979;300:1068-1073.
2. Gale RP, Horowitz MM. Graft-versus-leukemia in bone marrow transplantation: the Advisory Committee of the International Bone Marrow Transplant Registry. Bone Marrow Transplant. 1990; 6(suppl 1):94-97.
3. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75:555-562.
4. Carella AM, Beltrami G, Corsetti MT, et al. Reduced intensity conditioning for allograft after cytoreductive autograft in metastatic breast cancer. Lancet. 2005;366:318-320.
5. Bregni M, Dodero A, Peccatori J, et al. Nonmyeloablative conditioning followed by hematopoietic cell allografting and donor lymphocyte infusions for patients with metastatic renal and breast cancer. Blood. 2002;99:4234-4236.
6. Ueno NT, Cheng YC, Rondon G, et al. Rapid induction of complete donor chimerism by the use of a reduced-intensity conditioning regimen composed of fludarabine and melphalan in allogeneic stem cell transplantation for metastatic solid tumors. Blood. 2003;102:3829-3836.
7. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4:371-380.
8. Kawase T, Morishima Y, Matsuo K, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood. 2007;110:2235-2241.
9. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor: Japan Marrow Donor Program. N Engl J Med. 1998;339:1177-1185.
10. European Molecular Biology Laboratory–European Bioinformatics Institute. IMGT/HLA database. http://www.ebi.ac.uk/imgt/hla/. Accessed May 1, 2008.
11. 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.
12. Coviello V, Boffess M. Cumulative incidence estimation in the presence of compiting risks. Stata J. 2004;4:103-112.
13. Cox DR. Regression models and life-tables. J R Stat Soc Ser B. 1972;34:187-220.
14. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc. 1999;94:496-497.
15. Efron B. Bootstrap methods: another look at the jackknife. Ann Stat. 1979;7:1-26.

16. Manly BFJ. Randomization, Bootstrap and Monte Carlo Methods in Biology. London, United Kingdom: Chapman and Hall; 1997.
17. Shaw BE, Gooley TA, Malkki M, et al. The importance of HLA-DPB1 in unrelated donor hematopoietic cell transplantation. Blood. 2007;110: 4560-4566.
18. Morishima Y, Yabe T, Matsuo K, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. Biol Blood Marrow Transplant. 2007;13:315-328.
19. Weichold FF, Jiang YZ, Dunn DE, et al. Regulation of a graft-versus-leukemia effect by major histocompatibility complex class II molecules on leukemia cells: HLA-DR1 expression renders K562 cell tumors resistant to adoptively transferred lymphocytes in severe combined immunodeficiency mice/nonobese diabetic mice. Blood. 1997;90:4553-4558.
20. Gribben JG, Guinan EC, Boussiotis VA, et al. Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specific anergy: a method to ameliorate graft-versus-host disease and extend the donor pool. Blood. 1996;87:4887-4893.
21. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Gray GS, Vallera DA. Coblockade of the LFA1: ICAM and CD28/CTLA4:B7 pathways is a highly effective means of preventing acute lethal graft-versus-host disease induced by fully major histocompatibility complex-disparate donor grafts. Blood. 1995;85:2607-2618.
22. Hogan KT, Clayberger C, Bernhard EJ, et al. Identification by site-directed mutagenesis of amino acid residues contributing to serologic and CTL-defined epitope differences between HLA-A2.1 and HLA-A2.3. J Immunol. 1988;141:2519-2525.
23. Mattson DH, Shimojo N, Cowan EP, et al. Differential effects of amino acid substitutions in the beta-sheet floor and alpha-2 helix of HLA-A2 on recognition by alloreactive viral peptide-specific cytotoxic T lymphocytes. J Immunol. 1989;143: 1101-1107.
24. Shimojo N, Cowan EP, Engelhard VH, Maloy WL, Coligan JE, Biddison WE. A single amino acid substitution in HLA-A2 can alter the selection of the cytotoxic T lymphocyte repertoire that responds to influenza virus matrix peptide 55-73. J Immunol. 1989;143:558-564.
25. Tynan FE, Elhassen D, Purcell AW, et al. The immunogenicity of a viral cytotoxic T cell epitope is controlled by its MHC-bound conformation. J Exp Med. 2005;202:1249-1260.
26. Macdonald WA, Purcell AW, Mifsud NA, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. J Exp Med. 2003;198:679-691.
27. Shaw BE, Marsh SGE, Mayor NP, Madrigal JA. Matching status at amino acid positions 57 and 65 of the HLA-DPB1 beta chain determines outcome in recipients of unrelated donor haematopoietic stem cell transplants [abstract]. Blood. 2004;104:Abstract 827.
28. Steven GE, Peter P, Linda DB. The HLA Facts Book. London, United Kingdom: Academic Press; 2000.
29. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.
30. Yabe T, Matsuo K, Hirayasu K, et al. Donor killer immunoglobulin-like receptor (KIR) genotype-patient cognate KIR ligand combination and anti-thymocyte globulin preadministration are critical factors in outcome of HLA-C-KIR ligand-mismatched T cell-replete unrelated bone marrow transplantation. Biol Blood Marrow Transplant. 2008;14:75-87.

Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia

Yoshiko Atsuta,[1] Ritsuro Suzuki,[1] Tokiko Nagamura-Inoue,[2] Shuichi Taniguchi,[3] Satoshi Takahashi,[4] Shunro Kai,[5] Hisashi Sakamaki,[6] Yasushi Kouzai,[7] Masaharu Kasai,[8] Takahiro Fukuda,[9] Hiroshi Azuma,[10] Minoko Takanashi,[11] Shinichiro Okamoto,[12] Masahiro Tsuchida,[13] Keisei Kawa,[14] Yasuo Morishima,[15] Yoshihisa Kodera,[16] and Shunichi Kato,[17] for the Japan Marrow Donor Program and the Japan Cord Blood Bank Network

[1]Department of Hematopoietic Stem Cell Transplantation Data Management, Nagoya University School of Medicine, Nagoya; [2]Department of Cell Processing & Transfusion, Research Hospital, Institute of Medical Science, University of Tokyo, and Tokyo Cord Blood Bank, Tokyo; [3]Department of Hematology, Toranomon Hospital, Tokyo; [4]Department of Molecular Therapy, Institute of Medical Science, University of Tokyo, Tokyo; [5]Department of Transfusion Medicine, Hyogo College of Medicine, Nishinomiya; [6]Division of Hematology, Tokyo Metropolitan Komagome Hospital, Tokyo; [7]Department of Transfusion Medicine, Tokyo Metropolitan Fuchu Hospital, Tokyo; [8]Department of Hematology, Sapporo Hokuyu Hospital, Sapporo; [9]Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo; [10]Hokkaido Red Cross Blood Center, Sapporo; [11]Japanese Red Cross Tokyo Blood Center, Tokyo; [12]Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo; [13]Ibaraki Children's Hospital, Mito; [14]Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi; [15]Aichi Cancer Center Hospital, Nagoya; [16]BMT Center, Japanese Red Cross Nagoya First Hospital, Nagoya; and [17]Department of Cell Transplantation & Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan

We made a disease-specific comparison of unrelated cord blood (CB) recipients and human leukocyte antigen allele–matched unrelated bone marrow (BM) recipients among 484 patients with acute myeloid leukemia (AML; 173 CB and 311 BM) and 336 patients with acute lymphoblastic leukemia (ALL; 114 CB and 222 BM) who received myeloablative transplantations. In multivariate analyses, among AML cases, lower overall survival (hazard ratio [HR] = 1.5; 95% confidence interval [CI], 1.0-2.0, $P = .028$) and leukemia-free survival (HR = 1.5; 95% CI, 1.1-2.0, $P = .012$) were observed in CB recipients. The relapse rate did not differ between the 2 groups of AML (HR = 1.2; 95% CI, 0.8-1.9, $P = .38$); however, the treatment-related mortality rate showed higher trend in CB recipients (HR = 1.5; 95% CI, 1.0-2.3, $P = .085$). In ALL, there was no significant difference between the groups for relapse (HR = 1.4, 95% CI, 0.8-2.4, $P = .19$) and treatment-related mortality (HR = 1.0; 95% CI, 0.6-1.7, $P = .98$), which contributed to similar overall survival (HR = 1.1; 95% CI, 0.7-1.6, $P = .78$) and leukemia-free survival (HR = 1.2; 95% CI, 0.9-1.8, $P = .28$). Matched or mismatched single-unit CB is a favorable alternative stem cell source for patients without a human leukocyte antigen–matched related or unrelated donor. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients. (Blood. 2009;113:1631-1638)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) with bone marrow (BM) or peripheral blood, the curative treatment of choice for acute leukemia, is limited by the inadequate supply of human leukocyte antigen (HLA)–identical related donors. Bone marrow from HLA-matched unrelated donors has been a major alternative graft source.[1-3] Umbilical cord blood (CB), an alternative stem cell source to BM or peripheral blood stem cells, has been used primarily in children,[4-10] but its use in adults is increasing.[11,12]

Clinical comparison studies of cord blood transplantation (CBT) and bone marrow transplantation (BMT) for leukemia from unrelated donors in adult recipients showed comparable outcomes.[11-13] Recipients of CBT showed delayed neutrophil recovery and lower incidence of acute graft-versus-host disease (GVHD).[11-13] Overall treatment-related mortality (TRM) was reported to be similar[12] or higher[11] compared with HLA-matched BM. Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are different disease entities that require different chemotherapy regimens for treatment. However, previous comparison studies have included both diseases because of limitation in the number of CBTs given to adults.

In addition, the study periods of previous studies encompass the pioneering period of CBT, when the general practice was to use these grafts in patients in whom there were no other curative options and when the relevance of cell dose and HLA matching had not yet been recognized.[6,7,14]

Accumulation of a larger number of CBT results enabled us to make a controlled comparison with unrelated BMTs. To avoid the inclusion of the pioneering period of CBT, the subjects were limited to those who received transplantations in and after 2000.

Methods

Collection of data and data source

The recipients' clinical data were provided by the Japan Cord Blood Bank Network (JCBBN) and the Japan Marrow Donor Program (JMDP).[15]

Submitted March 21, 2008; accepted November 14, 2008. Prepublished online as *Blood* First Edition paper, December 22, 2008; DOI 10.1182/blood-2008-03-147041.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Peripheral blood stem cell donation from unrelated donors is not permitted in Japan. All 11 CB banks in Japan are affiliated to JCBBN. Both JCBBN and JMDP collect recipients' clinical information at 100 days after transplantation. Patients' information on survival, disease status, and long-term complications, including chronic GVHD and second malignancies, are renewed annually by follow-up forms. This study was approved by the data management committees of JMDP and JCBBN.

Patients

Between January 2000 and December 2005, a total of 1690 adult patients at least 16 years of age with acute leukemia (999 AML, 261 CB and 738 BM; and 691 ALL, 178 CB and 513 BM) received first HSCT with myeloablative conditioning either CB or BM from unrelated donors. Of these, patients who received a single CB unit with 0 to 2 HLA mismatches, or HLA-A, -B, -C, and DRB1 allele-matched BM from unrelated donors were analyzed. HLA matching of CB was performed using low-resolution molecular typing methods for HLA-A and -B, and high-resolution molecular typing for HLA-DRB1. Of 1023 BM recipients with complete HLA high-resolution data, the following recipients with HLA HLA-A, -B, -C, and DRB1 allele mismatches were excluded: 306 recipients with 1 of 8 mismatches (39 for HLA-A, 6 for HLA-B, 137 for HLA-C, and 124 for HLA-DRB1), 150 recipients with 2 of 8 mismatches (36 for 2 class I antigens, and 114 for class I and class II antigens), 33 recipients with 3 of 8 mismatches, and 1 recipient with 4 of 8 mismatches. Of 390 recipients of CB with complete HLA data, 95 recipients with 3 mismatches and 8 patients with 4 mismatches were excluded. A total of 484 patients with AML (173 CBTs and 311 BMTs) and 336 patients with ALL (114 CBTs and 222 BMTs) were the subjects for the analyses. Eighty-five centers performed 287 CBTs analyzed in this study, and 114 centers performed 533 BMTs.

Definitions

Neutrophil recovery was defined by an absolute neutrophil count of at least 500 cells/mm^3 for 3 consecutive points; platelet recovery was defined by a count of at least 50 000 platelets/mm^3 without transfusion support. Diagnosis and clinical grading of acute GVHD were performed according to the established criteria.[16] Relapse was defined as a recurrence of underlying hematologic malignant diseases. Treatment-related death was defined as death during a continuous remission. Leukemia-free survival (LFS) was defined as survival in a state of continuous remission.

Statistical analysis

Separate analyses were performed for AML and ALL. Descriptive statistical analysis was performed to assess patient baseline characteristics, diagnosis, disease classification, disease status at conditioning, donor-patient ABO mismatches, preparative regimen, and GVHD prophylaxis. The 2-sided χ^2 test was used for categorical variables, and the 2-sided Wilcoxon rank sum test was used for continuous variables. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of neutrophil and platelet recovery, acute and chronic GVHD, relapse, and TRM.[17] For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, death without GVHD and relapse were the competing events; for relapse, death without relapse was the competing event; and, for TRM, relapse was the competing event. Gray test was used for group comparison of cumulative incidence.[18] Overall survival (OS) and LFS were calculated using the Kaplan-Meier method. The log-rank test was used for group comparisons. Adjusted comparison of the stem cell source on OS and LFS was performed with the use of the Cox proportional-hazards regression model. For other outcomes, the Fine and Gray proportional-hazards model for subdistribution of a competing risk was used.[19] Adjusted probabilities of OS and DFS were estimated using the Cox proportional-hazards regression model, with consideration of other significant clinical variables in the final multivariate models. The variables considered were the patient's age at transplantation, patient's sex, donor-patient sex mismatch, donor-patient ABO mismatch, disease status at conditioning, and t(9;22) chromosome abnormality or others for ALL, cytogenetic information and French-American-British (FAB) classification of M5/M6/M7 or others for AML, the conditioning regimen, and the type of prophylaxis against GVHD. Factors differing in distribution between CB and BM recipients ($P < .10$) and factors known to influence outcomes (such as patient age at transplantation and chromosome abnormalities and FAB classification of leukemia) were included in the final models. Variables with more than 2 categories were dichotomized for the final multivariate model. The cutoff points of the variables were chosen to make optimal use of the information, with the proviso that smaller groups contain at least 20% of the patients. Variables were dichotomized as follows: patient age greater or younger than 45 years at transplantation, female donor to male recipient donor-recipient sex mismatch versus others for donor-recipient sex matching, donor-recipient ABO major mismatch versus others for ABO matching, M5/M6/M7 FAB classification versus others for classification of AML, chromosome abnormality other than favorable abnormalities for cytogenetics of AML, cyclophosphamide and total body irradiation (TBI) or busulfan and cyclophosphamide or others for conditioning regimen of AML, cyclophospohamide and TBI, or others for conditioning regimen of ALL, and cyclosporine-based versus tacrolimus-based prophylaxis against GVHD. Disease status at transplantation was categorized as first complete remission (1CR), second or later complete remission (2CR), or more advanced disease; which was included in the final model using dichotomized dummy variables. All P values were 2-sided.

The statistical power to detect hazard ratios (HRs) of 2.0 and 1.5 (a regression coefficient equal to 0.6931 and 0.4055, respectively) on Cox regression of the log hazard ratio at a .05 significance level adjusted for event rate were 99% and 78%, respectively, for 484 patients with AML and 97% and 60%, respectively, for 336 patients with ALL. The levels of statistical power for subgroup analyses were as follows: 54% and 22% for 1CR, 51% and 21% for 2CR, 96% and 58% for more advanced in AML patients, 62% and 26% for 1CR, 47% and 20% for 2CR, and 67% and 29% for more advanced in ALL patients.[20]

Results

Patient characteristics

The characteristics of the patients are shown in Table 1. There was no significant difference in recipients' age at transplantation in AML (median age, CB vs BM = 38 vs 38 years, $P = .61$) and in ALL (median age, CB vs BM = 34 vs 32 years, $P = .29$). The female/male ratio was higher (CB vs BM = 54% vs 38% in AML patients, and CB vs BM = 54% vs 38% in ALL patients, $P < .001$ and $P = .005$, respectively) in CB recipients, resulting in the lower donor-patient sex match rate (CB vs BM = 48% vs 69% in AML patients, and CB vs BM = 46% vs 65% in ALL patients, $P < .001$ and $P = .002$, respectively) in CB recipients. The proportion of ALL patients with Philadelphia chromosome abnormality was higher (CB vs BM = 38% vs 23%) in CB recipients. CB recipients were likely to have more advanced disease status at transplantation (relapse or induction failure, CB vs BM = 47% vs 31% in AML patients, and CB vs BM = 26% vs 19% in ALL patients), and the difference was significant in AML ($P = .003$). HLA-A, -B (low-resolution typing), and -DRB1 (high-resolution typing) was mismatched in 93% of both AML and ALL among CB recipients, whereas HLA -A, -B, -C, and -DRB1 were all genotypically matched for BM recipients. The ABO-matched donor-patient pair proportion was consistently lower for CB (CB vs BM = 34% vs 59% in AML patients and CB vs BM = 32% vs 58% in ALL patients).

A preparative regimen with TBI and cyclophosphamide was used in almost all patients, and cytosine arabinoside was supplemented for CB recipients with AML (36%) in addition to TBI and cyclophosphamide. For GVHD prophylaxis, tacrolimus (CB vs BM = 29% vs 56% in AML patients, and CB vs BM = 37% vs 53% in ALL patients) and

Table 1. Characteristics of recipients of cord blood or bone marrow from unrelated donors in 484 patients with acute myeloid leukemia and 336 patients with acute lymphoblastic leukemia

	Acute myeloid leukemia			Acute lymphoblastic leukemia		
Characteristic	**U-CBT**	**U-BMT**	***P***	**U-CBT**	**U-BMT**	***P***
No. of transplantations	173	311		114	222	
Median patient age at transplantation, y (range)	38 (16-69)	38 (16-60)	.61	34 (16-58)	32 (16-59)	.29
Patient sex, n (%)						
Male	80 (46)	194 (62)	< .001	52 (46)	137 (62)	.005
Female	93 (54)	117 (38)		62 (54)	85 (38)	
Sex matching, n (%)			< .001			.002
Matched	83 (48)	216 (69)		52 (46)	145 (65)	
Male to female	44 (25)	57 (18)		35 (31)	42 (19)	
Female to male	46 (27)	37 (12)		27 (24)	35 (16)	
Unknown	0 (0)	1 (0)		0 (0)	0 (0)	
Disease classification						
AML (French-American-British)			.045			
M0	17 (10)	26 (8)				
M1	30 (17)	38 (12)				
M2	52 (30)	88 (28)				
M3	4 (2)	25 (8)				
M4	27 (16)	55 (18)				
M5	23 (13)	41 (13)				
M6	3 (2)	18 (6)				
M7	2 (1)	5 (2)				
Others/unknown	15 (9)	15 (5)				
Cytogenetics			.042			
Favorable*	19 (11)	66 (21)				
Normal	74 (43)	116 (37)				
Other	57 (33)	95 (31)				
Unknown	23 (13)	34 (11)				
ALL cytogenetics						.022
t(9;22)				43 (38)	52 (23)	
t(4;11)				2 (2)	3 (1)	
Others				22 (19)	51 (23)	
Normal				27 (24)	85 (38)	
Unknown				20 (18)	31 (14)	
Disease status			.003			.33
First CR	50 (29)	130 (42)		63 (55)	130 (59)	
Second or after CR	39 (23)	82 (26)		21 (18)	48 (22)	
Relapse/induction failure	81 (47)	95 (31)		30 (26)	42 (19)	
Unknown	3 (2)	4 (1)		0 (0)	2 (1)	
HLA matching†						
0 mismatched loci	12 (7)			8 (7)		
1 mismatched locus	35 (20)			25 (22)		
2 mismatched loci	126 (73)			81 (71)		
ABO matching			< .001			< .001
Matched	59 (34)	185 (59)		37 (32)	128 (58)	
Minor mismatch	48 (28)	57 (18)		30 (26)	48 (22)	
Major mismatch	37 (21)	59 (19)		24 (21)	41 (18)	
Bidirectional	28 (16)	8 (3)		23 (20)	3 (1)	
Unknown	1 (1)	2 (1)		0 (0)	2 (1)	
Nucleated cells infused per 10^7/kg, median (range)	2.44 (1.65-5.49)	26.3 (2.10-58.8)	< .001	2.48 (1.51-4.06)	28.2 (2.30-79.0)	< .001
Preparative regimen			< .001			.38
CY + TBI	43 (25)	142 (46)		42 (37)	92 (41)	
CY + CA + TBI	62 (36)	41 (13)		31 (27)	53 (24)	
CY + BU + TBI	7 (4)	36 (12)		3 (3)	5 (2)	
Other TBI regimen	42 (24)	33 (11)		34 (30)	54 (24)	
BU + CY	18 (10)	55 (18)		4 (4)	12 (5)	
Other non-TBI regimen	1 (1)	4 (1)		0 (0)	6 (3)	
GVHD prophylaxisis			< .001			< .001
Cyclosporine A + sMTX	103 (60)	131 (42)		65 (57)	100 (45)	
Cyclosporine A ± other	20 (12)	4 (1)		6 (5)	3 (1)	
Tacrolimus + sMTX	34 (20)	168 (54)		26 (23)	106 (48)	
Tacrolimus ± other	15 (9)	5 (2)		16 (14)	11 (5)	
Others	1 (1)	3 (1)		1 (1)	2 (1)	

U-CBT, indicates unrelated cord blood transplantation; U-BMT, unrelated bone marrow transplantation; CR, complete remission; HLA, human leukocyte antigen; CY, cyclophosphamide; CA, cytarabine; BU, oral busulfan; TBI, total body irradiation; and sMTX, short-term methotrexate.

*Favorable abnormal karyotypes are defined as t(8;21), inv16,or t(15;17).

†Number of mismatches was counted among HLA-A, -B (low-resolution typing), and DRB1 (high-resolution typing).

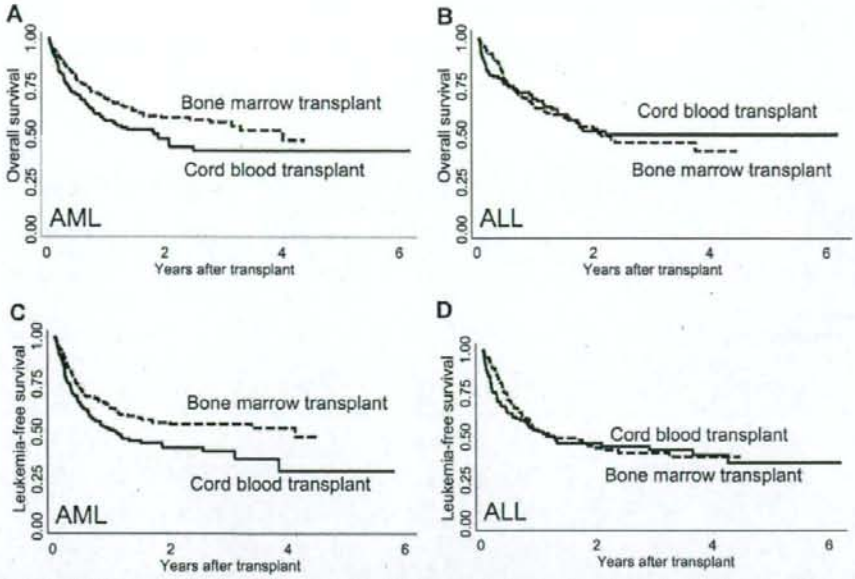

Figure 1. Adjusted OS and LFS of recipients with AML or ALL of CB or BM from unrelated donors. For patients with AML, adjusted probabilities of (A) OS (CB vs BM = 48% vs 59% at 2 years, $P = .010$) and (C) LFS (CB vs BM = 42% vs 54% at 2 years, $P = .004$) were both lower in CB recipients. For patients with ALL, the adjusted probabilities of (B) OS (CB vs BM = 52% vs 53% at 2 years, $P = .99$) and (D) LFS (CB vs BM = 46% vs 44% at 2 years, $P = .41$) were similar between CB recipients and BM recipients.

short-term methotrexate (CB vs BM = 80% vs 96% in AML patients, and CB vs BM = 80% vs 93% in ALL patients) were used preferentially in BM recipients. The median follow-up period for survivors was 1.9 years (range, 0.1-6.2 years) for CB recipients and 1.4 years (range, 0.3-4.5 years) for BM recipients.

Outcome

OS. For patients with AML, the unadjusted probabilities of OS were lower for CB recipients at 1 year (51% vs 69%) and 2 years (43% vs 60%) compared with BM recipients ($P < .001$). For patients with ALL, there were no significant differences between the 2 groups (CB vs BM = 66% vs 66% at 1 year, 49% vs 57% at 2 years, $P = .40$).

Among patients with AML, the use of CB remained a significant risk factor for overall mortality after adjustment for other factors (HR = 1.5; 95% confidence interval [CI], 1.0-2.0; $P = .028$; Table 2). However, in patients with ALL, the use of CB was not a significant factor for overall mortality on multivariate analysis (HR = 1.1; 95% CI, 0.7-1.6; $P = .78$). The adjusted probability of OS was significantly lower for CB recipients (57% vs 69% at 1 year, and 48% vs 59% at 2 years, $P = .010$; Figure 1A) compared with BM recipients for patients with AML, whereas the adjusted probability of OS was similar (69% vs 64% at 1 year, and 52% vs 53% at 2 years, $P = .99$; Figure 1B) between the groups for patients with ALL.

Results of the subgroup analyses showed that the difference in survival among AML patients was prominent in patients demonstrating 1CR at transplantation (RR = 2.9, 95% CI = 1.4-6.2, $P = .005$; Table 3).

LFS. For patients with AML, the unadjusted probabilities of LFS were significantly lower for CB recipients at 1 year (43% vs 62%) and 2 years (36% vs 54%) compared with BM recipients ($P < .001$). For patients with ALL, the unadjusted probabilities of LFS were lower with marginal significance for CB recipients at 1 year (52% vs 58%) and 2 years (45% vs 51%) compared with BM recipients ($P = .06$).

Among patients with AML, the use of CB remained as a significant risk factor for treatment failure (ie, relapse or death) after adjustment for other factors (HR = 1.5; 95% CI, 1.1-2.0; $P = .012$; Table 2). However, in patients with ALL, the use of CB was not a significant factor for treatment failure by multivariate analysis (HR = 1.2; 95% CI, 0.9-1.8; $P = .28$). The adjusted probability of LFS was significantly lower for CB recipients (51% vs 62% at 1 year, and 42% vs 54% at 2 years, $P = .004$; Figure 1C) compared with BM recipients for patients with AML, whereas the adjusted probability of LFS was similar (53% vs 53% at 1 year, and 46% vs 44% at 2 years, $P = .41$; Figure 1D) between the groups for patients with ALL.

Relapse

On univariate analyses, the cumulative incidence of relapse was higher for CB recipients with marginal significance in both AML (27% vs 20% at 1 year, and 31% vs 24% at 2 years) and ALL (27% vs 19% at 1 year, and 31% vs 24% at 2 years) ($P = .067$, and .085, respectively; Figure 2A,B).

On multivariate analyses adjusted by other factors, there was no significantly higher risk of relapse for CB recipients with either AML (RR = 1.2, 95% CI = 0.8-1.9, $P = .38$) or ALL (RR = 1.4, 95% CI = 0.8-2.4, $P = .19$; Table 2).

TRM

For patients with AML, the unadjusted cumulative incidence of TRM was significantly higher for CB recipients at 1 year (30% vs 19%) and 2 years (33% vs 22%) compared with those for BM recipients ($P = .004$; Figure 2C). For patients with ALL, the

Table 2. Results of multivariate analysis of outcomes in 173 recipients of cord blood and 311 recipients of bone marrow with acute myeloid leukemia, and 114 recipients of cord blood and 222 recipients of bone marrow with acute lymphoblastic leukemia

	Acute myeloid leukemia		Acute lymphoblastic leukemia	
Outcome	RR (95% CI)	P	RR (95% CI)	P
Overall survival*				
BM	1.00		1.00	
CB	1.45 (1.04-2.01)	.028	1.06 (0.71-1.57)	.78
Leukemia-free survival†				
BM	1.00		1.00	
CB	1.48 (1.09-2.01)	.012	1.22 (0.85-1.76)	.28
Relapse‡				
BM	1.00		1.00	
CB	1.21 (0.79-1.87)	.38	1.42 (0.84-2.41)	.19
TRM§				
BM	1.00		1.00	
CB	1.47 (0.95-2.28)	.085	1.01 (0.59-1.73)	.98
Neutrophil recovery‖				
BM	1.00		1.00	
CB	0.41 (0.33-0.51)	< .001	0.37 (0.29-0.48)	< .001
Platelet recovery¶				
BM	1.00		1.00	
CB	0.34 (0.27-0.44)	< .001	0.43 (0.33-0.56)	< .001
Acute GVHD#				
BM	1.00		1.00	
CB	0.80 (0.56-1.15)	.23	0.61 (0.39-0.95)	.028
Chronic GVHD**				
BM	1.00		1.00	
CB	0.94 (0.63-1.42)	.79	1.08 (0.66-1.77)	.77
Chronic GVHD, extensive type††				
BM	1.00		1.00	
CB	0.36 (0.18-0.72)	.004	0.58 (0.28-1.20)	.14

RR indicates relative risk; CI, confidence interval; BM, bone marrow; CB, cord blood; and GVHD, graft-versus-host disease.

*For overall survival, other significant variables for AML were patient age more than 45 years at transplantation, more advanced disease status at conditioning, M5/M6/M7 French-American-British classification, and female donor to male recipient donor-recipient sex mismatch; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and Philadelphia chromosome abnormality.

†For leukemia-free survival, other significant variables for AML were patient age more than 45 years at transplantation, more advanced disease status at conditioning, M5/M6/M7 French-American-British classification, and female donor to male recipient donor-recipient sex mismatch; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and Philadelphia chromosome abnormality.

‡For relapse, other significant variables for AML were more advanced disease status at conditioning, donor-recipient ABO major mismatch, chromosome abnormality other than favorable abnormalities, and cyclophosphamide and total body irradiation or busulfan and cyclophosphamide conditioning regimen; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and cyclophosphamide and total body irradiation conditioning.

§For TRM, other significant variables for AML were patient age more than 45 years at transplantation, second or after complete remission disease status, more advanced disease status, and chromosome abnormality other than favorable abnormalities; other significant variables for ALL were patient age more than 45 years at transplantation, more advanced disease status at conditioning, and conditioning other than cyclophosphamide and total body irradiation.

‖For neutrophil recovery, other significant variables for AML were second or after complete remission disease status and more advanced disease status; other significant variables for ALL were more advanced disease status at conditioning and cyclosporine-based GVHD prophylaxis.

¶For platelet recovery; other significant variables for AML were second or after complete remission disease status, more advanced disease status, female donor to male recipient donor-recipient sex mismatch, and tacrolimus-based GVHD prophylaxis; other significant variables for ALL were more advanced disease status at conditioning and conditioning other than cyclophosphamide and total body irradiation.

#For acute GVHD, no other significant variables were identified for both AML and ALL.

**For chronic GVHD, other significant variables for AML were more advanced disease status and conditioning other than cyclophosphamide and total body irradiation or busulfan and cyclophosphamide; there were no other significant variables identified for ALL.

††For extensive chronic GVHD, there were no other significant variables identified for AML; another significant variable for ALL was patient male sex.

cumulative incidence of TRM was similar between the 2 groups (CB vs BM = 21% vs 23% at 1 year, 24% vs 25% at 2 years, $P = .83$; Figure 2D).

On multivariate analyses adjusted by other factors, the risk for TRM was higher for CB recipients compared with that for BM recipients among patients with AML (RR = 1.5, 95% CI = 1.0-2.3, $P = .085$; Table 2) with marginal significance. For patients with ALL, the risk for TRM was similar between CB and BM recipients (RR = 1.0, 95% CI = 0.6-1.7, $P = .98$).

Cause of death

Recurrence of the primary disease was the leading cause of death in each group (CB vs BM = 37% vs 33% in patients with AML and 36% vs 41% in patients with ALL). The following causes were infection and organ failure in all groups (Table 4).

Other outcomes of transplantation

Neutrophil and platelet recovery. The unadjusted cumulative incidence of neutrophil recovery or platelet recovery at day 100 was significantly lower in CB recipients for both AML (77% vs 94%) and ALL (80% vs 97%) compared with that among BM recipients ($P < .001$ for both). On multivariate analyses, neutrophil recovery was significantly lower among CB recipients for both AML (RR = 0.4, 95% CI = 0.3-0.5, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.5, $P < .001$; Table 2).

Table 3. Results of multivariate analysis of overall survival according to disease status at transplantation

	First complete remission			Second or after complete remission			More advanced		
Overall survival	n	RR (95% CI)	P	n	RR (95% CI)	P	n	RR (95% CI)	P
AML									
UBMT	130	1.00		82	1.00		95	1.00	
UCBT	50	2.92 (1.38-6.18)	.005	39	1.24 (0.51-3.04)	.63	81	1.29 (0.84-1.98)	.25
ALL									
UBMT	130	1.00		48	1.00		42	1.00	
UCBT	63	1.60 (0.84-3.05)	.16	21	0.62 (0.22-1.74)	.36	30	0.80 (0.38-1.69)	.57

RR indicates relative risk; CI, confidence interval; UBMT, unrelated bone marrow transplantation; and UCBT, unrelated cord blood transplantation.

The unadjusted cumulative incidence of platelet recovery greater than 50 000/μL at 4 months was significantly lower among CB recipients for both AML (59% vs 85%) and ALL (61% vs 83%) compared with that of BM recipients ($P < .001$ for both). The difference was also significant on multivariate analyses for both AML (RR = 0.3, 95% CI = 0.3-0.4, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.6, $P < .001$; Table 2).

Acute GVHD. The unadjusted cumulative incidence of grade 2 to 4 acute GVHD was lower among CB recipients compared with that among BM recipients (32% vs 35% in AML, 28% vs 42% in ALL); the difference was significant in patients with ALL ($P = .39$ in AML, $P = .008$ in ALL). The difference was also significant on multivariate analyses in ALL (RR = 0.6, 95% CI = 0.4-1.0, $P = .028$). There was no significant difference in patients with AML (RR = 0.8, 95% CI = 0.6-1.2, $P = .23$; Table 2).

Chronic GVHD. The unadjusted cumulative incidence of chronic GVHD at 1 year after transplantation did not significantly differ between CB recipients and BM recipients in both AML (28% vs 32%, $P = .46$) and ALL (27% vs 30%, $P = .50$). The cumulative incidence of extensive-type chronic GVHD was significantly lower among CB recipients compared with that among BM recipients in both AML (8% vs 20%, $P < .001$) and ALL (10% vs 17%, $P = .034$). On multivariate analyses, the risk of developing chronic GVHD was similar in CB recipients and BM recipients in both AML (RR = 0.9, 95% CI = 0.6-1.4, $P = .79$) and ALL (RR = 1.1, 95% CI = 0.7-1.8, $P = .77$). The risk of developing extensive chronic GVHD was lower in CB recipients compared with BM recipients (RR = 0.4, 95% CI = 0.2-0.7, $P = .004$ in AML, and RR = 0.6, 95% CI, 0.3-1.2, $P = .14$ in ALL) and was significantly different in patients with AML (Table 2).

Discussion

The objective of our study was to investigate the outcomes of HLA-A, -B, low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit unrelated CBT in adult patients with acute leukemia compared with those of HLA-A, -B, -C, and -DRB1 (8 of 8) allele-matched unrelated BMT. Although AML and ALL are different diseases, previous comparisons of unrelated BMT and

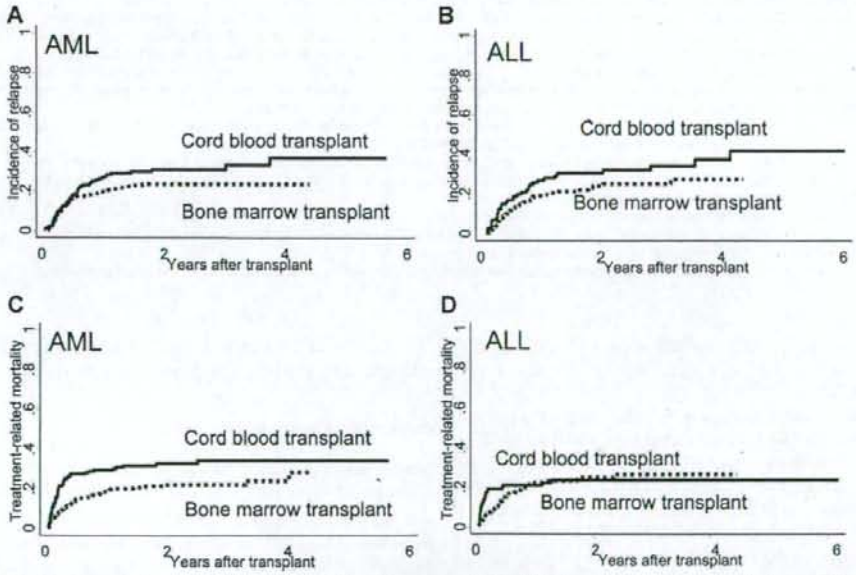

Figure 2. Cumulative incidence of relapse or TRM of recipients of CB or BM among patients with AML or ALL. For patients with AML, the cumulative incidence of (A) relapse (CB vs BM = 31% vs 24% at 2 years, $P = .068$) and (C) TRM (CB vs BM = 33% vs 22% at 2 years, $P = .004$) was higher in CB recipients. For patients with ALL, the cumulative incidence of relapse (B) was higher in CB recipients with marginal significance (CB vs BM = 31% vs 24% at 2 years, $P = .085$), but the incidence of TRM (D) was similar in CB and BM recipients (CB vs BM = 24% vs 25% at 2 years, $P = .83$).

Table 4. Causes of death after transplantation of unrelated cord blood or unrelated bone marrow among patients with acute myeloid leukemia or acute lymphoblastic leukemia

	Acute myeloid leukemia		Acute lymphoblastic leukemia	
Cause of death	UCBT	UBMT	UCBT	UBMT
Recurrence of disease	35 (37)	34 (33)	18 (36)	34 (41)
Graft failure/rejection	3 (3)	4 (4)	0 (0)	3 (4)
Graft-versus-host disease	6 (6)	7 (7)	3 (6)	5 (6)
Infection	22 (23)	19 (18)	13 (26)	11 (13)
Idiopathic pneumonia	4 (4)	4 (4)	2 (4)	6 (7)
Organ failure	17 (18)	17 (16)	8 (16)	10 (12)
Secondary cancer	0 (0)	1 (1)	0 (0)	0 (0)
Other causes	5 (5)	5 (5)	2 (4)	4 (5)
Unknown/data missing	2 (2)	13 (13)	4 (8)	10 (12)
Total	94 (100)	104 (100)	50 (100)	83 (100)

Data are presented as n (%).

UCBT indicates unrelated cord blood transplantation; and UBMT, unrelated bone marrow transplantation.

unrelated CBT did not separate these 2 diseases. Our report is the first to show the result of disease-specific analyses with a sufficient number of patients.

For AML patients, the recipients of CB were more likely to have advanced leukemia at the time of transplantation, as reported previously, suggesting that CB was used as an alternative stem cell source in the later phase of unrelated donor searches, especially in adults.[11,12,14] A larger proportion of CB recipients with ALL had the Philadelphia chromosome abnormality, which correlates with highly aggressive ALL and usually requires urgent transplantation, in which CB has an advantage over BM.[21]

Different outcomes of mortality were found between AML and ALL in a controlled comparison using multivariate analyses. Whereas significantly lower OS and LFS rates were observed in CB recipients with AML, rates of overall mortality and treatment failure were similar between CB and BM recipients with ALL. The relapse rate was not different between CBT and BMT in patients with both AML and ALL, which was consistent with previous reports.[11-13] In adult patients with ALL, a previous report showed no difference in the outcome of related compared with unrelated BM or peripheral blood transplantation in 1CR.[22] Favorable disease status at transplantation could be a more important factor affecting outcome rather than the type of stem cell source or donor type in patients with ALL. It is notable that TRM in HLA allele-matched unrelated BM recipients with AML was quite low in our study. This is probably associated with the low incidence of acute and chronic GVHD in the Japanese population, which is thought to be the result of genetic homogeneity.[23-26] Among patients with AML, although the difference was not statistically significant, a higher trend of TRM observed in CB recipients might be associated with higher overall and TRM rates in CB recipients. Reasons for higher TRM could include the graft source and delayed neutrophil recovery. Better supportive care is required after CBT for patients going through a prolonged neutropenic period. Development of better graft engineering or better conditioning regimens would help to decrease the TRM rate in CB recipients. Because relapse was the major cause of death in all groups, any attempt to decrease TRM should preserve the antileukemia effect to improve OS and LFS. Another reason for the higher TRM could be a higher risk patient population, higher risk for both disease status and comorbid conditions, requiring rapid transplantation. Searching for unrelated donors earlier and providing transplantation earlier in the disease course could help to decrease TRM in CB recipients.

Neutrophil and platelet recovery was slower in CB recipients with either AML or ALL, consistent with the results of previous reports.[11,12,27] Multiple studies have reported lower incidence of acute GVHD in CB recipients.[8-10,12,13] In our study, particularly in patients with ALL, the risk of developing grade 2 to 4 acute GVHD in CB recipients was lower compared with BM recipients, which was reported to be lower compared with the incidence reported from Western countries.[23-25] The risk of developing chronic GVHD was similar between CB and BM recipient with either disease, but the risk of developing extensive-type chronic GVHD was lower in CB recipients; the difference was significant in patients with AML. It is notable that there was no increase in the incidence of acute or chronic GVHD in CB recipients among patients with either AML or ALL, despite HLA disparity.

For differences in outcomes between AML and ALL, one possibility is a difference of treatment before conditioning therapy. Most AML patients received a more intense treatment for induction and consolidation therapy compared with that for ALL. There was no adjustment made for previous treatment, and this could be the reason for higher mortality in CBT, which requires a longer time for neutrophil recovery. Another possible cause of the difference in outcomes is the difference in conditioning regimens. Preparative regimens were similar between CB and BM recipients among ALL patients. However, in patients with AML, the proportion of standard regimens, such as cyclophosphamide and TBI or busulfan and cyclophosphamide, was smaller among CB recipients. These differences in the distribution of preparative regimens were also seen in a previous report.[11] Although the final model was adjusted for conditioning regimens, we cannot rule out the possibility of an effect that larger CB recipients received additional or different chemotherapeutic agents compared with BM recipients among patients with AML. Although the difference was small, the median age of CB recipients with AML was 4 years older than CB recipients with ALL (median age, 38 vs 34 years, $P = .021$), which might have affected the higher mortality rate among CB recipients with AML. It is also possible that some unknown biologic aspects have contributed to these differences, and this would require further evaluation in future studies.

Further subgroup analyses indicated that the superiority of HLA allele-matched BM versus CB for OS was mostly found in patients with AML showing 1CR at conditioning. However, because of the limited numbers of patients in these subgroup analyses and the possibility of an unidentified bias in stem cell source selection, our findings should be verified by further analysis in a larger population.

In conclusion, we found different outcomes between patients with AML and ALL, indicating the importance of disease-specific analyses in alternative donor studies. HLA-A, -B low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit CB is a favorable alternative stem cell source for patients without a suitable related or 8 of 8 matched unrelated BM donor. In the absence of a suitable donor, unrelated CBT should be planned promptly to transplant the patient while in a better disease status and better clinical condition. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients.

Acknowledgments

The authors thank all the staff members of the collaborating institutes of the Japan Cord Blood Bank Network and Japan Marrow Donor Program for their assistance and cooperation and

Dr Takakazu Kawase for validating human leukocyte antigen data of the Japan Marrow Donor Program.

This work was supported by a Research Grant for Tissue Engineering (H17-014), a Research Grant for Allergic Disease and Immunology (H20-015), and a Research Grant for Cancer (H19-1) from the Japanese Ministry of Health, Labor, and Welfare.

Authorship

Contribution: Y.A. and R.S. designed the study and wrote the paper; Y.A. analyzed results and made the figures; S. Kato and Y.M. designed the research; T.-N.I., H.A., and M. Takanashi reviewed and cleaned the Japan Cord Blood Bank Network data and reviewed the results; S. Taniguchi, S. Takahashi, S. Kai, H.S., Y. Kouzai, M.K., and T.F. submitted and cleaned the data; and S.O., M. Tsuchida, K.K., Y.M., and Y. Kodera reviewed and cleaned the Japan Marrow Donor Program data and reviewed the results.

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

A complete list of members from the Japan Marrow Donor Program and the Japan Cord Blood Bank Network can be found in the Supplemental Appendix (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Correspondence: Yoshiko Atsuta, Department of Hematopoietic Stem Cell Transplantation Data Management, Nagoya University School of Medicine, 1-1-20 Daiko-Minami, Higashi-ku Nagoya, 461-0047 Japan; e-mail: y-atsuta@med.nagoya-u.ac.jp.

References

1. Szydlo R, Goldman JM, Klein JP, 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, 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, et al. Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. N Engl J Med. 1998;338:962-968.
4. Wagner JE, Rosenthal J, Sweetman R, 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.
5. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. N Engl J Med. 1996;335:157-166.
6. Gluckman E, Rocha V, Boyer-Chammard A, 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.
7. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med. 1998;339:1565-1577.
8. Rocha V, Wagner JE Jr, Sobocinski KA, 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.
9. Rocha V, Cornish J, Sievers EL, et al. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. Blood. 2001;97:2962-2971.
10. Eapen M, Rubinstein P, Zhang MJ, 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.
11. Laughlin MJ, Eapen M, Rubinstein P, 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.
12. Rocha V, Labopin M, Sanz G, 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.
13. Takahashi S, Iseki T, Ooi J, 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.
14. Sanz MA. Cord-blood transplantation in patients with leukemia: a real alternative for adults. N Engl J Med. 2004;351:2328-2330.
15. Kodera Y, Morishima Y, Kato S, et al. Analysis of 500 bone marrow transplants from unrelated donors (UR-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. Bone Marrow Transplant. 1999;24:995-1003.
16. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant. 1995;15:825-828.
17. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med. 1999;18:695-706.
18. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat. 1988;16:1141-1154.
19. Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. J Am Stat Assoc. 1999;94:456-509.
20. Hsieh FY, Lavori PW. Sample-size calculations for the Cox proportional hazards regression model with nonbinary covariates. Control Clin Trials. 2000;21:552-560.
21. Barker JN, Krepski TP, DeFor TE, Davies SM, Wagner JE, Weisdorf DJ. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. Biol Blood Marrow Transplant. 2002;8:257-260.
22. Kiehl MG, Kraut L, Schwerdtfeger R, et al. Outcome of allogeneic hematopoietic stem-cell transplantation in adult patients with acute lymphoblastic leukemia: no difference in related compared with unrelated transplant in first complete remission. J Clin Oncol. 2004;22:2816-2825.
23. Morishima Y, Morishita Y, Tanimoto M, et al. Low incidence of acute graft-versus-host disease by the administration of methotrexate and cyclosporine in Japanese leukemia patients after bone marrow transplantation from human leukocyte antigen compatible siblings; possible role of genetic homogeneity. The Nagoya Bone Marrow Transplantation Group. Blood. 1989;74:2252-2256.
24. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor: Japan Marrow Donor Program. N Engl J Med. 1998;339:1177-1185.
25. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood. 2002;99:4200-4206.
26. Atsuta Y, Suzuki R, Yamamoto K, et al. Risk and prognostic factors for Japanese patients with chronic graft-versus-host disease after bone marrow transplantation. Bone Marrow Transplant. 2006;37:289-296.
27. Takahashi S, Ooi J, Tomonari A, et al. Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. Blood. 2007;109:1322-1330.

Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

Takakazu Kawase,[1,2] Yasuhito Nannya,[3-5] Hiroki Torikai,[1] Go Yamamoto,[3-5] Makoto Onizuka,[6] Satoko Morishima,[1] Kunio Tsujimura,[7] Koichi Miyamura,[5,8] Yoshihisa Kodera,[5,8] Yasuo Morishima,[5,9] Toshitada Takahashi,[10] Kiyotaka Kuzushima,[1] Seishi Ogawa,[3-5] and Yoshiki Akatsuka[1,5]

[1]Division of Immunology, [2]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya; [3]Department of Hematology/Oncology and [4]21st Century COE Program, Graduate School of Medicine, University of Tokyo, Tokyo; [5]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama; [6]Department of Genetic Information, Division of Molecular Life Science, Tokai University School of Medicine, Isehara; [7]Department of Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu; [8]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya; [9]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya; and [10]Aichi Comprehensive Health Science Center, Aichi Health Promotion Foundation, Chita-gun, Japan

Minor histocompatibility (H) antigens are the molecular targets of allo-immunity responsible both for the development of antitumor effects and for graft-versus-host disease (GVHD) in allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, despite their potential clinical use, our knowledge of human minor H antigens is largely limited by the lack of efficient methods of their characterization. Here we report a robust and efficient method of minor H gene discovery that combines whole genome association scans (WGASs) with cytotoxic T-lymphocyte (CTL) assays, in which the genetic loci of minor H genes recognized by the CTL clones are precisely identified using pooled-DNA analysis of immortalized lymphoblastoid cell lines with/without susceptibility to those CTLs. Using this method, we have successfully mapped 2 loci: one previously characterized (*HMSD* encoding ACC-6), and one novel. The novel minor H antigen encoded by *BCL2A1* was identified within a 26 kb linkage disequilibrium block on chromosome 15q25, which had been directly mapped by WGAS. The pool size required to identify these regions was no more than 100 individuals. Thus, once CTL clones are generated, this method should substantially facilitate discovery of minor H antigens applicable to targeted allo-immune therapies and also contribute to our understanding of human allo-immunity. (Blood. 2008;111: 3286-3294)

Introduction

Currently, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been established as one of the most effective therapeutic options for hematopoietic malignancies[1] and is also implicated as a promising approach for some solid cancers.[2] Its major therapeutic benefits are obtained from allo-immunity directed against patients' tumor cells (graft-versus-tumor [GVT] effects). However, the same kind of allo-immune reactions can also be directed against normal host tissues resulting in graft-versus-host disease (GVHD). In HLA-matched transplants, both GVT and GVHD are initiated by the recognition of HLA-bound polymorphic peptides, or minor histocompatibility (H) antigens, by donor T cells. Minor H antigens are typically encoded by dichotomous single nucleotide polymorphism (SNP) alleles, and may potentially be targeted by allo-immune reactions if the donor and recipient are mismatched at the minor H loci. Identification and characterization of minor H antigens that are specifically expressed in hematopoietic tissues, but not in other normal tissues, could contribute to the development of selective antileukemic therapies while minimizing unfavorable GVHD reactions, one of the most serious complications of allo-HSCT.[3,4] Unfortunately, the total number of such useful minor H antigens that are currently molecularly characterized is still disappointingly small, including HA-1,[5] HA-2,[6] ACC-1[Y] and ACC-2,[7] DRN-7,[8] ACC-6,[9] LB-ADIR-1F,[10] HB-1,[11] LRH-1,[12] and 7A7-PANE1,[13] limiting the number of patients eligible for such GVT-oriented immunotherapy.

Several techniques have been developed to identify novel minor H antigens targeted by CTLs generated from patients who have undergone transplantation. Among these, linkage analysis based on the cytotoxicity of the CTL clones against panels of lymphoblastoid cell lines (B-LCLs) from large pedigrees was proposed as a novel genetic approach,[14] and has been successfully applied to identify novel minor H epitopes encoded by the *BCL2A1* and *P2RX5* genes.[7,12] Nevertheless, the technology is still largely limited by its resolution, especially when large segregating families are not available. Linkage analysis using B-LCL panels from the Centre d'Etude du Polymorphisme Humain (CEPH) could only localize minor H loci within a range of 1.64 Mb to 5.5 Mb, which still contained 11 to 46 genes,[7,12,14] thus requiring additional selection procedures to identify the actual minor H genes.

On the other hand, clinically relevant minor H antigens might be associated with common polymorphisms within the human

Submitted October 22, 2007; accepted December 19, 2007. Prepublished online as *Blood* First Edition paper, January 4, 2008; DOI 10.1182/blood-2007-10-118950.

T.K. and Y.N. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

population, and therefore could be ideal targets of genetic association studies, considering recent advances of large-scale genotyping technologies and the assets of the International HapMap Project.[15,16] In this alternative genetic approach using the extensive linkage disequilibrium (LD) found within the human genome, target loci can be more efficiently localized within relatively small haplotype blocks without depending on limited numbers of recombination events, given the large number of genotyped genetic markers.[17] Moreover, since the presence of a target minor H allele in individual target cells can be determined by ordinary immunologic assays using minor H antigen-specific CTLs, the characterization of minor H antigens should be significantly more straightforward than identifying alleles associated with typical common complex diseases, for which typically weak-to-moderate genetic effects have been assumed.[18]

In this report, we describe a high-performance, cost-effective method for the identification of minor H antigens, in which whole genome association scans (WGASs) are performed based on SNP array analysis of pooled DNA samples constructed from cytotoxicity-positive (CTX^+) and cytotoxicity-negative (CTX^-) B-LCLs as determined by their susceptibility to CTL clones. Based on this method, termed WGA/CTL, we were able to map the previously characterized ACC-6 minor H locus to a 115-kb block containing only 4 genes, including *HMSD*.[9] Moreover, using the same approach, a novel minor H antigen encoded by the *BCL2A1* gene was identified within a 26-kb block containing only *BCL2A1* on chromosome 15q25. Surprisingly, the pool size required to identify these regions was no more than 100 individuals. Thus, this WGA/CTL method has significant potential to accelerate the discovery of minor H antigens that could be used in more selective, and thus more effective, allo-immune therapies in the near future.

Methods

Cell isolation and cell cultures

This study was approved by the institutional review board of the Aichi Cancer Center and the University of Tokyo. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-LCLs were derived from allo-HSCT donors, recipients, and healthy volunteers. B-LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate.

Generation of CTL lines and clones

CTL lines were generated from peripheral blood mononuclear cells (PBMCs) obtained after transplantation by stimulation with irradiated (33 Gy) recipient PBMCs harvested before HSCT, thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. IL-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[7] CTL-1B9 was isolated from PBMCs harvested on day 30 after transplantation from a patient receiving a marrow graft from his HLA-identical sibling (HLA A11, A24, B39, B51, Cw7, Cw14), and CTL-2A12 has been described recently.[9]

Chromium release assay

Target cells were labeled with 0.1 mCi (3.7 MBq) of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTL at the effector-to-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Percent specific lysis was calculated as follows: ((Experimental cpm − Spontaneous cpm) / (Maximum cpm − Spontaneous cpm)) × 100.

Immunophenotyping by enzyme-linked immunosorbent assay

B-LCL cells (20 000 per well, which had been retrovirally transduced with restriction HLA cDNA for individual CTLs, if necessary) were plated in each well of 96-well round-bottomed plates, and corresponding CTL clones (10 000 per well) were added to each well. After overnight incubation at 37°C, 50 μL supernatant was collected and released IFN-γ was measured by standard enzyme-linked immunosorbent assay (ELISA).

Construction of pooled DNA and microarray experiments

Genomic DNA was individually extracted from immunophenotyped B-LCLs. After DNA concentrations were measured and adjusted to 50 μg/mL using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR), the DNA specimens from CTX^+ and CTX^- B-LCLs were separately combined to generate individual pools. DNA pools were analyzed in pairs using Affymetrix GeneChip SNP-genotyping microarrays (Affymetrix, Tokyo, Japan) according to the manufacturer's protocol,[19,20] where 2 independent experiments were performed for each array type (for more detailed statistical analysis for generated microarray data, see Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Estimation of LD blocks

LD structures of the candidate loci were evaluated based on empirical data from the International Hap Map Project (http://www.hapmap.org/).[15] LD data for the relevant HapMap panels were downloaded from the HapMap web site and further analyzed using Haploview software (http://www.broad.mit.edu/mpg/haploview/).[21]

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-A*2402 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing full-length *BCL2A1* cDNA generated from either the patient or his donor using Trans IT-293 (Mirus, Madison, WI). B-LCLs of the recipient and his donor were used as positive and negative controls, respectively. Ten thousand CTL-1B9 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by ELISA.

SNP identification by direct sequencing

Complementary DNA prepared from B-LCLs was polymerase chain reaction (PCR) amplified for the coding region of *BCL2A1* using the following primers: sense: 5′-AGAAGATGACAGACTGTGAATTTGG-3′; antisense: 5′-TCAACAGTATTGCTTCAGGAGAG-3′.

PCR products were purified and directly sequenced with the same primer and BigDye Terminator kit (version 3.1) by using ABI PRISM 3100 (Applied Biosystems, Foster City, CA).

Confirmatory SNP genotyping

Genotyping was carried out using fluorogenic 3′-minor groove binding (MGB) probes in a PCR assay. PCR was conducted in 10-μL reactions containing both allelic probes, 500 nM each of the primers, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 μL (100 ng) DNA. PCR cycling conditions were as follows: predenature, 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 92°C for 15 seconds and 60°C for 1 minute in a GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analyzed on an ABI 7900HT with the aid of SDS 2.2 software (Applied Biosystems).

Epitope reconstitution assay

The candidate *BCL2A1*-encoded minor H epitope and its allelic counterpart (DYLQYVLQI) peptides were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled CTX^- donor B-LCLs were incubated with graded concentrations of the peptides and then used as targets in standard cytotoxicity assays.

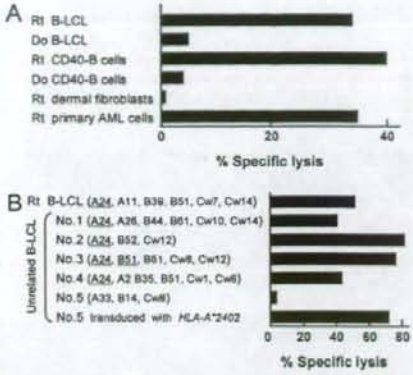

Figure 1. Specificity of CTL-1B9 against hematopoietic cells and its restriction HLA. (A) The cytolytic activity of CTL-1B9 was evaluated in a standard 4-hour ^{51}Cr release assay (E/T ratio, 20:1). Targets used were B-LCL, CD40-activated (CD40-B) B cells, dermal fibroblasts, and primary acute myeloid leukemia cells from the recipient (Rt), and B-LCL and CD40-B cells from his donor (Do). Rt dermal fibroblasts were pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (B) Cytolytic activity of CTL-1B9 against a panel of B-LCLs derived from unrelated individuals, each of whom shared 1 or 2 class I MHC allele(s) with the recipient from whom the CTL-1B9 was generated. The shared HLA allele(s) with the recipient are underlined. B-LCLs (no. 5) which did not share any HLA alleles with the recipient, were retrovirally transduced with HLA-A*2402 cDNA and included to confirm HLA-A*2402 restriction by CTL-1B9. Results are typical of 2 experiments and data are the mean plus or minus the standard deviation (SD) of triplicates.

Results

CTL-based typing and SNP array analysis of pooled DNA

CTL-2A12 and CTL-1B9 are CTL clones established from the peripheral blood of 2 patients with leukemia who had received HLA-identical sibling HSCTs. Each clone demonstrated specific lysis against the B-LCLs of the recipient but not against donor B-LCLs, indicating recognition of minor H antigen (Figure 1A and Kawase et al[9]). The minor H antigen for CTL-2A12 had been previously identified by expression cloning[9]; on the other hand, the target minor H antigen for the HLA-A24–restricted CTL-1B9 clone, which was apparently hematopoietic lineage–specific (Figure 1A) and present in approximately 80% of the Japanese population (data not shown), had not yet been determined. Using these CTL clones, a panel of B-LCLs expressing the restriction HLA (HLA-B44 for CTL-2A12 and HLA-A24 for CTL-1B9) endogenously or retrovirally transduced, were subjected to "immunophenotyping" for the presence or absence of the minor H antigen by ELISA and, if necessary, by standard chromium release assay (CRA). Based on the assay results, for CTL-2A12 we initially collected 44 cytotoxicity-positive (CTX^+) and 44 cytotoxicity-negative (CTX^-) B-LCLs after screening 132 B-LCLs, while 57 CTX^+ and 38 CTX^- B-LCLs were obtained from 121 B-LCLs for CTL-1B9. From these sets of B-LCL panels, pools of DNA were generated and subjected to analysis on Affymetrix GeneChip 100 K and 500 K microarrays in duplicate.[19,20]

Detection of association between minor H phenotypes and marker SNPs

Genetic mapping of the minor H locus was performed by identifying marker SNPs that showed statistically significant deviations in allele-frequencies between CTX^+ and CTX^- pools based on the observed allele-specific signals in the microarray experiments. For this purpose, we evaluated the deviations of observed allele ratios between CTX^+ and CTX^- pools for each SNP on a given array (Document S1). An SNP was considered as positive for association if its test statistic exceeded an empirically determined threshold that provided a "genome-wide" P value of .05 in duplicate experiments (Document S1, Figures S1,S2, and Table S1). Threshold values for different pool sizes are also provided in Table S2 for further experiments. The positive SNPs eventually obtained for both CTLs are summarized in Table 1, where the 10 SNPs showing the highest test statistics are listed for individual experiments.

Figure 2. Whole genome association scans performed with pooled DNA generated based on immunophenotyping with CTL-2A12. Pooled DNAs generated from 44 CTX^+ and 44 CTX^- B-LCLs were analyzed with 50 K *Xba*I (A), 50 K *Hind*III (B), 250 K *Nsp*I (C), and 250 K *Sty*I (D) arrays. Test statistics were calculated for all SNPs and plotted in the chromosomal order. In all SNP array types, a common association peak is observed at 18q21, to which the minor H antigen for CTL-2A12, encoded by the *HMSD* gene, had been mapped based on expression cloning[9] (arrows).

Mapping of the minor H loci by WGASs

All the SNPs significantly associated with susceptibility to CTL-2A12 were correctly mapped within a single 115 kb LD block at chromosome 18q21 containing the *HMSD* gene (Figures 2 and 3A), which had been previously shown to encode the ACC-6 minor H antigen recognized by CTL-2A12.[9] According to the above criteria, no false-positive SNPs were reported in any array types (Table 1). Confirmation genotyping of individual B-LCLs from both panels revealed none of the 44 that had been immunophenotyped as CTX^- were misjudged, while 8 of the 44 CTX^+ B-LCLs were found to actually carry no minor H-positive allele for ACC-6, which was likely due to the inclusion of individual B-LCLs showing borderline cytotoxicity (data not shown).

On the other hand, positive association of the target minor H antigen with CTL-1B9 was detected in 2 independent loci: SNP rs1879894 at 15q25.1 in 250 K *Nsp*I (Table 1, Figure 4A-B, and Figure S5) and SNP rs1842353 at 8q12.3 in 50 K *Hind*III (Table 1 and Figure S3A). We eventually focused on rs1879894, as it showed a much more significant genome-wide P value than SNP rs1842353 (Table 1). In contrast to the CTL-2A12 case, where many mutually correlated SNPs around the most significant one created a broad peak in the statistic plots (Figure 2 arrows and Figure S3), the adjacent SNPs (rs6495463 and rs2562756; Figure 3B solid arrows) around rs187894 (Figure 3B open arrow) did not show large test statistic values, reflecting the fact that no marker SNPs on 100 K and 500 K arrays exist in high LD (Figure 3B dashed red lines encompassing 26 kb) with this SNP according to the HapMap data. To further confirm the association, we generated additional B-LCL pools consisting of 75 CTX^+ and 34 CTX^-

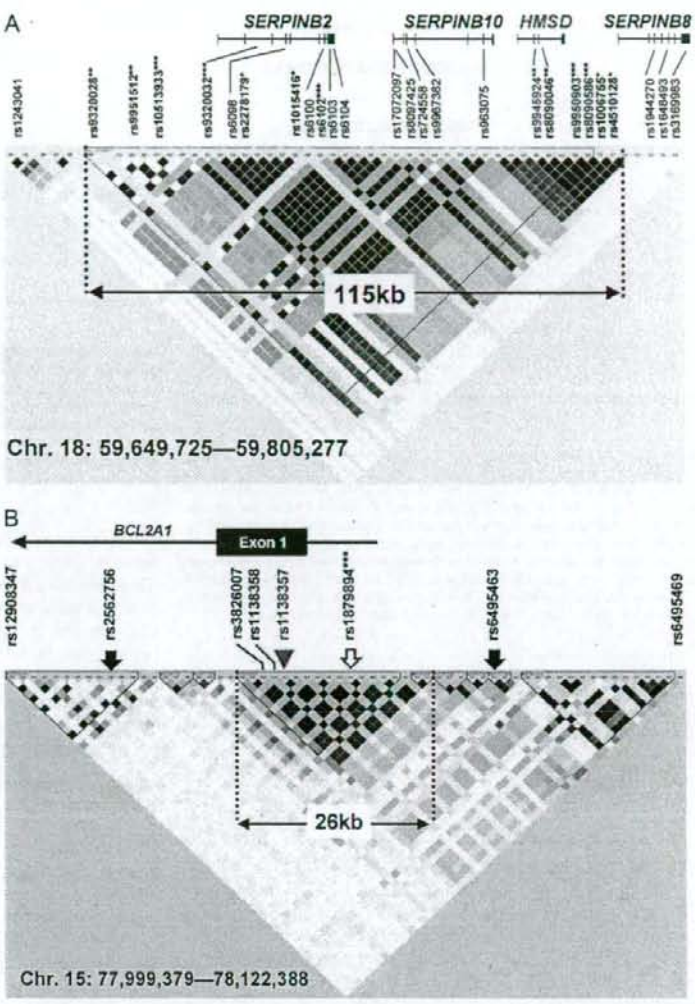

Figure 3. Linkage disequilibrium (LD) block mapped by CTL-2A12 and CTL-1B9. (A) An LD block map identified by pairwise r^2 plot from HapMap CEU data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and 4 genes in the 115 kb block. SNPs that emerged repeatedly in the 2 independent experiments are indicated in blue. The genomewide *P* values for positive SNPs are shown as follows: $*P < .05$; $**P < .01$; $***P < .001$. The intronic SNP (rs9945924) controlling the alternative splicing of *HMSD* transcripts and expression of encoded ACC-6 minor H antigen is indicated in red. (B) LD blocks identified by pairwise r^2 plot from HapMap JPT data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and exon 1 of the *BCL2A1* gene. The only SNP showing a high association with CTL-1B9 immunophenotypes (rs1879894) is shown as an open arrow. The nonsynonymous SNP (rs1138357) controlling the expression of the minor H antigen recognized by CTL-1B9 is indicated by a red arrowhead. ***SNP with genomewide $P < .001$. The 2 SNPs adjacent to the 26 kb LD block (rs2562756 and rs6495463) never gave a significant genomewide *P* value.

B-LCLs from another set of 128 B-LCLs, and performed a WGAS. As expected, the WGAS of the second pools also identified the identical SNP with the highest test statistic value in duplicate experiments, unequivocally indicating that this SNP is truly associated with the minor H locus of interest (Figure 4C,D and Table S3). The association was also detected when the references in the first and second pools were swapped (data not shown).

Identification of the minor H epitope recognized by CTL-1B9

The LD block containing SNP rs1879894 that was singled out from more than 500 000 SNP markers with 2 sets of DNA pools only encodes exon 1 of *BCL2A1* (Figure 3B). To our surprise, this was the region to which we had previously mapped an HLA-A24–restricted minor H antigen, ACC-1^{Y}.[7] We first confirmed that full-length *BCL2A1* cDNA cloned only from the recipient but not his donor could stimulate interferon-γ secretion from CTL-1B9 when transduced into donor B-LCL (Figure 5A), indicating that *BCL2A1* is a bona fide gene encoding minor H antigen recognized by CTL-1B9. We next genotyped 3 nonsynonymous SNPs in the *BCL2A1* exon 1 sequence (Figure 3B) and comparison was made between the genotypes and the susceptibility to CTL-1B9 of 9 HLA-A*2402^{+} B-LCLs, including ones generated from the recipient (from whom CTL-1B9 was established) and his donor. Susceptibility to CTL-1B9 correlated completely with the presence of guanine at SNP rs1138357 (nucleotide position 238, according to the mRNA sequence for NM_004049.2) and thymine at SNP rs1138358 (nucleotide position 299) (Table 2), suggesting that the expression of the minor H epitope recognized by CTL-1B9 is controlled by either of these SNPs. We searched for nonameric amino acid sequences spanning the 2 SNPs using BIMAS software,[22] since most reported HLA-A*2402 binding peptides contain 9 amino acid residues.[23] Among these, a nonameric peptide, DYLQ*C*VLQI (the polymorphic residue being underlined), has a predicted binding score of 75 and was considered as a candidate minor H epitope. As shown in Figure 5B, the DYLQ*C*VLQI was strongly recognized by CTL-1B9, whereas its allelic counterpart,

Table 1. Positive SNPs from pooled DNA analysis

CTL-2A12, Exp 1				CTL-2A12, Exp 2				CTL-1B9, Exp 1				CTL-1B9, Exp 2			
rsID	Chr	Position	$\Delta R_{A\pm R_B}$	rsID	Chr	Position	$\Delta R_{A\pm R_B}$	rsID	Chr	Position	$\Delta R_{A\pm R_B}$	rsID	Chr	Position	$\Delta R_{A\pm R_B}$
50K X bal															
rs10513933	18	59699669	0.366*	rs10513933	18	59699669	0.511†	rs1363258	5	103297593	0.239	rs10499174	6	131209689	0.352*
rs9320028	18	59668150	0.255‡	rs9320028	18	59668150	0.360*	rs726083	3	67093729	0.203	rs30058	5	122325602	0.240
rs6102	18	59721450	0.221	rs10485873	7	3503743	0.157	rs639243	5	31392931	0.198	rs150724	16	61960443	0.213
rs724533	23	116440574	0.137	rs219323	14	59510440	0.150	rs1936461	10	56519024	0.186	rs1993129	8	63618836	0.208
rs1341112	6	104919391	0.136	rs10506892	12	82478539	0.147	rs763876	12	94922502	0.186	rs356946	13	69066751	0.201
rs470490	18	61182216	0.136	rs10492269	12	97786333	0.144	rs958404	7	133054441	0.179	rs2869268	4	86421898	0.184
rs2826718	21	21471423	0.134	rs10483466	14	35986827	0.139	rs10486727	7	41672315	0.178	rs287002	12	40312537	0.183
rs10506697	12	73241741	0.128	rs5910124	23	116408616	0.137	rs2833488	21	32010112	0.176	rs1146808	13	67688608	0.182
rs10506891	12	82393029	0.127	rs10512545	17	66337079	0.134	rs379212	5	60977687	0.172	rs10501287	11	42446011	0.180
rs308995	14	59657919	0.125	rs295678	5	58186928	0.131	rs1954004	14	58627872	0.170	rs564993	5	31393476	0.177
50K *Hind*III															
rs9320032	18	59712191	0.486†	rs9320032	18	59712191	0.506†	rs1842353	8	63617543	0.244*	rs9300692	13	101216476	0.225‡
rs8090046	18	59773066	0.207‡	rs8090046	18	59773066	0.245*	rs10521202	17	12755289	0.201‡	rs1842353	8	63617543	0.210‡
rs1474220	2	108525317	0.193‡	rs10498752	6	41876488	0.210‡	rs7899961	10	59696431	0.198‡	rs10520983	5	31314700	0.195‡
rs10498752	6	41876488	0.178	rs1941538	18	37994337	0.176	rs9320974	6	124421441	0.197‡	rs1334375	13	80897038	0.173
rs2298578	21	21632551	0.167	rs7682770	4	152748018	0.174	rs10520983	5	31314700	0.179	rs10519164	15	75412758	0.163
rs7516032	1	91618962	0.165	rs1445862	5	3675257	0.169	rs1862446	5	147460749	0.170	rs9322063	6	146852196	0.152
rs5030938	10	70645922	0.164	rs4696976	4	21058616	0.167	rs1358778	20	13256796	0.169	rs8067384	17	37926265	0.150
rs1883041	21	44921845	0.158	rs5030938	10	70645922	0.165	rs1873790	4	83422480	0.166	rs10521202	17	12755289	0.147
rs3902916	4	189045176	0.155	rs3902916	4	189045176	0.165	rs1220724	4	70888705	0.162	rs7914904	10	62749969	0.141
rs1000551	20	58708208	0.154	rs1883041	21	44921845	0.164	rs9300692	13	101216476	0.157	rs1220724	4	70888705	0.141
250K *Nsp*I															
rs9950903	18	59781783	0.534†	rs9950903	18	59781783	1.036†	rs1879894	15	78055874	0.846†	rs1879894	15	78055874	1.072†
rs1463835	3	23539615	0.532†	rs8090586	18	59781864	0.518†	rs9646294	16	6110019	0.484†	rs6771859	3	190642054	0.387†
rs16975459	18	37802275	0.383*	rs6473170	8	80664840	0.338*	rs17734332	5	134945240	0.365†	rs10512261	9	98804394	0.299*
rs8090586	18	59781864	0.367*	rs4510128	18	59782312	0.310‡	rs566619	7	41381538	0.345*	rs12122772	1	60384564	0.287*
rs16872621	4	22081055	0.312‡	rs1006755	18	59782026	0.300‡	rs17737566	6	50345280	0.310*	rs2153155	4	26034162	0.248‡
rs870582	6	125097114	0.301‡	rs7039378	9	118735938	0.258	rs3849955	9	28350374	0.285*	rs17126896	14	53320494	0.246‡
rs1015416	18	59720363	0.270‡	rs1860563	16	6418899	0.258	rs4616156	13	86581518	0.273*	rs1328652	13	35607527	0.240
rs2155907	11	97599883	0.227	rs4699126	4	105709109	0.212	rs2484698	1	217474460	0.263*	rs7021551	9	27446645	0.237
rs2112948	5	50994294	0.222	rs10275055	7	156212079	0.204	rs17139603	11	79638632	0.262*	rs252817	5	106752487	0.237
rs2919747	2	129681506	0.217	rs1526411	7	124658309	0.201	rs2156737	4	100642529	0.246‡	rs10772587	12	12681356	0.235
250K *Sty*I															
rs6102	18	59721450	0.597†	rs6102	18	59721450	0.495†	rs9383925	6	151975774	0.819†	rs201204	6	104842863	0.688†
rs9951512	18	59690885	0.374*	rs9945924	18	59771746	0.407*	rs6497397	16	19646258	0.311‡	rs12556155	23	108836419	0.442†
rs6496897	15	90493249	0.320‡	rs9951512	18	59690885	0.317‡	rs917252	7	22219990	0.289‡	rs4791422	17	10605304	0.435†
rs9945924	18	59771746	0.315‡	rs1983205	3	157782892	0.314‡	rs1019403	3	7823997	0.260‡	rs7749012	6	106459559	0.336*
rs12707805	8	107404746	0.303‡	rs950865	5	2720684	0.307‡	rs17053134	5	155373544	0.259‡	rs509951	5	31385483	0.308‡
rs10971778	9	33893184	0.296‡	rs2278179	18	59715512	0.292‡	rs11710880	3	72214965	0.246	rs16879024	8	32225711	0.256‡
rs6565076	16	81487818	0.294‡	rs10427722	22	36417752	0.289‡	rs17167866	7	13919264	0.237	rs2100054	15	75293482	0.252
rs2278179	18	59715512	0.291‡	rs17156659	7	82046820	0.271	rs10867062	9	137935241	0.237	rs11811023	1	143805934	0.240
rs7806238	7	29906442	0.290‡	rs4502324	18	4811261	0.262	rs5925800	23	23278707	0.235	rs17382798	15	75256074	0.231
rs965888	18	38062658	0.283‡	rs1348428	2	225927288	0.260	rs2255831	4	146614313	0.234	rs2030302	17	12526591	0.231

Significant SNPs that appeared on both experiments are underlined.
*Genomewide $P < .01$.
†Genomewide $P < .001$.
‡Genomewide $P < .05$.

DYLQ*Y*VLQI, was not. Decameric peptide, QDYLQCVLQI, on the other hand, appeared to be weakly recognized; however, it is likely that the nonameric form was actually being presented after N-terminal glutamine cleavage by aminopeptidase in the culture medium. Because it was possible that the cystine might be cysteinylated, recognition of synthetic peptides DYLQCVLQI and cysteinylated DYLQC*VLQI were assayed using CTL-1B9. Half-maximal lysis for the former was obtained at a concentration of 200 pM, whereas recognition of the latter was several-fold weaker (Figure 5C). Thus, we concluded that DYLQCVLQI defines the cognate HLA-A*2402–restricted CTL-1B9 epitope, now designated ACC-1^C. This incidentally provides a second example of products from both dichotomous SNP alleles being recognized as HLA-A*2402–restricted minor H antigens, the first example being the HB-1 minor H antigen.[24] Finally, real-time quantitative PCR revealed that T cells carrying the complementarity-determining region 3 sequence identical to CTL-1B9 became detectable in the patient's blood at the frequencies of 0.22%, 0.91%, 1.07% and 0.01% among TCR$\alpha\beta^+$ T cells at days 30, 102, 196, and 395 after transplantation, respectively, suggesting that ACC-1^C minor H antigen is indeed immunogenic (Figure 5D).

Discussion

Recent reports have unequivocally demonstrated that WGASs can be successfully used to identify common variants involved in a wide variety of human diseases.[25-27] Our report represents a novel

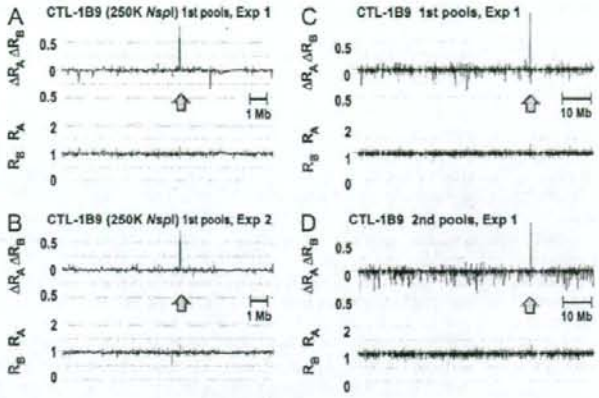

Figure 4. Reproducible detection of association with the immunophenotypes determined by CTL-1B9 at the *BCL2A1* locus. The maximum test statistic value was observed at a single SNP (rs1879894) within 15q25.1 in duplicate experiments for the first pools consisting of 57 CTX$^+$ and 38 CTX$^-$ B-LCLs (A-C). The peak association at the same SNP was reproduced in the experiments with the second pools consisting of 75 CTX$^+$ and 34 CTX$^-$ LCLs (D). Test statistic values ($\Delta R_A \Delta R_B$) are plotted by blue lines together with their R_A (red) and R_B (green) values. The expected $\Delta R_A \Delta R_B$ values multiplied by r^2 correlation coefficients for the adjacent SNPs within 500 kb from the SNP rs1879894 are overlaid by red lines (A,B).

application of WGASs to transplantation immunology, which provides a simple but robust method to fine-map the genetic loci of minor H antigens whose expression is readily determined by standard immunophenotyping with CTL clones established from patients who have undergone transplantation.

The current WGA/CTL method has several desirable features that should contribute to the acceleration of minor H locus mapping. In comparing the method to those of linkage analysis and other nongenetic approaches, including direct peptide sequencing of chemically purified minor H antigens[5,6,10,13] and conventional

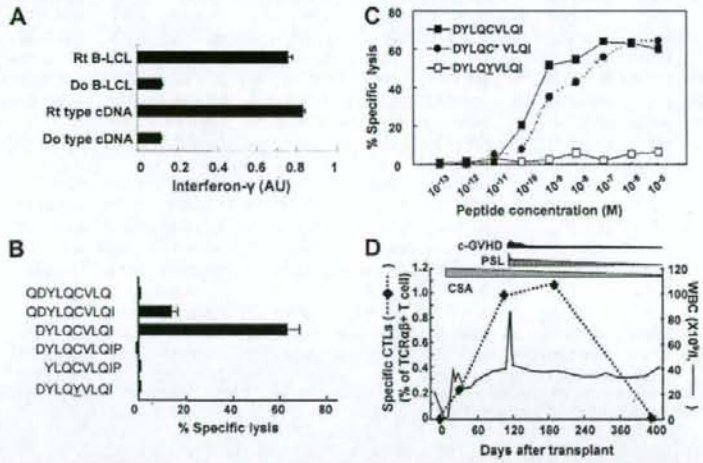

Figure 5. Identification of the CTL-1B9 minimal minor H epitope. (A) Interferon-γ production from CTL-1B9 against HLA-A*2402–transduced 293T cells transfected with plasmid encoding full-length *BCL2A1* cDNA cloned from either the recipient (Rt) from whom CTL-1B9 was isolated or his donor (Do). Rt B-LCL and Do B-LCL were used as positive and negative controls, respectively. Secreted interferon-γ was measured by ELISA and is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (B) A peptide reconstitution assay was conducted to determine the minimal epitope for CTL-1B9. Nonameric peptide (DYLQCVLQI), 2 nonameric peptides shifted by one amino acid to N- or C-terminus, N- and C-terminal extended decameric peptides, and its allelic counterpart (DYLQYVLQI) were synthesized and tested by adding to antigen-negative donor B-LCL at 10 nM in a standard ^{51}Cr release assay. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (C) Titration of the candidate minor H peptide by epitope reconstitution assay. Chromium-labeled donor B-LCLs were distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 minutes at room temperature, and then used as targets for CTL-1B9 in a standard ^{51}Cr release assay. A cysteinylated peptide (indicated by an asterisk) was included as an alternative form of the potential epitope. Results are typical of 2 experiments. (D) Tracking of ACC-1^C–specific T cells in the recipient's peripheral blood. In order to longitudinally analyze the kinetics of the ACC-1^C–specific CTLs in peripheral blood from the patient from whom CTL-1B9 was established, a real-time quantitative PCR was conducted. Complementary DNAs of peripheral blood mononuclear cells from the donor and patient before and after HSCT were prepared from the patient. Real-time PCR analysis was performed using a TaqMan assay as described previously.[9] The primers and fluorogenic probe sequences spanning the CTL-1B9 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-1B9. The primers and fluorogenic probe sequences spanning constant region of TCR beta chain (TCRBC) mRNA were used as internal control. Samples were quantified with the comparative CT method. The delta CT value was determined by subtracting the average CT value for TCRBC from the average CTL-1B9 CDR3 CT value. The standard curve for the proportion of CTL-1B9 among TCRαβ$^+$ T cells was composed by plotting mean delta CT values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-1B9 were calculated by using this standard curve. During this period, quiescent chronic GVHD, which required steroid treatment, developed; however, involvement of immune reaction to ACC-1^C minor H antigen was unlikely since its frequency increased even after resolution of most chronic GVHD symptoms. c-GVHD, chronic GVHD; CSA, cyclosporine A; PSL, prednisolone; WBC, white blood cell count.

Table 2. Correlation of *BCL2A1* sequence polymorphisms with susceptibility to CTL-1B9

	HLA-A*2402–positive B-LCLs								
	Rt	Do	UR1	UR2	UR3	UR4	UR5	UR6	UR7
Cytolysis by CTL-1B9	+	−	+	+	+	+	+	−	−
Detected SNP, position*									
rs1138357, 238	G/A	A	G	G	G/A	G/A	G/A	A	A
rs1138358, 299	T/G	G	T	T	T/G	T/G	T/G	G	G
rs3826007, 427	G	G/A	G	G	G	G	G/A	G/A	G

Rt indicates recipient; Do, donor; UR, unrelated; +, yes; and −, no.

*Nucleotide positions are shown according to the NM_004092.2 mRNA sequence, available at http://www.ncbi.nlm.nih.gov/ as GEO accession GSE10044.

expression cloning,[8,9,11] there are differences in terms of power, sensitivity, and specificity. Direct sequencing of minor H antigen peptide guarantees that the purified peptide is surely present on the cell surface as antigen, but it requires highly specialized equipment and personnel. Expression screening of cDNA libraries is also widely used and has become feasible with commercially available systems. However, it depends highly on the quality of the cDNA library and expression levels of the target genes. In addition, it often suffers from false-positive results due to the forced expression of cDNA clones under a strong promoter. The current method of WGA/CTL genetically determines the relevant minor H antigen locus, not relying on highly technical protein chemistry using specialized equipment, or repetitive cell cloning procedures. It is also not affected by the expression levels of the target antigens.

As a genetic approach, the current method based on genetic association has several advantages over conventional linkage analysis: the mapping resolution has been greatly improved from several Mb in the conventional linkage analysis to the average haplotype block size of less than 100 kb,[17,25-27] usually containing a handful of candidate genes, compared with the dozens as typically found in linkage analysis. This means that the effort needed for the subsequent epitope mapping will be substantially reduced. In fact, the 115 kb region identified for CTL-2A12 contains 4 genes compared with 38 genes as revealed by the previous linkage study (data not shown), and the candidate gene was uniquely identified within the 26 kb region for CTL-1B9, for which linkage analysis had failed due to very rare segregating pedigrees among the CEPH panels with this trait (now ACC-1[C]; data not shown).[15,16] In addition, before moving on to epitope mapping, it would be possible to evaluate the clinical relevance of the minor H antigens by examining the tissue distribution of their expression, based on widely available gene expression databases such as Genomic Institute of the Novartis Research Foundation (GNF, http://symatlas.gnf.org/SymAtlas/).[28]

Second, the required sample size is generally small, and should be typically no more than 100 B-LCLs for common minor H alleles. This is in marked contrast to the association studies for common diseases, in which frequently thousands of samples are required.[17,25-27] In the current approach, sufficiently high test statistic values could be obtained for the relevant loci with a relatively small sample size, since the minor H allele is correctly segregated between the CTX^+ and CTX^- pools by the highly specific immunologic assay. Combined with high accuracy in allelic measurements, this feature allows for the use of pooled DNAs in WGAS, which substantially saves cost and time, compared with the genotyping of individual samples. Unexpectedly, our method allows for a considerable degree of error in the immunophenotyping, indicating the robustness of the current method; in fact, the minor H locus for CTL-2A12 was successfully identified in spite of the presence of 8 (~10%) immunophenotyping errors. When the minor H allele has an extreme allele frequency (eg, $< 5\%$ or $> 95\%$), which could be predicted by preliminary immunophenotyping, WGAS/CTL may not be an efficient method of mapping, due to the impractically large numbers of B-LCLs that would need to be screened to obtain enough CTX^+ or CTX^- B-LCLs. However, such minor H antigens would likely have limited clinical impact or applicability.

Sensitivity of the microarray analysis seems to be very high when the target SNP has good proxy SNPs on the array, because we were able to correctly identify the single SNP correlated with the target of CTL-1B9 from more than 500 000 SNP markers. On the other hand, genome coverage of the microarray is definitely important. In our experiments on CTL-2A12, the association was successfully identified by the marker SNPs showing r^2 values of approximately 0.74 with the target locus of ACC-6. Since the GeneChip 500 K array set captures approximately 65% of all the HapMap phase II SNPs with more than 0.74 of r^2,[29] and higher coverage will be obtained with the SNP 6.0 arrays having more than 1 000 K SNP markers, these arrays can be satisfactorily used as platforms for the WGA/CTL method.

As shown in the current study, the intrinsic sensitivity and specificity of the WGA/CTL method in detecting associated SNPs were excellent. In other words, as long as target SNPs are captured in high r^2 values with one or more marker SNPs within the Affymetrix 500 K SNP set, there is a high likelihood of capturing the SNP with the current approach. To evaluate the probability of a given minor H antigen being captured in high r^2 with marker SNPs, we checked the maximum r^2 values of known minor H antigen SNPs with the Affymetrix 500 K SNPs, according to empirical data from the HapMap project (www.hapmap.org). Among 13 known minor H antigens, 7 have their entries (designated minor H SNP) in the HapMap phase II SNP set (HA-3,[30] HA-8,[31] HB-1,[11] ACC-1 and ACC-2,[7] LB-ADIR-1F,[10] and 7A7-PANE1[13]), and were used for this purpose (note that absence of their entries in the HapMap data set does not necessarily mean that they could not be captured by a particular marker SNP set). As shown in Table S4, all 7 minor H SNPs are captured by at least one flanking SNP that is included in the Affymetrix 500 K SNP set with r^2 values of more than 0.74 in at least one HapMap panel. The situation should be more favorable in the recently available SNP 6.0 array set with 1 000 K SNPs, indicating the genome coverage with currently available SNP arrays would be sufficient to capture typical minor H antigens with our approach.

Most patients who have received allo-HSCT could be a source of minor H antigen-specific CTL clones to be used for this assay, since the donor T cells are in vivo primed and many CTL clones could be established using currently available methods. In fact, substantial numbers of CTL clones have been established worldwide and could serve as the probes to identify novel minor H antigens.[32,33] Once constructed, a panel of B-LCLs, including those transduced with HLA cDNAs, could be commonly applied to immunophenotyping with different CTL clones, especially when

CTLs are obtained from the same ethnic group. In addition, by adopting other immunophenotyping readouts such as production of IL-2 from $CD4^+$ T cells, this method could be applied to identification of MHC class II–restricted minor H antigens which have crucial roles in controlling CTL functions upstream. This may open a new field in the study of allo-HSCT since MHC class II–restricted mHags have been technically difficult to identify by conventional methods.

Finally, the discovery of ACC-1^C as a novel minor H antigen indicates that all the mismatched transplants at this locus could be eligible for allo-immune therapies, since we have previously demonstrated that the counter allele also encodes a minor H antigen, ACC-1^Y, which is preferentially expressed and presented on blood components including leukemic cells and may serve as a target of allo-immunity.[7,34] Indeed, CTLs specific for ACC-2, an HLA-B44–restricted minor H antigen restricted by the third exonic SNP on *BCL2A1*,[7] was independently isolated from the peripheral blood of a patient with recurrent leukemia re-entering complete remission after donor lymphocyte infusion.[32] The number of eligible allo-HSCT recipients has now been effectively doubled, accounting for 50% of transplants with HLA-A24 or 20% of all transplantations performed in the Asian population. In conclusion, we have described a simple but powerful method for minor H mapping to efficiently accelerate the discovery of novel minor H antigens that will be needed to contribute to our understanding of the molecular mechanism of human allo-immunity.

Acknowledgments

The authors thank Dr W. Ho for critically reading the manuscript; Dr Keitaro Matsuo, Dr Hiroo Saji, Dr Etsuko Maruya, Dr Mamoru Ito, Ms Keiko Nishida, Dr Ayako Demachi-Okamura, Ms Yasuko Ogino, Ms Hiromi Tamaki, and the staff members of the transplant center, donor centers, and the Japan Marrow Donor Program office for their generous cooperation and expert technical assistance.

This study was supported in part by Scientific Research on Priority Areas (B01; no.17016089) from the Ministry of Education, Culture, Science, Sports, and Technology, Japan; Research on Human Genome, Tissue Engineering Food Biotechnology and the Second and Third Team Comprehensive 10-year Strategy for Cancer Control (no. 26), from the Ministry of Health, Labor, and Welfare, Japan; and a Grant-in-Aid from Core Research for Evolutional Science and Technology (CREST) of Japan.

Authorship

Contribution: T.K. performed most immunologic experiments and preparation of pooled DNA and quantative PCR, analyzed data, and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T. performed T-cell receptor analysis and designed q-PCR primers and probes; G.Y. contributed to the organization of software for linkage analysis and simulation; S.M. prepared the pooled DNA; M.O., K.M., Y.K, and Y.M. collected clinical data and specimens; T.T. and K.K. contributed to data analysis and interpretation, and to the writing of the article; S.O. and Y.A. supervised the entire project, designed and coordinated most of the experiments in this study, contributed to manuscript preparation, and are senior coauthors.

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

Correspondence: Seishi Ogawa, Department of Hematology and Oncology, Department of Regeneration Medicine for Hematopoiesis, The 21st Century COE Program, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; e-mail: sogawa-tky@umin.ac.jp; or Yoshiki Akatsuka, Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan; e-mail: yakatsuk@aichi-cc.jp.

References

1. Thomas ED Sr. Stem cell transplantation: past, present and future. Stem Cells. 1994;12:539-544.
2. Childs RW, Barrett J. Nonmyeloablative allogeneic immunotherapy for solid tumors. Annu Rev Med. 2004;55:459-475.
3. Goulmy E. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. Immunol Rev. 1997;157:125-140.
4. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4:371-380.
5. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science. 1998;279:1054-1057.
6. Pierce RA, Field ED, Mutis T, et al. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. J Immunol. 2001;167:3223-3230.
7. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. J Exp Med. 2003;197: 1489-1500.
8. Warren EH, Vigneron NJ, Gavin MA, et al. An antigen produced by splicing of noncontiguous peptides in the reverse order. Science. 2006;313: 1444-1447.
9. Kawase T, Akatsuka Y, Torikai H, et al. Alternative splicing due to an intronic SNP in HMSD generates a novel minor histocompatibility antigen. Blood. 2007;110:055-1063.
10. van Bergen CA, Kester MG, Jedema I, et al. Multiple myeloma-reactive T cells recognize an activation-induced minor histocompatibility antigen encoded by the ATP-dependent interferon-responsive (ADIR) gene. Blood. 2007;109:4089-4096.
11. Dolstra H, Fredrix H, Maas F, et al. A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. J Exp Med. 1999; 189:301-308.
12. de Rijke B, van Horssen-Zoetbrood A, Beekman JM, et al. A frameshift polymorphism in P2X5 elicits an allogeneic cytotoxic T lymphocyte response associated with remission of chronic myeloid leukemia. J Clin Invest. 2005;115:3506-3516.
13. Brickner AG, Evans AM, Mito JK, et al. The PANE1 gene encodes a novel human minor histocompatibility antigen that is selectively expressed in B-lymphoid cells and B-CLL. Blood. 2006;107:3779-3786.
14. Warren EH, Otterud BE, Linterman RW, et al. Feasibility of using genetic linkage analysis to identify the genes encoding T cell-defined minor histocompatibility antigens. Tissue Antigens. 2002;59:293-303.
15. Consortium TIH. The International HapMap Project. Nature. 2003;426:789-796.
16. Consortium TIH. A haplotype map of the human genome. Nature. 2005;437:1299-1320.
17. Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science. 1996;273:1516-1517.
18. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. Nat Rev Genet. 2005;6:95-108.
19. Kennedy GC, Matsuzaki H, Dong S, et al. Large-scale genotyping of complex DNA. Nat Biotechnol. 2003;21:1233-1237.
20. Matsuzaki H, Dong S, Loi H, et al. Genotyping over 100 000 SNPs on a pair of oligonucleotide arrays. Nat Methods. 2004;1:109-111.
21. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263-265.
22. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol. 1994;152:163-175.
23. Kubo RT, Sette A, Grey HM, et al. Definition of specific peptide motifs for four major HLA-A alleles. J Immunol. 1994;152:3913-3924.
24. Dolstra H, de Rijke B, Fredrix H, et al. Bi-directional allelic recognition of the human minor histocompatibility antigen HB-1 by cytotoxic T lymphocytes. Eur J Immunol. 2002;32:2748-2758.
25. Easton DF, Pooley KA, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature. 2007; 447:1087-1093.
26. Gudmundsson J, Sulem P, Manolescu A, et al.

Genome-wide association study identifies a second prostate cancer susceptibility variant at 8q24. Nat Genet. 2007;39:631-637.

27. Zeggini E, Weedon MN, Lindgren CM, et al. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. Science. 2007;316:1336-1341.
28. Su AI, Cooke MP, Ching KA, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci U S A. 2002;99:4465-4470.
29. Nannya Y, Taura K, Kurokawa M, Chiba S, Ogawa S. Evolution of genome-wide power of genetic association studies based on empirical data from the HapMap project. Hum Mol Genet. 2007;16:3494-3505.
30. Spierings E, Brickner AG, Caldwell JA, et al. The minor histocompatibility antigen HA-3 arises from differential proteasome-mediated cleavage of the lymphoid blast crisis (Lbc) oncoprotein. Blood. 2003;102:621-629.
31. Brickner AG, Warren EH, Caldwell JA, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. J Exp Med. 2001;193:195-206.
32. Kloosterboer FM, van Luxemburg-Heijs SA, van Soest RA, et al. Minor histocompatibility antigen-specific T cells with multiple distinct specificities can be isolated by direct cloning of IFNgamma-secreting T cells from patients with relapsed leukemia responding to donor lymphocyte infusion. Leukemia. 2005;19:83-90.
33. Tykodi SS, Warren EH, Thompson JA, et al. Allogeneic hematopoietic cell transplantation for metastatic renal cell carcinoma after nonmyeloablative conditioning: toxicity, clinical response, and immunological response to minor histocompatibility antigens. Clin Cancer Res. 2004;10:7799-7811.
34. Kenny JJ, Knobloch TJ, Augustus M, Carter KC, Rosen CA, Lang JC. GRS, a novel member of the Bcl-2 gene family, is highly expressed in multiple cancer cell lines and in normal leukocytes. Oncogene. 1997;14:997-1001.

Biology of Blood and Marrow Transplantation 14:75-87 (2008)

1083-8791/08/1401-0001$32.00/0
doi:10.1016/j.bbmt.2007.09.012

Donor Killer Immunoglobulin-Like Receptor (KIR) Genotype-Patient Cognate KIR Ligand Combination and Antithymocyte Globulin Preadministration Are Critical Factors in Outcome of HLA-C-KIR Ligand-Mismatched T Cell–Replete Unrelated Bone Marrow Transplantation

Toshio Yabe,[a] Keitaro Matsuo,[b] Kouyuki Hirayasu,[ac] Koichi Kashiwase,[a] Sumiyo Kawamura-Ishii,[d] Hidenori Tanaka,[a] Atsuko Ogawa,[a] Minoko Takanashi,[a] Masahiro Satake,[a] Kazunori Nakajima,[a] Katsushi Tokunaga,[e] Hidetoshi Inoko,[e] Hiroo Saji,[f] Seishi Ogawa,[g] Takeo Juji,[d] Takehiko Sasazuki,[h] Yoshihisa Kodera,[i] and Yasuo Morishima[j] for the Japan Marrow Donor Program

[a]Japanese Red Cross Tokyo Metropolitan Blood Center, Tokyo, Japan; [b]Aichi Cancer Center Research Institute, Division of Epidemiology and Prevention, Nagoya, Japan; [c]University of Tokyo, Department of Human Genetics, Tokyo, Japan; [d]Japanese Red Cross Central Blood Institute, Tokyo, Japan; [e]Tokai University School of Medicine, Division of Molecular Science, Isehara, Japan; [f]HLA Laboratory, NPO, Kyoto, Japan; [g]Tokyo University Hospital, Tokyo, Japan; [h]International Medical Center of Japan, Tokyo, Japan; [i]Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; and [j]Aichi Cancer Center Hospital, Department of Hematology and Cell Therapy, Nagoya, Japan

Correspondence and reprint requests: Toshio Yabe, PhD, Japanese Red Cross Tokyo Metropolitan Blood Center, 2-1-67 Tatsumi, Koto-ku, Tokyo 135-8639, Japan; Tel: +81-3-5534-7546; Fax: +81-3-5534-7547 (e-mail: to-yabe@tokyo.bc.jrc.or.jp).

Received July 18, 2007; accepted September 21, 2007

ABSTRACT

We previously reported the potent adverse effects of killer immunoglobulin-like receptor (KIR) ligand mismatch (KIR-L-MM) on the outcome of T cell–replete unrelated hematopoietic stem cell transplantation (UR-HSCT) through the Japan Marrow Donor Program. Other UR-HSCT studies have yielded inconsistent results. To address this discrepancy, we evaluated candidate factors contributing to the effects of KIR-L-MM on transplantation outcomes in retrospectively selected hematologic malignancy cases with uniform graft-versus-host disease (GVHD) prophylaxis (n = 1489). KIR-L-MM in the graft-versus-host direction (KIR-L-MM-G) was associated with a higher incidence of acute GVHD (aGVHD; $P < .002$) and a lower overall survival (OS; $P < .0001$) only without the preadministration of antithymocyte globulin (ATG). Furthermore, in KIR-L-MM-G, the donor *KIR2DS2* gene with the patient cognate C1 ligand was associated with a higher incidence of aGVHD ($P = .012$). Multivariate analysis by Cox proportional hazard models suggested that donor *2DS2* and ATG preadministration were critical factors in grade III-IV aGVHD (hazard ratio = 1.96; 95% confidence interval = 1.01-3.80; $P = .045$, and hazard ratio = 0.56; 95% confidence interval = 0.31-0.99; $P = .047$, respectively). These results indicate that the adverse effects of KIR-L-MM-G depend on combination of donor-activating *KIR* genotype-patient cognate KIR ligand type and no ATG preadministration, thereby suggesting the importance of these factors in UR-HSCT and in leukemia treatment using natural killer (NK) cell alloreactivity.

INTRODUCTION

Natural killer (NK) cell alloreactivity plays an important role in hematopoietic stem cell transplantation (HSCT,) and its therapeutic use in leukemia treatment has been considered because of its possible graft-versus-leukemia (GVL) effect [1]. The beneficial effects of NK cell receptor killer immunoglobulin-like receptor (KIR) ligand incompatibility between patient and donor in the HLA-mismatched related hematopoietic stem cell transplantation (R-HSCT) has been reported [2,3]. These effects in unrelated hematopoietic stem cell transplantation (UR-HSCT) have been controversial, however [4]. We recently reported the potent adverse effects of HLA-C-KIR ligand incompatibility