	beta-plate sheet			alpha helix
Position of HLA class I	9	99	116	156
Peptide-binding pocket	B C	A B D	F	D E
Amino acid substitution				
HLA-A	Tyr-Phe		Asn-Asp*	
HLA-C	Tyr-Ser	Tyr-Phe	Leu-Ser	Arg-Leu

Figure 2. Schematic presentation of HLA class I molecule and summary of features of significant amino acid substituted positions. Numbers in schema of HLA molecule indicate substituted amino acid positions that were elucidated as significant risk factor for severe aGVHD. Positions 9, 99, and 116 are located in the beta-plated sheet and positions 77, 80, and 156 in the alpha helix of HLA class I molecule (left). Positions 77 and 80 are associated with KIR2DL ligand in HLA-C molecule. Position 9 constitutes peptide-binding pockets B and C; position 99 constitutes A, B, and D pockets; position 116 constitutes F pocket; and position 156 constitutes D and E pockets (right). For example, Tyr-Phe indicated amino acid substitution at indicated position in HLA molecule at which donor had tyrosine and patient phenylalanine. Tyr indicates tyrosine; Phe, phenylalanine; Asn, asparagine; Asp, asparatic acid; Ser, serine; Lys, lysine; Leu, leucine; and Arg, arginine. *Result of base analysis was significant but result of validating analysis using bootstrap resampling was not. Results of analysis were thus judged not to be statistically significant.

sheet, and position 156 is in the alpha helix of HLA class I molecule (Figure 2).[26,27] Position 9 constitutes peptide-binding pockets B and C, position 99 constitutes A, B, and D pockets, position 116 constitutes F pocket, and position 156 constitutes D and E pockets.[28] As a result, all amino acid positions elucidated in this study were important positions for peptide binding and T-cell recognition, although all substituted positions including positions at which residues are not accessible in the vicinity of peptide binding sites were analyzed.

To our knowledge, amino acid substitutions at position 9 (Tyr9A-Phe9A and Tyr9C-Ser9C) and position 99 (Tyr99C-Phe99C) were newly identified in the present study as responsible for severe aGVHD.

Ferrara et al reported that the amino acid substitution at position 116 in HLA class I molecule increased the risk for aGVHD, although the substituted amino acid was not taken into consideration.[29] In our study, specific amino acid substitution at position 116 had a significant effect in HLA-C (Leu116C-Ser116C) and a marginal effect in HLA-A (Asn116A-Asp116A) for severe aGVHD (Table 5).

Position 156 of HLA molecule was certified to modify T-cell alloreactivity in vitro in HLA-A2,[30-32] HLA-B35,[33] and HLA-B44.[24] For example, in contrast to Asp156B in HLA-B*4402, the nonpolar nature of substituted Leu156B in HLA-B*4403 lost many interactions such as hydrogen bonds and van der Waals interactions with the other amino acid residues that constructed binding pockets. As a result, this substitution made the significant conformation change for alloreactivity.[24] In the HLA-B*3501 and HLA-B*3508 combination, Leu156B in HLA-B*3501 with nonpolar residue was substituted for Asp156B in HLA-B*3508 with polar residue, and induced strong alloreactivity.[33] In our study, the magnitude of the polar change of each substituted amino acid was calculated by "hydropathy scale,"[17] because the influence of this scale on the amino acid interaction was much greater than the influence of the isoelectric point.[34] Specific amino acid substitutions at position 9, 99, 116, and 156, which were not associated with KIR2DL ligand, were found to induce great polar changes except for Tyr9C-Ser9C. Generally speaking, the 3 major physicochemical properties of amino acids that play important roles in protein structure are the hydropathy scale, isoelectric point, and molecular weight, and molecular weight is reflected in the size of amino acids.[34] Indeed, although tyrosine and serine in Tyr9C-Ser9C show few differences in hydropathy scale and isoelectric point, their molecular weights are quite different and may well induce an important conformation change in the HLA molecule. Thus, the change in the conformation by the polar change of the HLA molecule might be one of the mechanisms inducing alloreactivity. These data serve to clarify the mechanisms of aGVHD based on the HLA molecule.

The analysis of HLA-B, -DRB1, -DPB1, and -DQB1 mismatch for the substitution of amino acid elucidated no responsible position for severe aGVHD, and the analysis of HLA-A elucidated only one position. We speculate that the reason for the above result in HLA class I was that in this population there were fewer HLA-mismatched pairs in HLA-A and -B than in HLA-C. Although the findings are due mainly to the HLA-C molecule, specific amino acid substitution at positions 9, 99, 116, and 156 on the HLA class I molecule may induce strong alloreactivity because the structures of HLA class I molecules are quite similar.[29] Indeed, position 9 is selected in HLA-A and -C concurrently, and position 116 had a significant effect on HLA-C and a marginal effect on HLA-A (Figure 2). In HLA class II, we speculated that the molecular base of aGVHD caused by the HLA class II mismatch might be different from that in HLA class I.

In conclusion, we clarified nonpermissive mismatch combinations of all major 6 HLA loci. These data would be beneficial for the selection of suitable donors and international donor exchange for UR-HSCT. Furthermore, we identified the positions and types of amino acid substitutions responsible for severe aGVHD and presented speculations for alloreactivity on the basis of the conformation change of the HLA molecule. These findings provide evidence to elucidate the mechanism of aGVHD on the basis of the HLA molecule.

Acknowledgments

This study was supported in part by Health and Labor Science Research Grant from Ministry of Health, Labor and Welfare of Japan (Research on Human Genome, Tissue Engineering), Grant-in-Aid B from the Japan Society for the Promotion of Science, and a Grant from Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan.

We thank the staff members of the transplant center, donor centers, and JMDP office for their generous cooperation; Ms Ryouko Yamauchi for the data management; and Dr Toshitada Takahashi and Dr Setsuko Kawase for their expert technical assistance.

Authorship

Contribution: T.S., Y.M., T.K., T.J., and Y.K. participated in the conception of this study; K.K., H.I., and H.S. performed the execution for histocompatibility; Y.M. and S.K. performed the execution for transplantation; T.K. and K.M. performed statistical

data analysis; T.K. and Y.M. wrote the paper; all authors checked the final version of the paper.

A complete list of the institutions participating and registering patients through the Japan Marrow Donor Program for the present study is available on the *Blood* website; see the Supplemental Appendix link at the top of the online article.

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

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Age-Related EBV-Associated B-Cell Lymphoproliferative Disorders Constitute a Distinct Clinicopathologic Group: A Study of 96 Patients

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Abstract **Purpose:** We have recently reported EBV+ B-cell lymphoproliferative disorders (LPD) occurring predominantly in elderly patients, which shared features of EBV+ B-cell neoplasms arising in the immunologically deteriorated patients despite no predisposing immunodeficiency and were named as senile or age-related EBV+ B-cell LPDs. To further characterize this disease, age-related EBV+ B-cell LPDs were compared with EBV-negative diffuse large B-cell lymphomas (DLBCL).

Experimental Design: Among 1,792 large B-cell LPD cases, 96 EBV+ cases with available clinical data set were enrolled for the present study. For the control group, 107 patients aged over 40 years with EBV-negative DLBCL were selected. We compared clinicopathologic data between two groups and determined prognostic factors by univariate and multivariate analysis.

Results: Patients with age-related EBV+ B-cell LPDs showed a higher age distribution and aggressive clinical features or parameters than EBV-negative DLBCLs: 44% with performance status >1, 58% with serum lactate dehydrogenase level higher than normal, 49% with B symptoms, and higher involvement of skin and lung. Overall survival was thus significantly inferior in age-related EBV+ group than in DLBCLs. Univariate and multivariate analyses further identified two factors, B symptoms and age older than 70 years, independently predictive for survival. A prognostic model using these two variables well defined three risk groups: low risk (no adverse factors), intermediate risk (one factor), and high risk (two factors).

Conclusions: These findings suggest that age-related EBV+ B-cell LPDs constitute a distinct group, and innovative therapeutic strategies such as EBV-targeted T-cell therapy should be developed for this uncommon disease.

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Received 11/28/06; revised 5/8/07; accepted 6/21/07.

Grant support: Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan and in part by the Health and Labor Science Research Grants.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-06-2823

Diffuse large B-cell lymphoma (DLBCL) is the largest category of aggressive lymphomas and regarded as a heterogeneous group of lymphomas in terms of clinicopathologic profiles and biological properties (1). Recent advance in the lymphoma research shed the light on the distinct subgroups such as *de novo* CD5+ DLBCL (2), intravascular large B-cell lymphoma (Asian variant; ref. 3), primary effusion lymphoma (4), and pyothorax-associated lymphoma (5) under the nosologic term of DLBCL. In addition, we have recently identified a series of elderly patients of EBV+ B-cell lymphoproliferative disorders (LPD) and/or large-cell lymphomas without predisposing immunodeficiencies and named those senile or age-related EBV+ B-LPDs (6).

EBV is a ubiquitous γ-herpesvirus that infects more than 90% of worldwide adult population (7, 8). In contrast to its high prevalence, EBV is also well recognized as an apparent oncogenic agent (9). It transforms B cells into lymphoblastoid cell lines *in vitro*, and many human cancers, including Burkitt lymphoma (BL) (10), Hodgkin lymphoma (7), immunodeficiency-associated LPDs (11), and a part of diffuse large B-cell lymphoma (12), have close relation with EBV. Although the precise mechanism is not fully clarified, it is widely accepted that the T cell plays a crucial role for the suppression of EBV-associated oncogenesis (7). In fact, the use of strong

immunosuppressive agents in organ transplantation settings such as tacrolimus or cyclosporin A, or HIV infection, sometimes causes EBV-positive B-cell LPDs (13, 14). Four clinical settings of immunodeficiency associated with an increased incidence of lymphoma and other LPDs are recognized by the WHO classification: (*a*) primary immunodeficiency syndromes and other primary immune disorders; (*b*) infection by the HIV; (*c*) iatrogenic immunosuppression in patients who have received solid organ or bone marrow allografts; and (*d*) iatrogenic immunosuppression associated with methotrexate treatment, most commonly in patients with autoimmune disease (15).

We have highlighted the over-profile of senile EBV+ B-cell LPDs appearing analogous in many respects to that of immunodeficiency-associated B-cell LPDs, which were exemplified by a marked propensity to involve extranodal sites and a morphologic spectrum ranging from the precursor polymorphous proliferation of lymphoid cells to diverse types of lymphomas, although no evidence of underlying immunodeficiency was found (6). Therefore, it is speculated that this disease is related to an immunologic deterioration derived from the aging process, i.e., senescence in immunity. However, the detailed clinicopathlogic features and follow-up information of age-related EBV+ B-cell LPDs remain limited because of an inclusion of a small number of patients and the lack of the comparison with EBV-negative DLBCL. To address these issues further, we retrospectively assessed the clinicopathologic features of 96 cases with age-related EBV+ B-cell LPDs as a collaborative study.

Materials and Methods

Diagnosis. The diagnosis of age-related EBV-associated B-cell LPDs was made when more than 50% of the proliferating, often neoplastic-appearing cells showed both of the expression of one or more pan–B-cell antigens (CD20/CD79a) and/or light-chain restriction and positive signal for *in situ* hybridization using EBV-encoded small nuclear early region (EBER) oligonucleotides on paraffin section (Fig. 1) for patients more than 40 years of age without predisposing immunodeficiency such as HIV infection or past history of immunosuppressive agents (6) The cases <40 years old were excluded because we could not deny the possibility that they may be associated with any primary immune disorder or chronic active EBV infection (16, 17). In addition, pyothorax-associated lymphoma and EBV-associated lymphomas of T- or natural killer-cell phenotype were excluded from the present series because they were considered to constitute distinct clinicopathologic groups (5, 18). In particular, attention was given to the differential diagnoses of peripheral T-cell lymphoma with Reed-Sternberg–like cells of B-cell or angioimmunoblastic T-cell lymphoma with proliferation of large B cells (19). Only well-documented cases that had paraffin sections available for immunohistochemistry were included in this study. Each case was reviewed by five pathologists (authors K.T., K.O., N.N. T.Y., and S.N.) to confirm the diagnosis and immunophenotype. Among 149 cases fulfilling these criteria (Supplementary Table S1), 96 cases with available clinical data set were enrolled for the present study, including the 22 cases of senile EBV+ B-cell LPDs previously reported by us (6). For the control group, 107 patients aged over 40 years with EBV-negative DLBCL were selected from malignant lymphoma cases treated consecutively at Aichi Cancer Center between 1993 and 2000. This study was done by following the Ethical Guidelines for Clinical Studies and the Ethical Guideline for Epidemiological Research in Japan. The Institutional Review Board of the Aichi Cancer Center and the other institutes involved approved this study.

Histopathology. Tissue samples were fixed in 10% formalin and embedded in paraffin. Sections (5 μm thick) were stained with H&E, Elastica-van Gieson, silver impregnation, periodic acid-Schiff, May-Gruenwald-Giemsa, and methyl green-pyronine staining.

Immunohistochemistry. Immunoperoxidase studies were done on formalin-fixed paraffin sections with the avidin-biotin peroxidase complex method. A panel of monoclonal antibodies against human immunoglobulin light and heavy chains, CD3, CD8, UCHL-1/CD45RO, L26/CD20, Ber-H2/CD30, CD79a, latent membrane protein-1 (LMP-1), EBV-encoded nuclear antigen-2 (EBNA2; DAKO); CD2, CD4, CD5, CD56 (Novocastra Laboratories); LeuM1/CD15, Leu7/CD57

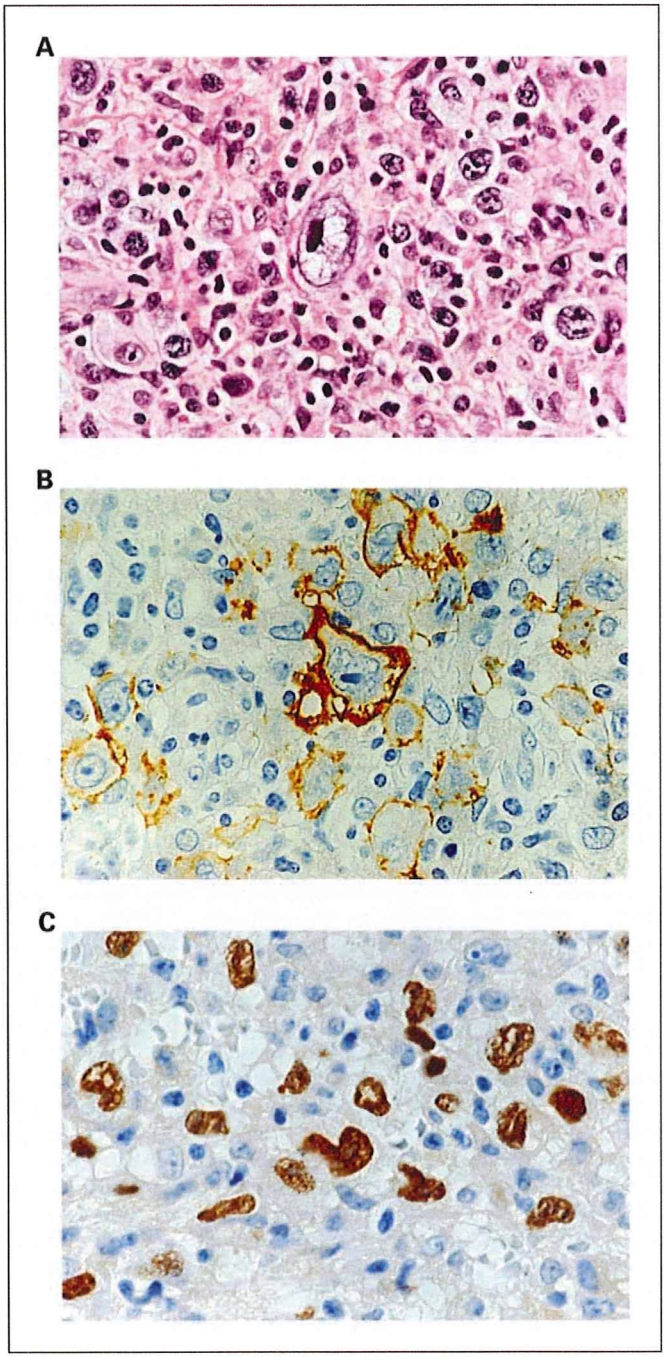

Fig. 1. Senile EBV-associated B-cell LPD, polymorphic subtype, arising in a 62-year-old male. The lesion reveals scattered distribution of Hodgkin and Reed-Sternberg – like giant cells (*A*, ×150), which are positive for CD20 (*B*, ×125). These large cells showed the expression of EBNA2 (*C*, ×125) in addition to the positive signals for EBV-encoded small RNAs (EBERs) *in situ* hybridization, indicating latency III status.

Table 1. Patient characteristics at diagnosis of age-related EBV-positive B-LPDs and EBV-negative DLBCL

Variable	Age-related EBV-positive LPD (n = 96)	EBV-negative DLBCL (n = 107)	P
Sex (male/female)	56/40 (1.4)	54/53 (1.02)	0.26
Age, median (range), y	71 (45-92)	62 (41-85)	<0.0001
	Number of cases (%)	Number of cases (%)	
Older than 60	79 (82%)	56 (52%)	<0.0001
ECOG PS 2-4	36 (44%)	18 (17%)	<0.0001
B-symptoms, presence	38 (49%)	18 (20%)	<0.0001
LDH level high	47 (58%)	46 (43%)	0.041
Ann Arbor stage III/IV	48 (58%)	49 (46%)	0.10
Extranodal involvement (>1 site)	28 (33%)	30 (28%)	0.43
Extranodal sites	n = 93	n = 107	
Skin	12 (13%)	5 (5%)	0.037
Lung	8 (9%)	3 (3%)	0.073
Pleural effusion	8 (9%)	5 (5%)	0.26
Stomach	8 (9%)	14 (13%)	0.31
Tonsil	7 (8%)	20 (19%)	0.021
Breast	0 (0%)	7 (7%)	0.012
IPI, High intermediate/high	43 (54%)	39 (37%)	0.017
Anti-EBV antibody titer category,*	18 (67%)	23 (24%)	<0.0001
Treatment			<0.0001
None or radiation only	9 (12%)	1 (1%)	
Ctx without anthracycline	7 (9%)	2 (2%)	
Ctx with anthracycline	62 (79%)	104 (97%)	
Response, in cases underwent Ctx with anthracycline			<0.0001
CR	37 (66%)	93 (91%)	
PR	8 (14%)	8 (8%)	
SD or PD	11 (20%)	1 (1%)	

Abbreviations: PS, performance status; LDH, lactate dehydrogenase; IPI, International Prognostic Index; Ctx, chemotherapy; CR indicates complete response; PR, partial response; SD, stable disease; PD, progressive disease.
*Cases were determined as having abnormal serum anti-EBV antibody titer if anti-EBV viral capsid antigen antibody was 640-fold or higher, or anti-EBV nuclear antigen antibody was negative.

(Becton Dickinson); TIA-1 (Coulter Immunology); and granzyme B (Monosan) were used. All antibodies were applied after antigen retrieval following microwave oven heating treatment.

In situ *hybridization study.* The presence or absence of EBV small RNAs was assessed by means of *in situ* hybridization using EBER oligonucleotides and done on formalin-fixed paraffin embedded sections. Briefly, a DAKO hybridization kit was used with a cocktail of FITC-labeled EBER oligonucleotides (one oligonucleotide corresponding to EBER1 and one to EBER2, both 30 bases long; DAKO A/S code Y 017). Hybridization products were detected with mouse monoclonal anti-FITC (DAKO M878) and a Vectastain ABC Kit (Vector). RNase A or DNase I pretreatment was used for the negative controls and EBER-positive Hodgkin's disease specimens for positive controls.

Statistical analysis. Variables related to patients, treatment, and disease were compared among the two groups with the use of the χ^2 test or Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. The probability of survival was calculated with the use of the Kaplan-Meier estimator, and the log-rank test was used for comparisons. Univariate and multivariate analyses were done with the Cox proportional hazard regression model. All P values are two sided, with a type I error rate fixed at 0.05. Statistical analyses were done with the STATA version 9.

Results

Case selection. From the files of six collaborating institutions, during the period from January 1990 to December 2004, the positive signals for B-cell [pan-B-cell antigens (CD20/CD79a) and/or light-chain restriction] and EBV were detected on more than 50% of cells in 243 (14%) of 1,792 large B-cell LPD cases, mainly consisting of DLBCL, by EBERs *in situ* hybridization. They contained HIV-associated lymphomas (n = 17), autoimmune disease-associated LPDs (n = 10), secondary lymphoma with prior chemotherapy (n = 7), post-transplant LPDs (n = 10), pyothorax-associated lymphoma (n = 30), BL (n = 13), and cases without any documentation for predisposing immunodeficiency (n = 156; Supplementary Table S1). EBV was detected in 10% of HIV-negative patients with BL in this study, which was comparable to the reported frequency in nonendemic BL (20). A bimodal age distribution with an incidence peak in the 10- to 19-year range and a second peak in older adult aged 70 to 79 was evident for EBV-positive B-cell LPD patients without predisposing immunodeficiency (Supplementary Fig. S1*A*). The positive percentages of this group for all cases examined became higher in parallel with the elder patient populations (≥40 years), showing the highest peak at ages >90 years (Supplementary Fig. S1*B*).

Patient characteristics for age-related EBV-positive B-cell LPDs and EBV-negative DLBCL. In comparison with EBV-negative DLBCL, patients with age-related EBV-positive B-cell LPDs showed higher age distribution (median, 71 versus 62 years: $P < 0.0001$) and a closer association with aggressive clinical features or parameters: 79 patients older than 60 (82%,

$P < 0.0001$), 36 with performance status (PS) >1 (44%, $P < 0.0001$), 47 with serum lactate dehydrogenase (LDH) level higher than normal (58%, $P = 0.041$), 48 with stage III/IV disease at diagnosis (58%, $P = 0.10$), and 38 with B symptoms (49%, $P < 0.0001$; Table 1). As a result, the International Prognostic Index (IPI) score for patients with age-related EBV-positive B-cell LPDs was significantly higher than that for patients with EBV-negative DLBCL ($P = 0.0017$), with 43 (54%) of the EBV-positive group categorized in the IPI high or high intermediate-risk group. There was no statistical difference between two groups in the incidence of having more than one extranodal site.

At diagnosis, 67% of the cases with age-related EBV-positive B-cell LPDs showed abnormal anti-EBV antibody titer, which was defined if anti-EBV VCA immunoglobulin G (IgG) antibody was 640-fold or higher, or anti-EBNA antibody was negative, as compared with only 24% of cases with DLBCL that showed abnormality ($P < 0.0001$).

Sites of extranodal involvement. In 17 patients (20%) of the current EBV-positive series, the disease was limited to extranodal sites. Twenty-seven patients (31%) had only lymphadenopathies without extranodal involvement, and the remaining 43 (49%) had lymphadenopathies with extranodal involvement. The total incidence of extranodal involvement was similar between age-related EBV-positive B-cell LPDs and EBV-negative DLBCL (69% and 72%, respectively).

The main sites of extranodal involvement in age-related EBV-positive B-cell LPDs was skin ($n = 12$; 13%), lung ($n = 8$; 9%), pleural effusion ($n = 8$; 9%), stomach ($n = 8$; 9%), and tonsil ($n = 7$; 8%) in an order of the incidence (Table 1). A comparison with EBV-positive and EBV-negative groups showed that the incidence of cutaneous involvement was significantly higher in age-related EBV-positive B-cell LPDs than those of EBV-negative DLBCLs ($P = 0.027$, respectively). There is a tendency of difference in lung involvement, but no statistical significance (9% versus 3%, $P = 0.073$). Involvement of breast and tonsil occurred less frequently in age-related EBV-positive B-cell LPDs than in EBV-negative DLBCL ($P = 0.012$ and.021, respectively). There were no significant differences between these two groups in the incidence of involvement in the other extranodal sites (Supplementary Table S2).

Histologic features. Age-related EBV-positive LPDs generally showed a diffuse and polymorphic proliferation of large lymphoid cells with a varing degree of reactive components such as small lymphocytes, plasma cells, histocytes, and epithelioid cells and were sometimes accompanied by necrosis and an angiocentric pattern. These tumor cells were often featured by a broad range of B-cell maturation, containing morphologic centroblasts, immunoblasts, and Hodgkin and Reed-Sternberg (HRS)-like giant cells with distinct nucleoli (Fig. 1*A*). According to the previous report (6), the present series were morphologically divided into two subtypes: large-cell lymphoma (LCL) and polymorphic LPD subtypes. The former ($n = 34$) was characterized by having areas where large lymphoid cells with relatively monomorphic appearance were notably dominant. The remaining 62 cases were simply categorized as polymorphous subtype with the scattered distribution of large cells in the polymorphous composition. The histology was frequently varied from area to area, indicating a continuous spectrum between these two subgroups because several LCL cases had areas of polymorphic LPD in the same or other tissues. In contrast to morphologic divergence, there was no significant difference in any clinical characteristics and immunophenotype between these two groups (Supplementary Table S3).

We detected clonal B-cell population in 10 cases out of 12 cases tested: eight cases by PCR analysis, one case by Southern blot analysis, and one by lambda light-chain restriction. Polyclonal pattern was observed in one case, and no band was detected in the other. As to polymorphic LPDs, the presence of clonal B-cell population was identified in five cases out of seven samples.

Phenotypic features. According to the definition adopted for this study, all patients with age-related EBV-positive B-cell LPDs were positive for EBV and B-cell markers (CD20 and/or CD79a; Fig. 1*B*). Immunohistologic studies for the EBV-latent gene products on paraffin sections showed that LMP1 was positive on the large atypical cells in 67 (94%) out of 71 tested cases. EBNA2 was also detected in the nuclei of 16 (28%) of 57 tested cases (Fig. 1*C*, Supplementary Table S4), indicating latency type III. CD30 was stained more common in age-related EBV-positive B-cell LPDs than in EBV-negative DLBCL (75% versus 13%, $P < 0.0001$). In addition, a comparison of adjacent sections often disclosed an overlapping staining pattern of LMP1 and CD30. There was also a statistically significant difference in the incidence of CD10 expression (18% and 38%, respectively. $P = 0.015$), but not others (CD19, CD20, or CD79a) between age-related EBV-positive B-cell LPDs and DLBCLs (Supplementary Table S4).

Response to treatment and Kaplan-Meier survival estimates. Treatment of age-related EBV-positive B-cell LPDs consisted of chemotherapeutic regimens containing anthracycline for 62 patients (79%) and without anthracycline for 7 patients (9%; Table 1). A total of 40 (63%) of 64 evaluable patients with age-related EBV-positive B-cell LPDs achieved a complete remission (CR) with initial therapy, and the rest of the 24 cases (38%) failed to have a CR with initial chemotherapy. On the other hand, 95 (91%) of 104 evaluable cases with DLBCL achieved a CR, and only 9 cases (9%) were refractory (PR, SD, or PD) to initial chemotherapy ($P < 0.0001$). This difference, in response to treatment, was still in a significant level when compared in cases who received chemotherapy with anthracycline ($P < 0.0001$, Table 1).

In this study, we observed 57 deaths in 96 cases of age-related EBV-positive B-cell LPDs and 34 deaths in 107 cases of DLBCL. The data on the causes of death were available for 47 cases for age-related EBV-positive B-cell LPDs and 29 for DLBCL. Deaths due to disease progression and complications such as infections were observed in 38 and 9 cases, respectively, in age-related EBV-positive B-cell LPDs, whereas 23 and 6 cases in EBV-DLBCL. The observed differences between two disease groups were not significant ($P = 0.870$). As to the cases of more than 70 years of age, 24 and 5 cases were dead due to disease progression, and seven and one were from complications in age-related EBV-positive B-cell LPDs and in DLBCL, respectively. Even in cases more than 70 years old, the observed differences were not significant ($P = 0.747$).

Unadjusted overall survival curves of both groups were shown in Fig. 2*A*. Age-related EBV-positive B-cell LPDs showed strikingly inferior survival to DLBCLs (median survival time, 24 months versus not reached, respectively;

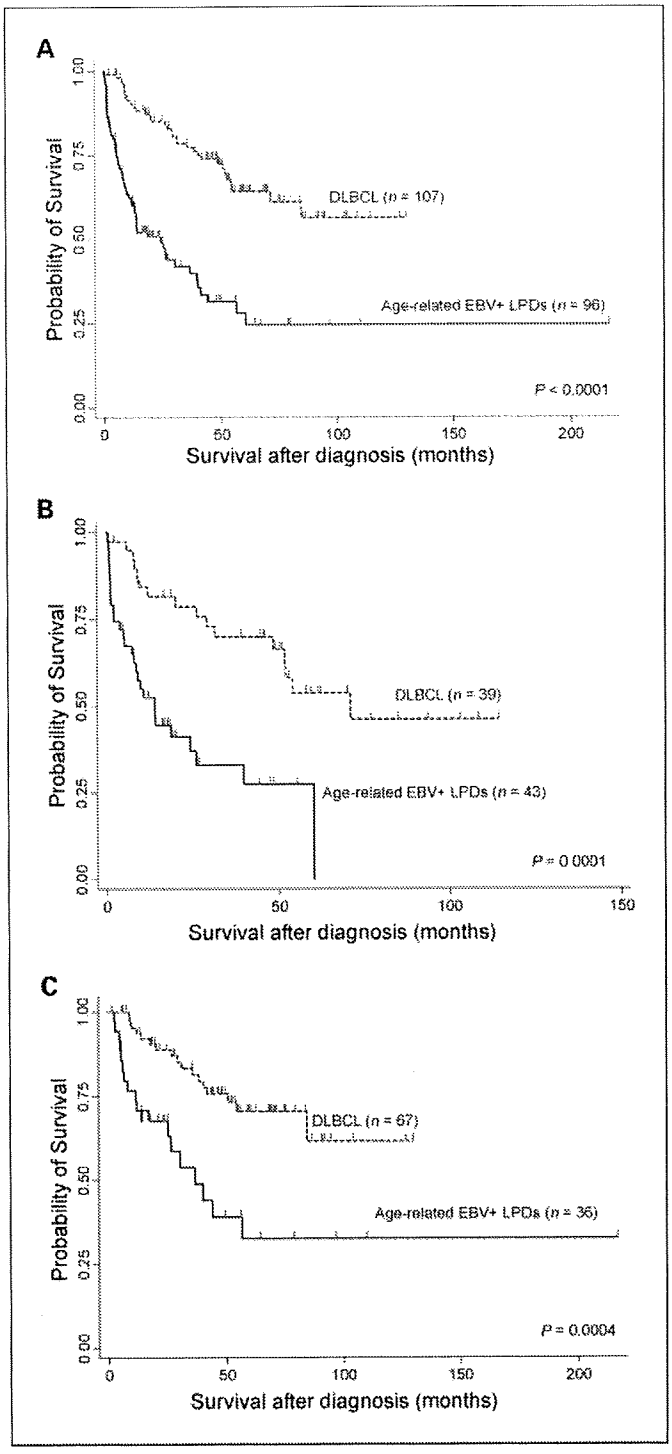

Fig. 2. Overall survival for patients with age-related EBV+ B-cell LPDs and EBV-negative DLBCLs. Age-related EBV+ B-cell LPDs (n = 96) show significantly worse survival than DLBCLs (n = 107) in all patients (*A*), patients with high-intermediate and high IPI risk (n = 43 and n = 39, respectively; *B*), and patients with low and low-intermediate IPI risk group (n = 36 and n = 67, respectively; *C*).

$P < 0.0001$). A significant difference was still found even when accounting for age (age $\leq$ 60, 60 < age $\leq$ 75, or age > 75) by performing the stratified log-rank test ($P < 0.0001$). Overall survival curves according to IPI are shown in Fig. 2*B* and *C*. Survival for age-related EBV-positive B-cell LPDs was significantly inferior to that for DLBCLs in both IPI subgroups. In this series, the IPI failed to separate age-related EBV+ B-cell LPD patients into groups with significantly different survivals (P = 0.1; Fig. 3*A*).

Univariate and multivariate analysis for survival. Among a total of 203 patients with EBER-positive (age-related EBV-positive B-cell LPDs) and EBV-negative diseases (DLBCLs), univariate Cox analysis identified the following as prognostic factors: age > 60 years, clinical stage, PS, extranodal involvement of more than one site, LDH, IPI, B symptoms, and EBV association (Table 2). Multivariate analysis, including five IPI factors, B symptoms, and EBV association, showed high LDH level, the presence of B-symptoms, and EBV association to be significant factors (Table 2). When multivariate analysis was done for EBV association and IPI categories, both of them were recognized as independent significant prognostic factors (Table 2).

Among patients with age-related EBV+ B-cell LPDs, the clinical parameters associated with reduced survival in univariate analysis are listed in Table 3: age older than 70

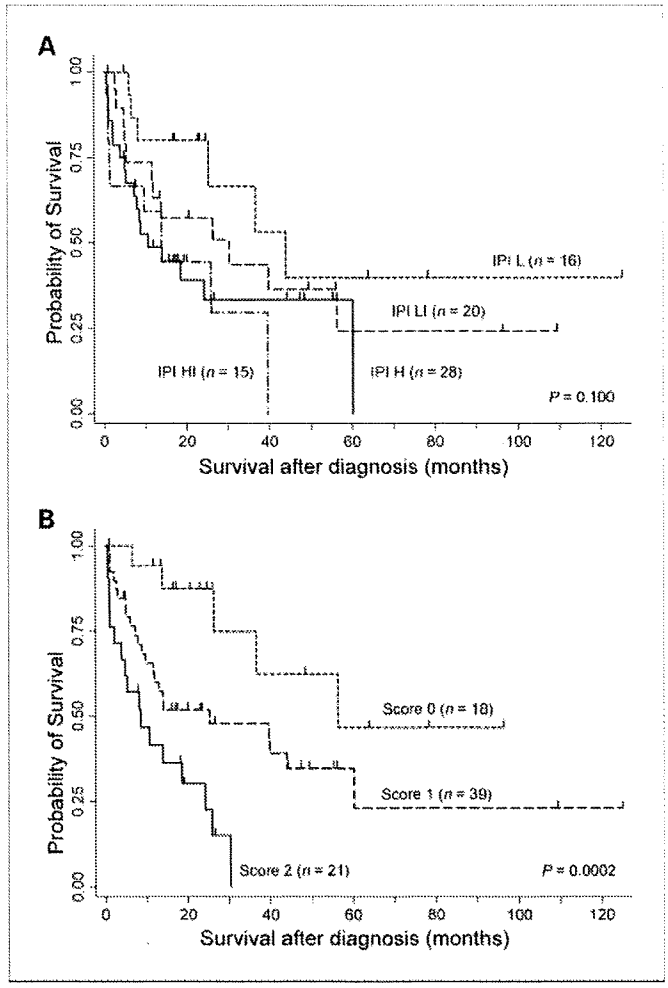

Fig. 3. Overall survival according to IPI (*A*) and prognostic model based on two simple clinical variables of age older than 70 y and the presence of B symptoms (*B*) in age-related EBV+ B-cell LPDs. This prognostic model is able to efficiently identify three groups of patients with different outcomes; patients with a score of 0 (*Score 0*, n = 18), no adverse factors; patients with a score of 1 (*Score 1*, n = 39), one factor; and patients with a score of 2 (*Score 2*, n = 21), two factors. Their median survival times were 56.3, 25.2, and 8.5 mo, respectively.

Table 2. Prognostic factors affecting overall survival of total entry series

Variables	Unfavorable factors	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Comparison with risk factors					
EBV	Positive	3.5 (2.3-5.5)	<0.0001	2.5 (1.5-4.1)	0.001
B symptom	Present	3.2 (2.0-5.1)	<0.0001	2.0 (1.2-3.5)	0.008
LDH	>normal	2.6 (1.6-4.1)	<0.0001	2.0 (1.2-3.4)	0.011
PS	2-4	2.4 (1.6-3.8)	<0.0001	—	—
Age	>60 y	2.0 (1.2-3.1)	0.006	—	—
Stage	III/IV	1.8 (1.1-2.8)	0.010	—	—
Extranodal disease	>1 site	1.5 (0.9-2.3)	0.083	—	—
Comparison with IPI category					
IPI	HI/H	2.1 (1.4-3.3)	0.001	2.0 (1.3-3.1)	0.003
EBV	Positive			3.3 (2.1-5.3)	<0.0001

Abbreviations: CI, confidential interval; LDH, lactate dehydrogenase; PS, performance status; IPI, International Prognostic Index.

years (P = 0.0008), the presence of B symptoms (P = 0.0058), and LDH level equal to or more than normal value (P = 0.040). Clinical stage, PS, and extranodal involvement of more than one site were nonsignificant factors. In multivariate analysis, the factors that turned out to correlate significantly with survival were B symptom (P = 0.0026) and age (P = 0.0045). Because the relative risk associated with each of the two factors was comparable, we constructed a prognostic model by combining these prognostic variables in the following way: patients with a score of 0 (n = 18), no adverse factors; patients with a score of 1 (n = 39), one factor; and patients with a score of 2, two factors (n = 21). This prognostic model for age-related EBV+ B-cell LPDs was able to efficiently identify three groups of patients with different outcomes (Fig. 3*B*; P < 0.0001). For the patients with scores of 0, 1, and 2, the median overall survival times were 56.3, 25.2, and 8.5 months, respectively.

Discussion

We recently have documented 22 cases named as senile EBV-associated B-cell LPDs arising in elderly patients aged ≥60 years without predisposing immunodeficiencies, suggesting that this disease has a relationship with an immunologic deterioration derived from the aging process (6). Among 1,792 large B-cell LPD cases examined by EBERs *in situ* hybridization, 156 cases harbored EBV without underlying immunodeficiency-related diseases. This larger series revealed that 149 (96%) of these patients are more than 40 years of age, the increasing positive percentages of which were observed in parallel with the elder patient populations (≥40 years) for all cases examined and reached the highest peak at ages ≥90 years. These data provided additional evidence that EBV-positive B-cell LPDs without predisposing immunodeficiency mainly occur in elderly patients, although seven patients were found to be <40 years of age. Considering these rare cases, the term of "age related" may be more appropriate than that of senile for further understanding the overall age distribution of EBV-positive B-cell LPDs without predisposing immunodeficiency.

This study was predominantly a comparison of clinical features in age-related EBV+ B-cell LPDs and EBV-negative DLBCLs. An analysis of 96 patients with age-related EBV-positive B-cell LPDs, in which the clinical data were available, highlighted the clinical features of this disease—high age at onset, frequent association with poor prognostic components of IPI, and aggressive clinical course. These features were significantly different from those of EBV-negative DLBCL besides more frequent involvement of the skin, supporting the concept that age-related EBV-associated B-cell LPDs constitute a distinct disease with a broad spectrum. However, it could not be definitively concluded whether this disease

Table 3. Prognostic factors affecting overall survival of age-related EBV-positive B-cell LPDs

Variables	Unfavorable factors	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
B symptoms	Present	2.3 (1.3-4.3)	0.0058	2.6 (1.4-4.8)	0.0026
Age	>70 y	2.4 (1.4-4.3)	0.0008	2.5 (1.3-4.8)	0.0045
LDH	>normal	1.9 (1.0-3.4)	0.040	—	—
Stage	III/IV	1.8 (1.0-3.2)	0.062	—	—
PS	2-4	1.2 (0.7-2.1)	0.57	—	—
Extranodal disease	>1 site	1.3 (0.7-2.3)	0.38	—	—
IPI category	HI/H	1.8 (1.0-3.2)	0.064	—	—

Abbreviations: LPDs, lymphoproliferative disorders; CI, confidence interval; EBV, Epstein-Barr virus; LDH, lactate dehydrogenase; PS, performance status; IPI, International Prognostic Index.

represented a heterogeneous group of disorders including several lymphoma subtypes.

The morphologic spectrum of age-related EBV+ B-cell LPDs seems to be broader than has been previously realized (data not shown). This disease comprised a spectrum ranging from polymorphic proliferation, sometimes suggestive of a reactive process, to large-cell lymphomas mostly consisting of transformed cells and, therefore, was subdivided into two subtypes, i.e., polymorphic and large-cell lymphomas, based on morphology and conventional immunophenotyping in our previous report (6). However, in the present study, we failed to show any statistical difference in the clinical profiles between these two subgroups. Indeed, several cases had areas that seem more monomorphic in the same or other tissues, thus indicating a continuous spectrum between polymorphic and large-cell lymphoma subtypes. The results that we found in the histologic subgrouping of age-related EBV+ B-cell LPDs seemed to parallel those of the post-transplant LPDs, in which current classification schemes are not fully predictive of prognosis (15, 21). Further investigation should be done to refine the distinction of age-related EBV+ B-cell LPDs into more homogeneous categories with prognostic relevance.

The prognosis of age-related EBV+ B-cell LPDs was significantly poorer than that of EBV-negative tumors. One possible explanation is that the EBV association as a biological marker seemed to be closely associated with the higher IPI index because 35% of patients with this disease were categorized in the high-risk IPI group, which is higher than 15% of the present series of EBV-negative DLBCL or 19% of DLBCL reported by the Non-Hodgkin's Lymphoma Classification project (22, 23). The other is the age distribution and performance status of the patients (Table 1). Due to higher age or poorer PS, many patients with age-related EBV-positive B-cell LPDs might not maintain the intensity of chemotherapy. However, subgroup analyses by age or the IPI also showed that age-related EBV-positive B-LPDs had lower CR rate and inferior overall survival compared with EBV-negative DLBCLs. Multivariate analysis in all cases further identified EBV association and IPI category as an independent prognostic factor. These findings emphasized that age-related EBV-positive B-cell LPDs merits separate consideration because of the diagnostic and therapeutic problems it poses.

Indeed, in multivariate analysis, two host-related factors, i.e., age older than 70 years and the presence of B symptoms, were prognostically significant. In the present series of age-related EBV+ B-cell LPDs, the IPI scoring system did not seem to work with the same efficacy as in DLBCLs for identifying subsets of patients with different prognoses. However, the extension of the disease (clinical stage and extranodal involvement of more than one site) and the biology or cell turnover of the tumor (LDH level) were no longer significant. These findings further supported our assertion that this disease is distinct from DLBCLs and significantly influenced by the host immune status in outcome of patients. Our prognostic model based on the two simple clinical variables of age older than 70 years and the presence of B symptoms also seemed to better define the clinical outcome of age-related EBV+ B-cell LPDs categorized as a single group with an overall superior predictive capacity as compared with IPI (log-rank, 0.0002 versus 0.1). Of course, an external validation study should be done on the larger series of cases in the future.

It is presumed that the pathogenesis of age-related EBV-positive B-cell LPDs has a close relation with an immunologic deterioration or senescence in immunity derived from the aging process because this disease seemed analogous in many respects to that immunodeficiency-associated LPDs, such as EBV association, waxing and waning of disease, and polymorphic proliferation of large bizarre B cells (16). Aging in humans is known to be associated with impaired immune status such as increased infections, the more global phenomenon termed "immune senescence" (24). Indeed, in the present series, 28% of the age-related EBV+ B-cell LPD cases examined were immunohistochemically positive for EBNA2, indicating the reduced immunity to EBV, i.e., type III latency which is believed to occur only in the setting of profound immunodeficiency (25). EBV DNA in peripheral blood mononuclear cells was more frequently detected in healthy individuals older than 70 years of age (8 of 9, 89%) than in ones <70 years (1 of 11, 9%) using real-time PCR (26). Yanagi et al. also showed that EBNA-2 IgG antibodies evoked in young children by asymptomatic primary EBV infections remain elevated throughout life using sera, suggesting the intervention of reactivation of latent and/or exogenous EBV superinfection (27). These data provided additional support on the speculation that age-related decline in immunity may be contributing to the pathogenesis of age-related EBV+ B-cell LPDs.

Biological interfaces may be assumed between age-related EBV+ B-cell LPDs and other EBV-associated B-cell neoplasms such as lymphomatoid granulomatosis and plasmablastic lymphoma, the distinction of which is currently based on the constellation of clinical, morphologic, and immunophenotypic features (28, 29). In our series, nine cases showed pulmonary involvement and four ones had gingival lesions at presentation, posing the differential diagnostic problems from lymphomatoid granulomatosis and plasmablastic lymphoma, respectively, although they were not prototypic in morphology as the latter two. Classic Hodgkin lymphoma (CHL) is also well known to have EBV harboring in 30% to 50% of the cases with achieving a general consensus of the B-cell derivation of the H-RS cells in most (30, 31). Interestingly, three population-based studies of Clarke et al. (32), Stark et al. (33), and more recently, Jarrett et al. (34), without selection bias documented that a marked survival disadvantage in older EBV-positive CHL patients as compared with EBV-negative CHL cases, which was contrasted with no effect of EBV status on the clinical outcome of HL patients selectively enrolled in clinical trails, with a tendency of their relatively younger age distribution (35, 36). As the interpretation for this age-related influence of EBV on clinical outcome of CHL patients, Gandhi et al. (37) and Jarrett et al. (34) clearly indicated that a decline in cellular immunity to EBV with age may contribute to the pathogenesis of EBV+ CHL in older patients. This standpoint is tempting to speculate that EBV+ CHL and age-related EBV+ B-cell LPDs may constitute a continuous spectrum. Our study may also raise an even more fundamental question: whether biological properties, such as an interaction or balance between latent EBV infection and host immunity, precede the morphologic and immunophenotypic evaluation for further understanding the overall clinicopathologic profiles of EBV-associated B-cell LPDs and/or lymphomas. Much still needs to be learned about the detailed clinicopathologic

features, the immunology, and the molecular biology of these diseases in a further study.

Innovative therapeutic strategies such as immunotherapy against EBV should be explored for age-related EBV+ B-cell LPD patients (38, 39), because conventional combination chemotherapy had only a limited effect in an analysis of this larger series. For poor risk patients with aggressive lymphomas such as DLBCL, the superiority of high dose chemotherapy with stem cell support over conventional method is now under confirmation (40–42). This therapeutic approach may not, however, be suitable for age-related EBV+ B-cell LPDs because the older age distribution of the patients, many (70%) of which were more than 65 years old, made the application of high-dose chemotherapy difficult enough. Rituximab is a non-cytotoxic drug that showed efficacy when adding to cyclophosphamide-Adriamycin-vincristine-prednisone (CHOP) on elderly patients with DLBCL (43). In our present series, only one case was documented to have received chemotherapy combined with rituximab for an initial treatment, preliminarily providing a good efficacy of this agent on age-related EBV+ B-cell LPD. Now, we are conducting prospective clinical trials to test the efficacy of chemotherapy with rituximab as a multi-institutional study on age-related EBV+ B-cell LPD patients.

In conclusion, the current study elucidates that age-related EBV-associated B-cell LPDs constitute a distinct clinicopathologic group in contrast with EBV-negative DLBCLs, in which conventional chemotherapy has a limited efficacy for this disease. A study to test the efficacy of rituximab with chemotherapy for age-related EBV+ is now ongoing. In the future, less toxic treatment strategy such as a cell therapy for EBV-specific viral antigens will be needed and should be evaluated in clinical trials.

Acknowledgments

The authors are grateful to Dr. Masao Seto for his scientific discussion and encouragement to prepare this manuscript.

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bjh research paper

Chronic graft-*versus*-host disease after allogeneic bone marrow transplantation from an unrelated donor: incidence, risk factors and association with relapse. A report from the Japan Marrow Donor Program

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Summary

Chronic graft-*versus*-host disease (GVHD) remains the major cause of late morbidity and mortality after allogeneic stem cell transplantation. We retrospectively analysed 2937 patients who underwent bone marrow transplantation from an unrelated donor (UR-BMT) facilitated by the Japan Marrow Donor Program (JMDP) and survived beyond day 100 after transplantation. The cumulative incidence of chronic GVHD (limited + extensive) or extensive chronic GVHD at 5 years post-transplant was 45·8% and 28·2%, respectively. On multivariate analysis, seven variables predicting chronic GVHD were identified: recipient age over 20 years, donor age over 30 years, primary diagnosis of chronic myeloid leukaemia, human leucocyte antigen (HLA)-A or -B mismatch, total body irradiation-containing regimen, platelet count not having reached 50×10^9/l by day 100, and prior acute GVHD. Among 2609 patients with haematological malignancy, overall survival was significantly higher in patients with limited chronic GVHD but lower in patients with extensive chronic GVHD compared with those without chronic GVHD. The cumulative incidence of relapse among patients with limited or extensive chronic GVHD was significantly lower than that among patients without chronic GVHD. Our results suggest that limited chronic GVHD provides a survival benefit to patients with haematological malignancies by reducing the risk of relapse without increasing the risk of death from chronic GVHD.

Keywords: chronic graft-*versus*-host disease, unrelated bone marrow transplantation, Japan Marrow Donor Program, relapse, graft-*versus*-leukaemia effect.

Received 16 October 2006; accepted for publication 23 January 2007

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†A complete list of the centres that participated in the bone marrow transplantations facilitated by the Japan Marrow Donor Program (JMDP) appears in Appendix 1.

Haematopoietic stem cell transplantation (HSCT) has become established as one of the curative therapies for haematological malignancies and other haematological or immunologic disorders (Armitage, 1994). However, various late complications of HSCT rather than relapse decrease the quality of life of HSCT recipients (Socie *et al*, 1999; Kiss *et al*, 2002). Among late complications that may occur beyond 100 d post-transplant, chronic graft-*versus*-host disease (GVHD) affects approximately 30–70% of long-term survivors depending on the degree of human leucocyte antigen (HLA)-mismatch with the donor and the source of the stem cells, and remains a major cause of late morbidity and mortality post-transplantation (Atkinson *et al*, 1990; Sullivan *et al*, 1991; Vogelsang, 2001; Lee *et al*, 2002; Farag, 2004). Despite improvements in other areas of supportive care, little significant progress has been made in the management of chronic GVHD (Vogelsang, 2001). Patients with chronic GVHD have decreased performance status, impaired quality of life, and increased risk of mortality (Duell *et al*, 1997; Socie *et al*, 1999). In spite of its adverse effects, chronic GVHD is associated with a lower incidence of leukaemia relapse by a graft-*versus*-leukaemia (GVL) effect that is comparable or greater than that ascribed to acute GVHD (Weiden *et al*, 1981; Sullivan *et al*, 1989; Kataoka *et al*, 2004).

Bone marrow transplantation (BMT) from an unrelated volunteer donor (UR-BMT) has become established as an accepted treatment for patients in need of HSCT who do not have a HLA-matched sibling donor (Kernan *et al*, 1993; Hansen *et al*, 1998; Kodera *et al*, 1999; Davies *et al*, 2000). The incidence of chronic GVHD is assumed to be higher after UR-BMT than after transplants from an HLA-matched sibling donor. Previous studies have identified the incidence and risk factors for chronic GVHD after sibling transplant (Storb *et al*, 1983; Ringden *et al*, 1985; Atkinson *et al*, 1990; Remberger *et al*, 2002); however, there are no definite data available on the incidence and risk factors for chronic GVHD among patients who have undergone UR-BMT. The Japan Marrow Donor Program (JMDP) was established in December 1991. We previously analysed the data of 1298 patients who underwent UR-BMT facilitated by the JMDP between 1993 and 1998 to identify the effect of HLA matching on acute GVHD, chronic GVHD, engraftment, survival and relapse (Morishima *et al*, 2002). In that study, HLA-A and/or HLA-B allele mismatch and patient age were found to be significant risk factors for the occurrence of chronic GVHD. The current study extended the analysis to include the data of 2937 patients who underwent UR-BMT facilitated by the JMDP between January 1993 and June 2004 and survived for at least 100 d post-transplant to clarify the incidence and risk factors for chronic GVHD, and the effect of chronic GVHD on survival and relapse in UR-BMT recipients.

Patients and methods

Patients and transplant procedure

Between January 1993 and June 2004, 2937 Japanese patients who underwent UR-BMT through the JMDP, engrafted and survived for at least 100 d after UR-BMT were included in this analysis. We excluded patients who survived <100 d after UR-BMT to exclude the effect of early mortality. Because peripheral blood stem cell harvest has not been performed through the JMDP, all transplants were BMTs. Baseline characteristics and follow-up data were obtained using standard report forms designed by the JMDP. Follow-up reports were submitted at 100 d, 1 year, and annually thereafter post-transplantation. A final clinical survey of these patients was performed on 1 November 2004. The median follow-up time was 822 d (range, 100–4129 d). Informed consent was obtained from the patients and donors according to the Declaration of Helsinki.

The characteristics of the patients and donors are summarised in Table I. The median age of the patients was 27 years and the median age of the donors was 33 years. As much as 59·7% of the patients and 59·5% of the donors were male. The number of patients with a haematological malignancy was 2667 (90·8%). Transplantation was performed according to the protocol of each centre, and therefore the conditioning regimen and GVHD prophylaxis varied among patients. A conditioning regimen containing anti-thymocyte globulin (ATG) was used in 203 patients (6·9%), and a conditioning regimen containing total body irradiation (TBI) was used in 2329 patients (79·3%). Only 14 patients (0·5%) received T cell-depleted marrow.

HLA matching and typing

According to the donor selection criteria of the JMDP, patients received marrow transplants from serologically HLA-A, -B and -DR antigen completely matched or serologically 1 antigen mismatched donors. Genomic typing of HLA-A, -B and -DR antigens was also performed. 68·5% of the donors were fully HLA-matched by both serological and genomic typing.

Statistical analysis

The incidence of chronic GVHD was the primary endpoint of our study. Diagnosis of chronic GVHD and its clinical grading were performed according to the standard criteria at each institution (Atkinson, 1990). Chronic GVHD was graded as limited (localised skin or single organ involvement) or extensive (generalised skin or multiple organ involvement). The cumulative incidence of chronic GVHD was calculated from the time of transplantation. To evaluate potential risk factors for developing chronic GVHD, the time-dependent

Table I. Characteristics of the patients who underwent UR-BMT and donors.

Number of patients	2937
Median age of patients, years (range)	27 (0–67)
Patient sex (male/female), *n*	1753/1184
Diagnosis, *n*	
Haematological malignancy	
AML	793
ALL	768
CML	604
MDS	285
NHL	168
Others	49
Non-malignant disease	
AA	191
Hereditary disorders	68
Conditioning, *n*	
ATG	203
TBI	2329
GVHD prophylaxis, *n*	
CsA + MTX	1545
FK506 + MTX	1118
Others	274
Median age of donors, years (range)	33 (20–52)
Donor sex (male/female), *n*	1748/1189
Sex (recipient/donor), *n*	
Male/male	1151
Male/female	602
Female/female	587
Female/male	597
HLA disparity, *n*	
Full match	2012
Class I one locus or one allele mismatch	286
Class II one locus or one allele mismatch	473
Others	166
Blood-type disparity, *n*	
Match	1535
Major mismatch	677
Minor mismatch	616
Major–minor mismatch	72
Bone marrow treatment, *n*	
No	1529
Yes	
Removal of red blood cells	764
Removal of plasma	750
T cell depletion	14
Time from diagnosis to BMT, months	
<13	1180
13–24	865
≥25	865
Median time from BMT to WBC = $1{\cdot}0 \times 10^9$/l, d (range)	17 (1–99)
Platelet count = 50×10^9/l by day 100 from BMT, *n*	
Yes	2714
No	223
Prior acute GVHD, *n*	
No	884
Grade I	915
Grade II	793
Grade III	281
Grade IV	64

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia, CML, chronic myeloid leukaemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; AA aplastic anaemia; ATG, anti-thymocyte globulin; TBI, total body irradiation; GVHD, graft-*versus*-host disease; CsA, ciclosporin A; MTX, methotrexate; FK506, tacrolimus; HLA, human leucocyte antigen; BMT, bone marrow transplantation; WBC, white blood cell count.

Cox proportional hazard regression model was used for univariate and multivariate analyses (Cox, 1972). Factors with a *P*-value of 0·2 or less in the univariate analysis were included in the multivariable analysis. Factors that remained significant were retained in the final model.

Patients were also analysed for overall survival (OS) and relapse. To illustrate the effect of chronic GVHD on relapse and survival, semi-landmark plots were constructed (Baron *et al*, 2005). In patients who developed chronic GVHD, the post-transplant day of development of chronic GVHD was defined as the landmark day; in patients who did not develop chronic GVHD, post-transplant day 112, which was the median day of occurrence of chronic GVHD, was defined as the landmark day. OS was calculated from the landmark day to death from any cause or date of last contact. Relapse was defined on the basis of evidence of the respective malignancy and its cumulative incidence was plotted as a function of time since the landmark day.

Survival analyses were performed by the Kaplan–Meier method (Kaplan & Meier, 1958) and the log-rank test was used for univariate comparisons. The cumulative incidences of chronic GVHD and relapse were calculated using the Gray method, considering death without chronic GVHD or death without relapse, respectively, as the competing risk (Gray, 1988). For most of the statistical analyses, the Statistical Package for the Social Sciences (SPSS) software version 11 (SPSS Inc., Chicago, IL, USA) was used. Analyses of cumulative incidences were carried out with package 'cmprsk' of the R statistical software 2.1.0 (the R Foundation for Statistical Computing, Vienna, Austria; available at http://www.r-project.org). All *P*-values were two-sided and differences were considered to be statistically significant when $P < 0{\cdot}05$. Differences with *P*-values > 0·10 are reported as not significant (NS), whereas differences with *P*-values between 0·05 and 0·1 are reported in detail.

Results

Incidence and severity of chronic GVHD

Among the 2937 patients, 1267 (43·1%) developed chronic GVHD, of whom 268 patients (21·2%) had *de novo* onset of

chronic GVHD. The median time to onset of chronic GVHD was 112 d following transplant. The 5-year cumulative incidence of chronic GVHD was 45·8%, and that of extensive chronic GVHD was 28·2% (Fig 1A). Fig 1B shows the cumulative incidences of chronic GVHD according to the primary diagnosis.

Risk factors for developing chronic GVHD

Multivariate analysis for risk factors for the development of chronic GVHD included the 2909 patients in whom data on the variables with $P \leq 0{\cdot}2$ in the univariate analysis were available (Table II). Recipient age ≥20 years, donor age ≥30 years, primary diagnosis of chronic myeloid leukaemia (CML), HLA-A or -B mismatch by serological or genomic typing, total body irradiation (TBI)-containing regimen, platelet count $<50 \times 10^9$/l by day 100, and prior acute GVHD remained in the optimal model on multivariate analysis and increased the risk of chronic GVHD significantly. Aplastic anaemia (AA) and hereditary disorders were significantly associated with a low incidence of chronic GVHD.

When the patients were divided by age decade, the incidence of chronic GVHD was significantly lower in recipient groups aged <10 years and 10–19 years; however, among recipients aged ≥20 years, there were no differences in the incidence of chronic GVHD (Fig 2). When the donors were divided by age decade, the cumulative incidence of chronic GVHD was significantly lower among patients transplanted from donors aged 20–29 years than among patients transplanted from donors aged ≥30 years ($P = 0{\cdot}005$, method of Gray). No differences in the incidence of chronic GVHD were found among patients transplanted from donors aged ≥30 years.

Prior acute GVHD was the strongest risk factor for chronic GVHD (Table II and Fig 1C). Among patients with no history of acute GVHD ($n = 870$), risk factors for chronic GVHD on multivariate analysis were recipient age ≥20 years {hazard ratio (HR) = 1·45 [95%confidence interval (95% CI), 1·06–1·98], $P = 0{\cdot}019$}, donor age ≥30 years [HR = 1·54 (95%CI, 1·19–2·00), $P = 0{\cdot}001$] and one locus mismatch or one allele mismatch at HLA-A/-B loci [*versus* full match, HR = 1·50 (95% CI, 1·02–2·20) $P = 0{\cdot}039$]. Among patients with a history of grade II–IV acute GVHD ($n = 1107$), platelet count $<50 \times 10^9$/l by day 100 [HR = 1·30 (95%CI, 1·00–1·67), $P = 0{\cdot}048$] was the only risk factor on multivariate analysis.

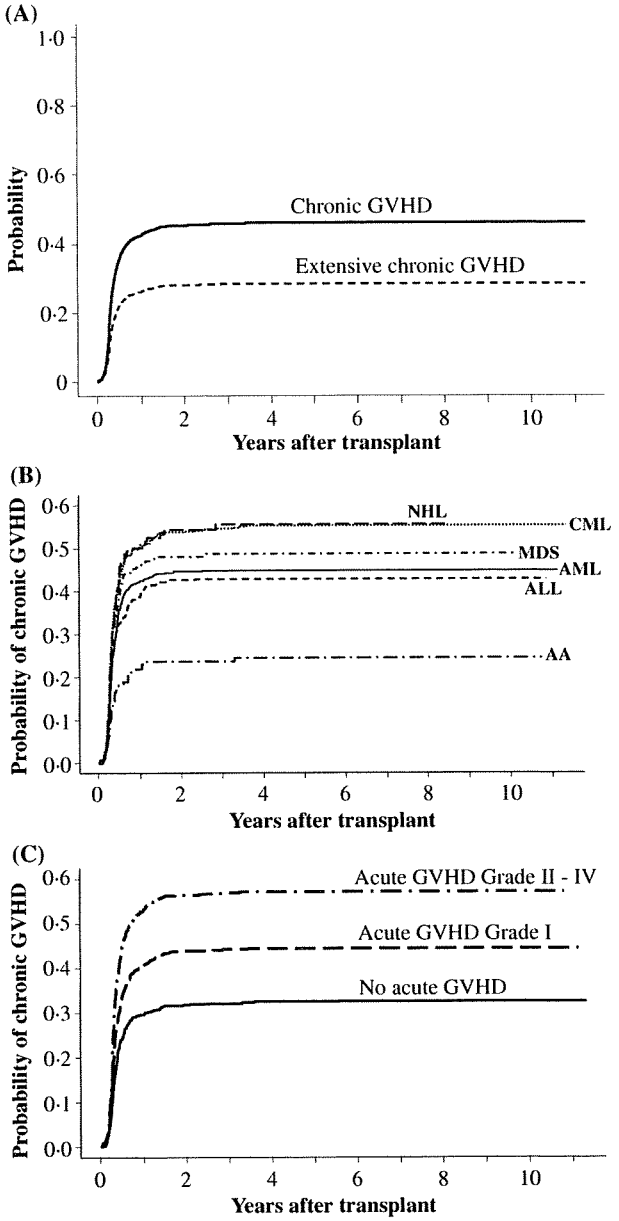

Fig 1. Cumulative incidence of chronic GVHD after UR-BMT. (**A**) Cumulative incidences of chronic GVHD (limited + extensive) and extensive chronic GVHD. The 5-year cumulative incidence of chronic GVHD was 45·8% (95% CI, 43·9–47·7%) and that of extensive chronic GVHD was 28·2% (95% CI, 26·5–29·9). Competing risks were death without chronic GVHD and death without chronic extensive GVHD (19·3% and 24·4%, respectively). (**B**) Cumulative incidences of chronic GVHD according to the primary diagnosis. The 5-year cumulative incidence and competing risk were 44·7% and 25·4% among patients with acute myeloid leukaemia (AML, solid line), 42·9% and 25·7% among patients with acute lymphoblastic leukaemia (ALL, dashed line), 49·0% and 16·1% among patients with myelodysplastic syndrome (MDS, dot-dash line), 55·3% and 12·8% among patients with chronic myeloid leukaemia (CML, dotted line), 55·7% and 11·5% among patients with non-Hodgkin lymphoma (NHL, long-dash line), and 24·4% and 6·1% among patients with aplastic anaemia (AA, dot-long dash line), respectively. (**C**) Cumulative incidences of chronic GVHD according to the severity of prior acute GVHD. The 5-year cumulative incidence was 32·4% among patients without a history of acute GVHD (solid line), 44·4% among patients with a history of grade I acute GVHD (dashed line), and 57·3% among patients with a history of grades II–IV acute GVHD (dot-dashed line). Competing risks were 20·0% without prior acute GVHD, 20·2% for grade I, and 17·9% for grades II–IV.

Table II. Univariate and multivariate analyses of risk factors for the development of chronic GVHD.

	Univariate analysis			Multivariate analysis (n = 2909)		
Factor	n	HR (95% CI)	P-value	n	HR (95% CI)	P-value
Recipient age						
0–19 years	972	1·0		961	1·0	
≥20 years	1965	1·41 (1·24–1·59)	<0·0001	1948	1·19 (1·04–1·36)	0·013
Recipient sex						
Female	1184	1·0				
Male	1753	1·11 (0·99–1·25)	0·07			NS
Donor age						
20–29 years	1007	1·0		994	1·0	
≥30	1930	1·28 (1·14–1·45)	<0·0001	1915	1·20 (1·07–1·36)	0·003
Sex matching						
Match	1738	1·0		1721	1·0	
Female to male	602	1·01 (0·87–1·16)	0·94	595	1·05 (0·91–1·22)	NS
Male to female	597	0·89 (0·77–1·03)	0·11	593	0·85 (0·74–0·99)	0·03
Diagnosis						
AML	793	1·0		787	1·0	
ALL	768	0·92 (0·79–1·08)	0·31	764	0·89 (0·76–1·04)	NS
MDS	285	1·13 (0·92–1·38)	0·25	283	1·11 (0·90–1·36)	NS
CML	604	1·27 (1·09–1·48)	0·002	602	1·19 (1·02–1·39)	0·03
NHL	168	1·32 (1·04–1·67)	0·02	166	1·18 (0·93–1·50)	NS
AA	191	0·43 (0·32–0·60)	<0·0001	190	0·51 (0·37–0·71)	0·0001
Other haematological malignancies	49	1·05 (0·67–1·65)	0·83	49	0·94 (0·60–1·48)	NS
Hereditary disorders	68	0·47 (0·29–0·77)	0·003	68	0·56 (0·34–0·93)	0·02
Time from diagnosis to BMT						
<13 months	1180	1·0				
13–24 months	865	1·05 (0·92–1·20)	0·45			
≥25 months	865	0·98 (0·85–1·12)	0·71			
Blood type disparity						
Match	1535	1·0				
Major mismatch	677	1·03 (0·89–1·18)	0·73			
Minor mismatch	616	1·08 (0·94–1·24)	0·30			
Major minor mismatch	72	1·12 (0·79–1·58)	0·54			
HLA disparity						
Full match	2012	1·0		1991	1·0	
Class I one mismatch	286	1·26 (1·05–1·51)	0·01	285	1·26 (1·05–1·52)	0·01
Class II one mismatch	473	1·03 (0·88–1·20)	0·73	468	0·90 (0·77–1·05)	NS
≥2 mismatches	166	1·31 (1·05–1·64)	0·02	165	1·14 (0·91–1·43)	NS
Preparative regimen TBI for conditioning						
Non-TBI regimen	608	1·0		601	1·0	
TBI-based regimen	2329	1·23 (1·06–1·42)	0·005	2308	1·16 (1·00–1·35)	0·04
ATG for conditioning						
No	2718	1·0				
Yes	203	0·58 (0·44–0·75)	0·0001			NS
GVHD prophylaxis						
CsA + MTX	1545	1·0				
FK506 + MTX	1118	1·00 (0·88–1·19)	0·93			
Treatment of bone marrow						
No	1529	1·0				
Yes	1384	1·06 (0·95–1·19)	0·29			
Platelet recovery (50×10^9/l or more by 100 d from BMT)						
Yes	2714	1·0		2688	1·0	
No	223	1·33 (1·10–1·61)	0·003	221	1·34 (1·10–1·63)	0·004

Table II. *Continued*

Factor	Univariate analysis			Multivariate analysis (n = 2909)		
	n	HR (95% CI)	P-value	n	HR (95% CI)	P-value
Days from BMT to WBC recovery						
<Day 18	1622	1·0				
≥Day18	1314	0·90 (0·80–1·00)	0·049			NS
Prior acute GVHD						
No	884	1·0		869	1·0	
Grade I	915	1·54 (1·31–1·80)	<0·0001	911	1·47 (1·25–1·72)	<0·0001
Grade II–IV	1138	2·28 (1·98–2·64)	<0·0001	1129	2·08 (1·80–2·42)	<0·0001

CI, confidence interval; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; AA aplastic anaemia; ATG, antithymocyte globulin; TBI, total body irradiation; GVHD, graft-*versus*-host disease; CsA, ciclosporin A; MTX, methotrexate; FK506, tacrolimus; HLA, human leucocyte antigen; BMT, bone marrow transplantation; WBC recovery, the first of three consecutive days with a persistent white blood cell count >1·0 × 10^9/l; HR, hazard ratio.

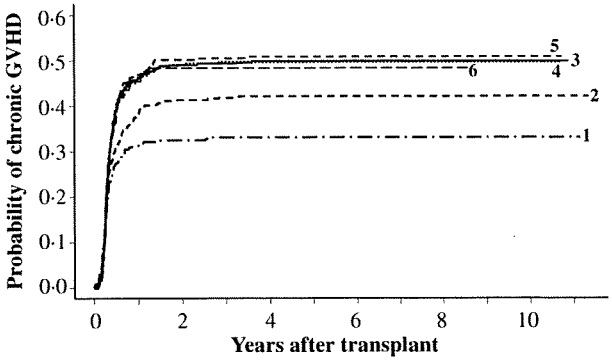

Fig 2. Cumulative incidence of chronic GVHD according to recipient's age decade. The competing risk was death without chronic GVHD. The 5-year cumulative incidence and competing risk were: 32·9% and 14·1% among patients aged 0–9 years (line 1; dot-dash line), 42·1% and 18·8% among those aged 10–19 years (line 2; dash line), 49·1% and 15·1% among those aged 20–29 years (line 3; solid line), 49·4% and 23·1% among those aged 30–39 years (line 4; dotted line), 51·0% and 23·2% among those aged 40–49 years (line 5; dash line), and 48·3% and 25·6% among those aged >50 years (line 6; long-dash line), respectively.

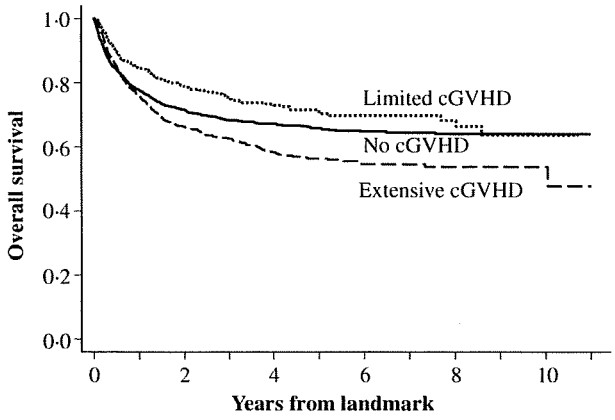

Fig 3. Overall survival according to chronic GVHD grading. The OS of all patients who survived beyond 100 d post-transplant according to chronic GVHD grade (n = 2877), is shown. The 5-year OS rate was 71·1% (95% CI, 66·4–75·8) among those with limited chronic GVHD (n = 489), 56·4% (95% CI, 52·3–60·5) among those with extensive chronic GVHD (n = 771), and 65·9% (95% CI, 63·2–68·5) among patients who did not develop chronic GVHD (n = 1617). The landmark day was the day of onset of chronic GVHD for patients with chronic GVHD, and it was day 112 from transplant, which was the median day of the onset of chronic GVHD, for patients without chronic GVHD. No chronic GVHD, solid line; limited chronic GVHD, dotted line; extensive chronic GVHD, dashed line.

Influence of chronic GVHD on OS and relapse

We analysed how chronic GVHD affects the prognosis after UR-BMT among 2877 patients (Fig 3). Patients with limited chronic GVHD had significantly better prognosis than patients with extensive chronic GVHD (log-rank test, P < 0·0001) or patients without GVHD (P = 0·009), whereas patients with extensive chronic GVHD had significantly poorer prognosis (*versus* without chronic GVHD, P = 0·003). The same tendencies were observed among 2609 patients with a haematological malignancy. On multivariate analysis using the Cox proportional hazard model with chronic GVHD as a time-dependent covariate, patients with extensive chronic GVHD had significantly increased mortality and patients with limited chronic GVHD had a survival advantage compared with those without chronic GVHD (Table III). However, patients with chronic GVHD had a lower cumulative incidence of relapse than patients without chronic GVHD (*versus* limited chronic GVHD, P = 0·049; *versus* extensive chronic GVHD, P = 0·009). There was no difference in relapse rate between patients with limited chronic GVHD and those with extensive chronic GVHD. The 5-year probability of relapse was 15·8% (95% CI, 12·1–19·5) among patients with limited chronic GVHD, 15·3% (95% CI, 12·3–18·4) among patients with extensive chronic GVHD, and 21·0% (95% CI, 18·5–23·6) among patients without chronic GVHD.

Table III. Multivariate analysis of prognostic factors in patients with haematological malignancies.

Factor	HR (95% CI)	*P*-value
Recipient age		
≥20 years	1·54 (1·30–1·83)	<0·0001
Donor age		
≥40 years	1·18 (1·18–1·38)	0·04
Diagnosis		
CML (*versus* AML)	0·67 (0·55–0·82)	0·0001
HLA disparity		
Class I one mismatch (*versus* full-match)	1·58 (1·27–1·97)	0·0001
≥2 mismatches (*versus* full-match)	1·52 (1·14–2·01)	0·0038
Platelet recovery (≥50 × 10^9/l by day 100 from BMT)		
No	1·58 (1·24–2·01)	0·0002
Prior acute GVHD		
Grade II–IV (*versus* No prior acute GVHD)	1·60 (1·31–1·95)	<0·0001
Relapse		
Yes	11·62 (10·06–13·41)	<0·0001
Secondary malignancies		
Yes	6·23 (3·28–11·83)	<0·0001
Chronic GVHD		
Limited (*versus* No)	0·67 (0·54–0·83)	0·0003
Extensive (*versus* No)	1·21 (1·03–1·43)	0·02

CI, confidence interval; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; GVHD, graft-*versus*-host disease; HLA, human leucocyte antigen; HR, hazard ratio.

Discussion

In the present study, the 5-year cumulative incidence of chronic GVHD was 45·8% and that of extensive chronic GVHD was 28·2%. These cumulative incidences, especially the cumulative incidence of extensive chronic GVHD, are slightly lower than those of the data of the National Marrow Donor Program (NMDP) (Kollman *et al*, 2001) and other previous reports (Sullivan, 1999). Notably, nearly 100% of the recipient and donor pairs in the present study were composed of a single ethnic population of Japanese people. Recently, Oh *et al* (2005) reported that Japanese and Scandinavian people had significantly lower incidences of acute GVHD than American and Irish people in HLA-identical sibling BMT. Because Japanese people have been geographically isolated for a long period of time historically, Japanese people are genetically more similar than people of the USA. or Western countries and it is unclear whether our results apply to other more diverse genetic groups.

Our previous study revealed two significant risk factors for chronic GVHD by multivariate analysis: HLA-A/-B allele mismatch and patient age (Morishima *et al*, 2002). In the current extended analysis, seven risk factors were found to be significant for the development of chronic GVHD on multivariate analysis.

Zecca *et al* (2002) reported that the incidence of chronic GVHD in children after HSCT was 27%, which was assumed to be lower than that in adult recipients. In the current analysis, the incidence of chronic GVHD among patients <20 years of age was significantly lower than that among patients over 20 years of age. However, there was no significant difference in the incidence of chronic GVHD when adult patients over 20 years of age were grouped by age decade, although the OS rate was significantly lower in older adults than in younger adults, probably because of an increased incidence of death from other causes rather than chronic GVHD.

Donor age ≥30 years was a significant risk factor for the development of chronic GVHD and it also tended to decrease the survival rate. Kollman *et al* (2001) also reported that younger donor was a significant predictor of lack of development of chronic GVHD. Although the reason for this is not well understood, our findings suggest that donors of younger age may be preferable when selecting from comparably HLA-matched volunteer donors.

In our previous study (Morishima *et al*, 2002), HLA-C allele mismatch also tended to increase the incidence of chronic GVHD, while HLA-DR/-DQ mismatch showed no effect. Petersdorf *et al* (2004) showed that a single HLA-C mismatch conferred increased risk of mortality compared with matches. Greinix *et al* (2005) also showed that HLA class I mismatch, as detected by high-resolution typing, had a significant impact on the development of chronic GVHD and survival of UR-BMT recipients. The present study returned the same result as that in the previous report, although the effect of HLA-C was not analysed.

Previous analysis of risk factors for chronic GVHD after HLA-identical sibling BMT (Atkinson *et al*, 1990) revealed that the strongest risk factor for chronic GVHD was the existence of prior acute GVHD. In that report, several risk factors including recipient age >20 years predicted a higher risk of chronic GVHD in patients with a history of grade I acute GVHD or without a history of acute GVHD; however, among patients with a history of moderate to severe acute GVHD, no other risk factor predicted the development of chronic GVHD. In our study, recipient age and donor age were important risk factors for *de novo* onset of chronic GVHD, whereas in patients with a history of moderate to severe acute GVHD, patient age and donor age were not risk factors for chronic GVHD. These results are similar to the results of the other report (Atkinson *et al*, 1990). Remberger *et al* (2002) revealed that CML was a risk factor for chronic GVHD. We also identified that the incidence of chronic GVHD among patients with CML was significantly higher than that among patients with acute myeloid leukaemia (AML).

Whether the primary disease was a haematological malignancy or not significantly affected the development of chronic GVHD. In our previous study, among patients with AA who underwent UR-BMT, the incidence of chronic GVHD was

30% (Kojima *et al*, 2002), and it was 24·4% in the present extended analysis. Moreover, we found that the incidence of chronic GVHD among patients with hereditary disorders was significantly low in multivariate analysis. This finding might be due to the difference in treatment strategies for patients with haematological malignancy and those with AA. Immunosuppressive agents might be stopped or decreased earlier in patients with haematological malignancy than in those with non-malignant disease in order to induce the GVL effect.

Limited chronic GVHD had a significant impact on increasing patient survival, whereas patients with extensive chronic GVHD had a poor prognosis. In patients with a haematological malignancy, we found no significant difference in relapse rates between patients with limited chronic GVHD and those with extensive chronic GVHD, indicating that extensive chronic GVHD does not provide a strong GVL effect compared with limited chronic GVHD.

We used the grading system of limited and extensive chronic GVHD, which was originally proposed in 1980 based on the clinicopathological findings in 20 patients (Shulman *et al*, 1980). However, this grading system has several limitations. Akpek *et al* (2001, 2003) proposed a new prognostic model by analysing GVHD-specific survival and suggested that three factors, i.e. skin involvement, platelet count and progressive-type onset, significantly influence the survival of patients who developed chronic GVHD. However, a recent Japanese report showed that Japanese patients could not be accurately classified when these proposed prognostic models were used because the manifestation of chronic GVHD differed between Japanese and Western ethnic populations (Atsuta *et al*, 2006). We have started to collect more detailed information on Japanese patients with chronic GVHD, such as organ involvement, treatment strategy, and treatment outcome, to establish prognostic models.

In conclusion, this large-scale study demonstrated the incidence of chronic GVHD after UR-BMT in a single Japanese ethnic population and provides strong evidence for seven risk factors for chronic GVHD after UR-BMT. This study also suggests that limited chronic GVHD provides a survival benefit to patients with a haematological malignancy by reducing the risk of relapse without increasing the risk of death from chronic GVHD. Extended intervention and clinical trials are necessary to overcome extensive chronic GVHD.

Acknowledgements

We express our deep gratitude to those who have volunteered to donate bone marrow to offer patients a second chance at life. We also appreciate the staff of the JMDP for their assistance and all physicians and nursing staffs of all transplantation teams in Japan for providing excellent patient care and reporting transplant data.

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