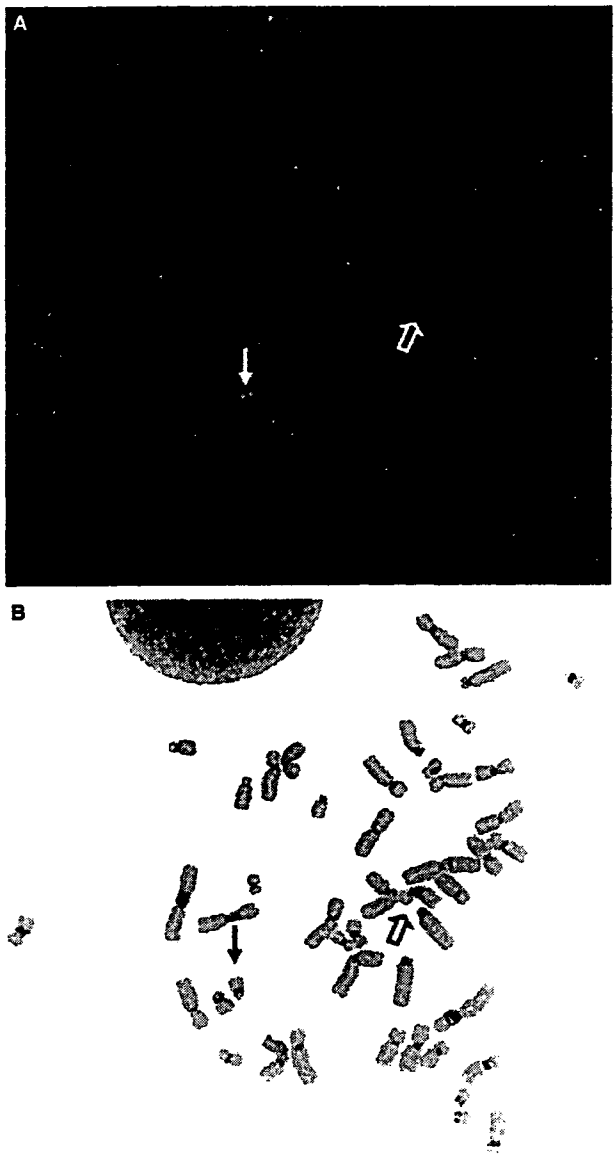

Figure 5 DC-FISH with the *IGλ* probe in the cell line HBL2. Red and green signals are originating from RP11-1152K19 and RP11-165G5, respectively. The green signal (open arrow) was detected on der(22). No red signal was detected, possibly due to the physiological VJ rearrangement of the *IGL* gene. (B) DAPI staining.

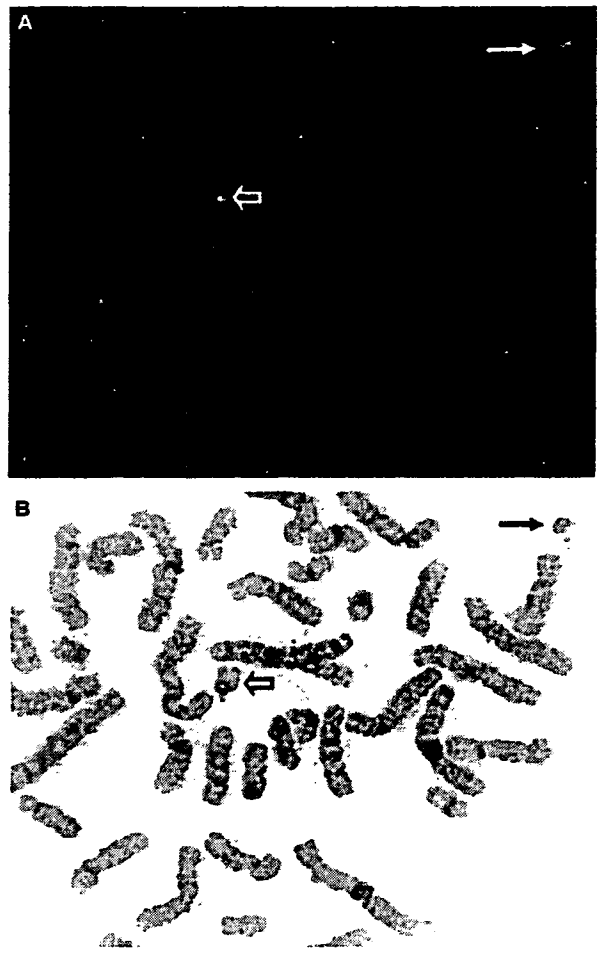

Figure 6 (A) DC-FISH with the *IGλ* probe in the lymphoma cell line HBL6. A fusion signal was detected on normal chromosome 22, as indicated by the open arrow. Only a green signal was noted on der(22), as indicated by the arrow. No V-region (red signal) was detected. (B) DAPI staining.

patients, seven were female and two were male. The histological subtypes of NHL were diffuse large B-cell lymphoma (DLBCL) in four patients, follicular lymphoma (FL) grade 2 in three patients, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue in one patient, and mantle cell lymphoma in one patient. Surface light chains were identified in all samples, and seven patients showed kappa light chain expression.

Discussion

IGL translocation was detected in two of seven cell lines and nine of 40 patients with B-cell lymphoma. We demonstrated two significant findings in this study. First, *IGL* and/or *IGH* double translocation was detected in three of 40 patients, accounting for 7.5% of B-cell lymphoma cases. Secondly, we identified novel translocation partners of *IGL* translocations in four patients. There have been a number of previous reports describing cases of double *IGH* translocation, or that of both *IGH* and *IGL* in B-cell malignancies (14–23). However, double *IGL* translocation has not been reported until now. Our case was identified as having double *IGL* translocation only through the use of FISH, indicating that FISH should be recommended for the detection of double *IGL* translocations, because the polymerase chain reaction

Table 3 Clinical and histological findings in nine patients with *IGL* translocation

Case no.	Age/Gender	Diagnosis	Surface light chain	Chemotherapy	Response	Survival (mo)
1	63/M	MCL	λ	R-CHO	PR	24
2	65/F	FL	λ	R-CHOP	CR	36+
3	52/F	DLBCL	κ	R-CHOP+Hi-MTX	CR	30+
4	64/F	DLBCL	κ	R-CHOP	CR	41+
5	68/F	MALT lymphoma	κ	R-CHOP	CR	40+
6	77/F	DLBCL	κ	R-CHOP	CR	17+
7	76/M	DLBCL	κ	R-CHOP	CR	6+
8	64/F	FL	κ	R-CHOP	CR	26+
9	73/F	FL	κ	R-CHOP	CR	51+

MCL, mantle cell lymphoma; FL, follicular lymphoma; MALT lymphoma, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; DLBCL, diffuse large B-cell lymphoma; CR, complete response; PR, partial response; LN, lymph node; Hi-MTX, high-dose methotrexate.

method is not able to detect double *IGL* translocation with an unknown partner.

Regarding previous reports, the most frequent partner gene of *IGH* translocation is *BCL2*, while that of *IGL* is *c-MYC*. *c-MYC* has been supposed to be implicated in tumour progression such as the development from FL carrying t(14;18) to Burkitt's or Burkitt-like lymphoma (15, 16, 24–31). In these previous reports, tumour progression is possibly associated with the co-existence of *c-MYC* and *BCL2*, but not with the co-existence of translocations of *IGH* and *IGL*. FISH with the *IGL/c-MYC* probe may possibly predict the therapeutic response and prognosis.

We identified chromosomal bands 1p13, 6p25, 17p11.2 and 17q21 as partners in *IGL* translocation. Although these breakpoints should be narrowed down by FISH specific for each locus-band probe to identify candidate genes located in these loci, samples are not available for further studies because of the small sample size. Cell lines exhibiting these abnormalities will be required for identifying genes involved in *IGL* translocation associated with the development as well as progression of lymphoma.

Histologically, four and three of the nine patients showed DLBCL and FL subtypes, respectively. Regarding the clinical outcome, eight of nine patients achieved a CR with a cyclophosphamide, hydroxydaunorubicin, Oncovin, prednisolone (CHOP)-like regimen. *IGL/BCL6* translocation cases in our study are compatible with those of a previous study in terms of the clinical outcome, confirming that patients with *non-IG/BCL6* tend to show a poorer clinical outcome than those with *IG/BCL6* (32). In the current study, *IGL* translocation was associated with neither a histological nor clinical subtype of NHL, although further studies involving a large number of patients will be required to draw definitive conclusions.

In conclusion, FISH analysis suggests that *IGL* translocation may be associated with lymphoma development, although *IGL* and/or *IGH* translocation was not correlated with a specific subtype of NHL in the current study. The cytogenetic findings described herein warrant the identification of novel genes as partners of *IGL* translocation associated with lymphomagenesis.

Acknowledgements

We thank Miss Minako Gotoh and Kayoko Kurita for their technical assistance.

References

1. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting - Airlie House, Virginia. *J Clin Oncol* 1999;**17**:3835–49.
2. Willis TG, Dyer MJS. The role of immunoglobulin translocations in the pathogenesis of B-cell malignancies. *Blood* 1999;**96**:808–22.
3. Taniwaki M, Nishida K, Ueda Y, Misawa S, Nagai M, Tagawa S, Yamagami T, Sugiyama H, Abe M, Fukuhara S. Interphase and metaphase detection of the breakpoint of 14q32 translocations in B-cell malignancies by double-color fluorescence in situ hybridization. *Blood* 1995;**85**:3223–8.
4. Kanda-Akano Y, Nomura K, Fujita Y, *et al.* Molecular-cytogenetic characterization of non-Hodgkin's lymphoma with double and cryptic translocations of the immunoglobulin heavy chain gene. *Leuk Lymphoma* 2004;**45**:1559–67.
5. Nomura K, Kanda-Akano Y, Shimizu D, *et al.* An additional segment at 1p36 derived from der(18)t(14;18) in patients with diffuse large B-cell lymphomas transformed from follicular lymphoma. *Ann Haematol* 2005;**84**:474–6.
6. Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M. Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res* 1971;**31**:1860–1.

7. Elghetany MT, Kurec AS, Schuehler K, Forbes BA, Duggan DB, Davey FR. Immunophenotyping of non-Hodgkin's lymphomas in paraffin-embedded tissue sections. A comparison with genotypic analysis. *Am J Clin Pathol* 1991;**95**:517–25.
8. Taniwaki M, Matsuda F, Jauch A, Nishida K, Takashima T, Tagawa S, Sugiyama H, Misawa S, Abe T, Kashima K. Detection of 14q32 translocations in B-cell malignancies by in situ hybridization with yeast artificial chromosome clones containing the human IgH gene locus. *Blood* 1994;**83**:2962–9.
9. ISCN. An International System for Human Cytogenetic Nomenclature. In: Shaffer LG, Tommerup N, eds. *Recommendations of the International Standing Comittee on Human Cytogenetic Nomenclature*. Basel: Karger, 2005.
10. Martin-Subero JI, Harder L, Gesk S, Schlegelberger B, Grote W, Martinez-Climent JA, Dyer MJ, Novo FJ, Calasanz MJ, Siebert R. Interphase FISH assays for the detection of translocations with breakpoints in immunoglobulin light chain loci. *Int J Cancer* 2002;**98**:470–4.
11. Poulsen TS, Silahtaroglu AN. Detection of illegitimate rearrangements within the immunoglobulin light chain loci in B cell malignancies using end-sequenced probes. *Leukemia* 2002;**16**:2156–8.
12. Iida S, Rao PH, Nallasivam P, Hibshoosh H, Butler M, Louie DC. The t(9;14)(p13;q32) chromosomal translocation associated with lymphoplasmacytoid lymphoma involves the PAX-5 gene. *Blood* 1996;**88**:4110–7.
13. Tamura A, Miura I, Iida S, Yokota S, Horiike S, Nishida K. Interphase detection of immunoglobulin heavy chain gene translocations with specific oncogene loci in 173 patients with B-cell lymphoma. *Cancer Genet Cytogenet* 2001;**29**:1–9.
14. D'Achille P, Seymour JF, Campbell LJ. Translocation (14;18)(q32;q21) in acute lymphoblastic leukemia: a study of 12 cases and review of the literature. *Cancer Genet Cytogenet* 2006;**171**:52–6.
15. De Jong D, Voetdijk BM, Beverstock GC, van Ommen GJ, Willemze R, Kluin PM. Activation of the c-myc oncogene in a precursor-B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. *N Engl J Med* 1988;**318**:1373–8.
16. Thangavelu M, Olopade O, Beckman E, Vardiman JW, Larson RA, McKeithan TW, Le Beau MM, Rowley JD. Clinical, morphologic, and cytogenetic characteristics of patients with lymphoid malignancies characterized by both t(14;18)(q32;q21) and t(8;14)(q24;q32) or t(8;22)(q24;q11). *Genes Chromosomes Cancer* 1990;**2**:147–58.
17. Koduru PR, Offit K. Molecular structure of double reciprocal translocations: significance in B-cell lymphomagenesis. *Oncogene* 1991;**6**:145–8.
18. Hebert J, Romana SP, Hillion J, Kerkaert JP, Bastard C, Berger R. Translocation t(3;22)(q27;q11) in non-Hodgkin's malignant lymphoma: chromosome painting and molecular studies. *Leukemia* 1993;**7**:1971–4.
19. Karsan A, Gascoyne RD, Coupland RW, Shepherd JD, Phillips GL, Horsman DE. Combination of t(14;18) and a Burkitt's type translocation in B-cell malignancies. *Leuk Lymphoma* 1993;**10**:433–41.
20. Berger R, Flexor M, Le Coniat M, Larsen CJ. Presence of three recurrent chromosomal reaarrangements, t(2;3)(p12;q37), del(8)(q24), and t(14;18), in an acute lymphoblastic leukemia. *Cancer Genet Cytogenet* 1996;**86**:76–9.
21. Fujii S, Miyata A, Takeuchi M, Yoshino T. Acute lymphoblastic leukemia (L3) with t(2;3)(p12;q27), t(14;18)(q32;q21), and t(8;22)(q24;q11). *Rinsho Ketsueki* 2005;**46**:134–40.
22. Bentley G, Palutke M, Mohamed AN. Variant t(14;18) in malignant lymphoma: a report of seven cases. *Cancer Genet Cytogenet* 2005;**157**:12–7.
23. Küppers R, Dalla-Favera R. Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene* 2001;**20**:5580–94.
24. Aventin A, Mecucci C, Guanyabens C, Brunet S, Soler J, Bordes R, van den Berghe H. Variant t(2;18) translocation in a Burkitt conversion of follicular lymphoma. *Br J Haematol* 1990;**4**:367–9.
25. Macpherson N, Lesack D, Klasa R, Horsman D, Connors JM, Barnett M, Gascoyne RD. Small noncleaved, non-Burkitt's (Burkit-Like) lymphoma: cytogenetics predict outcome and reflect clinical presentation. *J Clin Oncol* 1999;**17**:1558–67.
26. Klein G. Comparative action of myc and bcl-2 in B-cell malignancy. *Cancer Cells* 1991;**3**:141–3.
27. McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). *Nature* 1991;**349**:254–6.
28. Höglund M, Sehn L, Connors JM, Gascoyne RD, Siebert R, Säll T, Mitelman F, Horsman DE. Identification of cytogenetic subgroups and karyotypic pathways of clonal evolution in follicular lymphomas. *Genes Chromosomes Cancer* 2004;**39**:195–204.
29. Lee JT, Innes DJ Jr, Williams ME. Sequential bcl-2 and c-myc oncogene rearrangements associated with the clinical transformation of non-Hodgkin's lymphoma. *J Clin Invest* 1989;**84**:1454–9.
30. Yano T, Jaffe ES, Longo DL, Raffeld M. MYC rearrangements in histologically progressed follicular lymphomas. *Blood* 1992;**80**:758–67.
31. Martín-Subero JI, Odero MD, Hernandez R, *et al.* Amplification of IGH/MYC fusion in clinically aggressive IGH/BCL2-positive germinal center B-cell lymphomas. *Genes Chromosomes Cancer* 2005;**43**:414–23.
32. Akasaka T, Akasaka H, Ueda C, Yonetani N, Maesako Y, Shimizu A. Molecular and clinical features of non-Burkitt's, diffuse large-cell lymphoma of B-cell type associated with the c-MYC/immunoglobulin heavy-chain fusion gene. *J Clin Oncol* 2000;**18**:510–8.

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HEMATOLOGY

Epigenetic Regulation of CD20 Protein Expression in a Novel B-Cell Lymphoma Cell Line, RRBL1, Established from a Patient Treated Repeatedly with Rituximab-Containing Chemotherapy

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Abstract

Rituximab is a chimeric monoclonal antibody to the surface antigen CD20 and has provided better outcomes against $CD20^+$ B-cell lymphomas than chemotherapy with conventional antitumor drugs alone. Treatment with rituximab poses a considerable problem, however, because of $CD20^-$ tumor transformation and subsequent disease progression. We have established a $CD20^-$ lymphoma cell line, RRBL1, from a diffuse large B-cell lymphoma with $CD20^-$ transformation from $CD20^+$ follicular lymphoma after treatment with rituximab. RRBL1 was $CD10^+$, $CD19^+$, and $CD20^-$ by flow cytometry. CD20 expression was not detected by immunohistochemistry. Immunoblotting with whole RRBL1 cell lysate showed a very faint CD20 band only with longer exposures. The level of CD20 messenger RNA (mRNA) expression detected by quantitative reverse transcriptase–polymerase chain reaction analysis was almost 100 times lower than that in $CD20^+$ lymphoma cells. When we treated RRBL1 cells with trichostatin A, an epigenetic drug that modulates histone-acetylation status, we detected dramatically increased CD20 mRNA and protein expression, suggesting that epigenetic mechanisms may explain the $CD20^-$ phenotype in RRBL1 cells. Thus, RRBL1 may be useful not only for analyses of mechanisms for the absence of CD20 expression in vitro but also for exploration of therapies against $CD20^-$ B-cell malignancies in vivo.
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Key words: CD20; Rituximab; B-cell lymphoma; Epigenetics

1. Introduction

Rituximab is a chimeric monoclonal antibody that targets the B-cell–specific surface antigen CD20 [1,2]. CD20 is widely expressed on B-cell malignant cells, including indolent follicular lymphoma and aggressive diffuse large B-cell lymphoma (DLBCL). Recently, rituximab has been used as a new molecular targeting drug in combination with some forms of conventional chemotherapy to treat B-cell malignancies [3-8]. Although the conventional chemotherapy CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) has been administered to patients with B-cell lymphomas for more than 25 years, the cure rate has not improved, even with additional drugs. Recent reports have indicated that the cure and disease-free survival rates for $CD20^+$ B-cell lymphoma improved significantly when rituximab was used in combination with specific conventional chemotherapies, including the CHOP regimen [3-8].

Although almost 5 years have passed since clinical hematologists first used rituximab, the cure rate for $CD20^+$ lymphomas is still unsatisfactory, and considerable numbers of patients treated with rituximab show tumor progression and/or histologic transformation to a rituximab-resistant phenotype [9-12]. Two major mechanisms of resistance against rituximab have been considered in recent reports: (1) CD20-binding abnormalities (ie, problems from CD20 expression, antibody accessibility, and antibody metabolism),

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and (2) post–CD20-binding abnormalities (ie, abnormal B-cell signaling and/or apoptosis, inhibition of complement-dependent cytotoxicity, and/or antibody-dependent cell-mediated cytotoxicity) [13]. Recently, Terui et al confirmed genetic mutations in the CD20-coding sequence in the lymphoma cells of 10 of 48 patients, and 3 of these patients showed clinical features of rituximab resistance [10]. A conformational change in the CD20 protein caused by genetic mutation has been speculated to contribute to rituximab resistance. Importantly, the relationship between genetic mutations in the CD20-coding sequence and rituximab resistance as a clinical feature is still unclear.

Recently, we had a $CD20^+$ B-cell lymphoma patient who acquired a rituximab-resistant $CD20^-$ phenotype after repeated treatment with rituximab-containing combination chemotherapy. We have established a $CD20^-$ B-cell lymphoma cell line, RRBL1, from that patient. The level of CD20 protein in RRBL1 cells was very low in immunostaining, flow cytometry, and immunoblotting analyses. In contrast, CD20 messenger RNA (mRNA) expression as confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis was significantly weaker than in $CD20^+$ B-cell lymphoma cells. Interestingly, after treating RRBL1 cells with trichostatin A (TSA), an epigenetic drug that modulates histone-acetylation status, we detected CD20 protein by immunoblotting. In this report, we propose that one mechanism of resistance to rituximab is the aberrant down-regulation of CD20 mRNA expression. We suggest that epigenetic regulation is one mechanism for decreasing the levels of CD20 mRNA and protein in $CD20^-$ B-cell lymphoma cells. RRBL1 may thus be useful not only for analyzing in vitro and in vivo mechanisms of the negative CD20 phenotype but also for exploring new strategies for the treatment of $CD20^-$ rituximab-resistant B-cell lymphoma.

2. Materials and Methods

2.1. Case Report

A 43-year-old man was admitted to the hospital in 1993 for bilateral cervical lymph node swelling and night sweating. Axillary and inguinal lymph node swelling and splenomegaly (5 cm below the left costal margin) were also observed. Blood tests showed a low hemoglobin count (9.9 g/dL) and thrombocytopenia (76×10^9/L). The patient's white blood cell count and lactate dehydrogenase level were within the normal limits. A biopsy of the left cervical lymph node was performed, and the diagnosis was follicular small cleaved B-cell lymphoma with $CD10^+$, $CD19^+$, $CD20^+$, and immunoglobulin κ chain $(Igk)^+$ features by flow cytometry (Table 1). In addition, a bone marrow aspirate revealed bone marrow invasion. Repeated conventional chemotherapy produced partial remission (Figure 1). Beginning in 1997, rituximab was combined with conventional chemotherapy and administered repeatedly. Another partial remission was obtained, and chemotherapy with rituximab was administered again when the disease progressed. In June 2004, just after rituximab chemotherapy, we observed aggressive tumor progression in the lower abdomen, including bilateral inguinal lymph nodes, and the splenomegaly progressed significantly. A bone marrow aspirate revealed a massive invasion of lymphoma cells that were $CD10^+$, $CD19^+$, Igk^+, and $CD20^-$ (Table 1). At this point, the patient's diagnosis was $CD20^-$ DLBCL histologically transformed from a follicular small cleaved B-cell

Table 1.
Surface Antigen Analyses of Primary Lymphoma Cells and RRBL1 Cells*

	Primary Lymphoma Cells, %			
Surface Antigen	Lymph Node (Sep 1993)	Bone Marrow (Jun 2004)	Peripheral Blood (Sep 2004)	RRBL1 Cell Line (Oct 2005), %
B-cell markers				
CD10	55.1	88.3	100.0	98.0
CD19	49.3	51.2	82.2	97.7
CD20	58.7	0.1	4.1	3.3
CD23				1.8
κ Chain		82.8	90.1	0.7
λ Chain		0.1	1.8	1.2
T/NK cell markers				
CD2	18.0	12.4	3.8	0.7
CD3	17.9	12.1	1.7	1.3
CD4	12.3			1.5
CD5	18.1	14.8	3.0	2.2
CD7	15.6	12.6	5.2	2.6
CD8	5.3			13.9
CD56				2.1
Other markers				
CD11c				2.4
CD16				1.5
CD25	3.7			40.4
CD30				2.3
CD34	0.4			1.2

*Data are presented as the percentage of cells expressing the marker. T/NK cell indicates T-cell/natural killer cell.

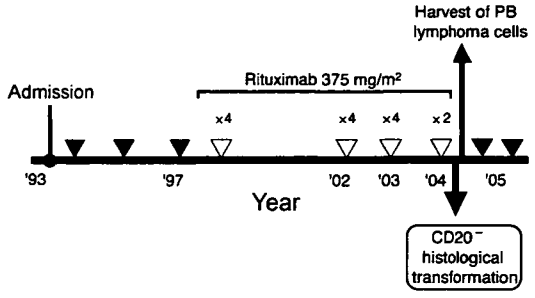

Figure 1. Clinical course of the patient, the source of the RRBL1 cell line. He was first admitted in 1993, and conventional chemotherapy (black triangles) and rituximab-containing chemotherapy (white triangles) were carried out repeatedly as his disease progressed. Histologic transformation into $CD20^-$ diffuse large B-cell lymphoma was observed in June 2004, and $CD20^-$ lymphoma cells gradually appeared in the patient's peripheral blood (PB). Tumor cells were harvested from the PB in July 2004 and cultured to establish the cell line. Despite intensive salvage chemotherapy without rituximab, the patient's disease progressed to the leukemic state, and he died in February 2005.

lymphoma. Although salvage chemotherapy without rituximab was carried out, the disease progressed. In October 2004, $CD20^-$ lymphoma cells appeared in the peripheral blood and gradually increased in number. Despite repeated intensive chemotherapy, the disease progressed to $CD20^-$ lymphoid cell leukemia, and the patient died of respiratory failure from pneumonia in February 2005. After informed consent was obtained from his family, an autopsy was performed.

2.2. Establishment of the Cell Line and Cell Culture

Peripheral blood (20 mL) containing $CD20^-$ lymphoma cells was harvested from the patient in October 2004. Mononuclear cells were purified with Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 20% fetal calf serum in a humidified incubator with 5% carbon dioxide. The medium was changed every 1 to 2 weeks, and after observing a growth advantage, we replaced the incubation medium with RPMI 1640 containing 10% fetal calf serum.

2.3. Cytogenetic Analysis

The chromosomes of RRBL1 cells and the patient's primary cells and were analyzed with the G-banding technique (Mitsubishi Kagaku Bio-Clinical Laboratories, Kyoto, Japan).

2.4. Cell Surface Marker Analysis and Immunohistochemistry

Cell surface antigens of primary lymphoma cells and RRBL1 cells were analyzed by means of flow cytometry with monoclonal antibodies, as indicated previously [14]. May-Giemsa staining and immunostaining with anti-CD20 B1 antibody (Beckman Coulter, Fullerton, CA, USA) were used to observe the morphologic features of cell lines in Cytospin specimens [15].

2.5. Preparation of Genomic DNA and Analysis of $BCL2$-J_H Breakpoint DNA Sequences

Genomic DNA from clinical samples and RRBL1 cells was prepared with a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) and used in PCR analyses [16]. Specific primers for *BCL2* at 18q21 and mixed primers for the *immunoglobulin heavy chain joining region* (J_H) gene at 14q32 were used for the PCR, as indicated previously [17-19]. A fragment of the $BCL2$-J_H breakpoint sequence was amplified with the following primers: *BCL2*-F, 5′-CAG CCT TGA AAC ATT GAT GG-3′; J_H-R mixed universal primer, 5′-CTC/T ACC TGA G/AGA GAC G/AGT GAC C-3′. Amplified PCR fragments were purified with a PCR purification kit (Qiagen) and directly sequenced with an ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.6. Immunoblot Analysis

Cells (5×10^4) were lysed in 100 μL of lysis buffer (50 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.5% Nonidet P-40, 300 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and a Complete Mini Protease Inhibitor Cocktail Tablet [Roche Diagnostics, Indianapolis, IN, USA]). After centrifugation at 10,000*g* for 10 minutes, the supernatants were put into new tubes, and 100 μL of 2× sodium dodecyl sulfate (SDS) sample buffer was added. After boiling for 5 minutes, samples were separated by SDS–polyacrylamide gel electrophoresis. Immunoblotting was carried out as described previously [16,20,21] with anti-CD20 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody (Santa Cruz Biotechnology).

2.7. RNA Preparation, Quantitative RT-PCR, and Conventional Semiquantitative RT-PCR

We used the Blood RNA Extraction Kit (Qiagen) to isolate total RNA from RRBL1, Raji, 293T, and Jurkat cells and from the primary lymphoma cells of patients [16]. Complementary DNA (cDNA) was prepared with SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen). Quantitative RT-PCR (TaqMan real-time PCR) was performed with the ABI Prism 7000 instrument (Applied Biosystems) as reported previously [16]. Probes and primers for human CD20 (Hs00544818_m1) were purchased from Applied Biosystems. Endogenous GAPDH mRNA was measured as an internal control. For conventional semiquantitative RT-PCR analyses of CD5, CD20, CD34, histone deacetylase 3 (HDAC3), and β-actin mRNAs, we used the following primers: CD5-U, 5′-TAT GAC CCA GAT TTC CAG GC-3′; CD5-L, 5′-GTG TTG TCT TCT GGG GTT CT-3′; CD20-U, 5′-ATG AAA GGC CCT ATT GCT ATG-3′; CD20-L, 5′-GCT GGT TCA CAG TTG TAT ATG-3′; CD34-U, 5′-ACA CCT AGT ACC

CTT GGA AG-3'; CD34-L, 5'-GGG TTT AGT GGG AGA TGT TG-3'; HDAC3-U, 5'-GTG GTG GTT ATA CTG TCC GA-3'; HDAC3-L, 5'-AAT CTC CAC ATC GCT TTC CTT-3'; β-actin-U, 5'-TCA CTC ATG AAG ATC CTC A-3'; and β-actin-L, 5'-TTC GTG GAT GCC ACA GGA C-3'. Semiquantitative RT-PCR with *LA Taq* polymerase (TaKaRa Bio, Otsu, Japan) was performed as described previously [16].

2.8. Mouse Transplantation Procedure

The mouse transplantation procedure has been described previously [14]. In brief, 2×10^6 RRBL1 cells were injected subcutaneously into the dorsal region or intravenously into the tail of nonirradiated 7- to 8-week-old male nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (CLEA Japan, Tokyo, Japan). The major and minor axes of the subcutaneous tumors were measured, and the weights of the tumors were calculated as indicated previously [14]. Animal experiments were approved by the intramural ethics committee for Laboratory Animal Research, Nagoya University School of Medicine, and were performed according to the guidelines of the institution.

2.9. Pathologic Examinations

To evaluate pathologic status, we fixed extracted tissues with 10% formaldehyde, mounted 4-μm sections on glass slides, and stained the slides with hematoxylin and eosin.

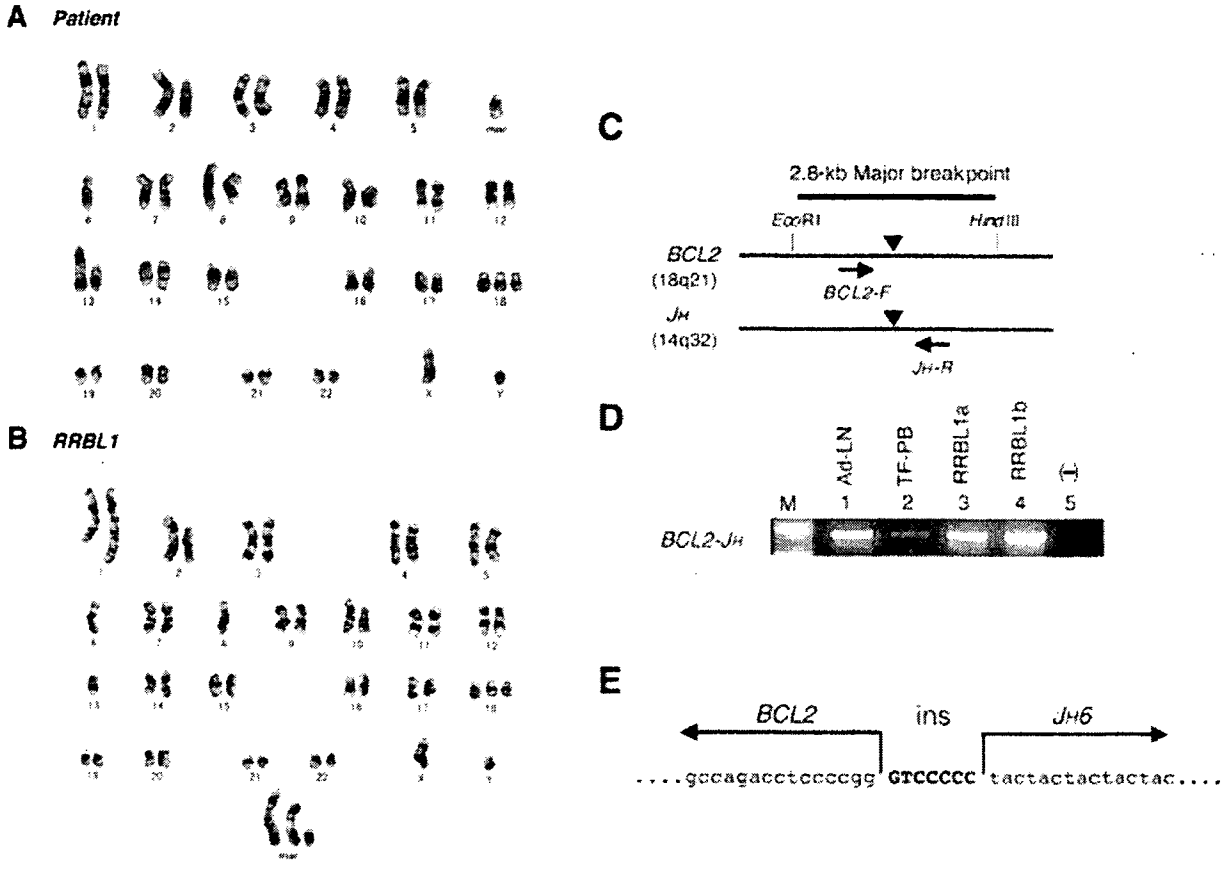

Figure 2. Chromosomal and genetic abnormalities were conserved in both RRBL1 cells and the patient. Chromosomal abnormalities of the original cells obtained from the patient's peripheral blood cells (A) and RRBL1 cells (B) were analyzed by the G-banding method. The abnormalities were as follows: A, 47,XY,t(1;7)(p36;p15),t(2;8)(p12;q24),–6,add(6)(q21),t(14;18)(q32;q21),+18,der(18)t(14;18)(q32;q21),+der(?)t(?;13)(?;q12),+mar2. B, 47,XY,der(1)t(1;?7)(p36;p15),dup(1)(q21q42),add(2)(p11),–6,del(6)(q15q21),add(7)(p11),–8,add(10)(p13),–13,t(14;18)(q32;q21),+18,der(18)t(14;18)(q32;q21),+3mar. C, Breakpoints of t(14;18)(q32;q21) are indicated. The major breakpoints of the *BCL2* gene at 18q21 and of the J_H region at 14q32 are illustrated with black triangles. Breakpoints in the J_H region are found in 6 different regions (*J1* to *J6*), as reported previously [22,31]. Polymerase chain reaction (PCR) primers for the amplification of the breakpoint are indicated by black arrows (*BCL2*-F and J_H-R). D, DNA from RRBL1 cells and the patient's samples were used in the PCR to detect the *BCL2*-J_H breakpoint. A single band was detected in each sample, indicating that the same lymphoma clone exists in 4 samples (lanes 1-4). As a negative control, genomic DNA from mononuclear cells of a healthy donor was used (lane 5). Ad-LN indicates lymphoma cells from the patient's cervical lymph node on admission; TF-PB, lymphoma cells from the patient's peripheral blood on transformation in July 2004; RRBL1a and RRBL1b, RRBL1 cells cultured for 90 days and 120 days, respectively. E, Genomic DNA sequence of the *BCL2*-J_H6 breakpoint (GenBank accession no. DQ979790). Sequences from 18q21 and 14q32 are indicated as *BCL2* and J_H6, respectively. The unspecified insertion sequence between *BCL2* and J_H6 is indicated in capital letters.

These specimens were also used for immunostaining with anti-CD20 (L-26; Dako, Carpinteria, CA, USA) and anti-CD79a (Dako) antibodies.

3. Results

3.1. Establishment of RRBL1 Cells

The clinical course of the patient is depicted in Figure 1. Peripheral blood mononuclear cells containing $CD20^-$ lymphoma cells were separated and cultured as indicated in "Materials and Methods." Cells were cultured for more than 1 year and thus were considered to be a cell line, which we designated RRBL1 (rituximab-resistant B-cell lymphoma 1). The chromosomal abnormalities of the original patient and the RRBL1 cells are indicated in Figures 2A and 2B. A G-banding analysis indicated that the translocations between chromosomes 1 and 7 (t[1;7][p36;p15]) and between chromosomes 14 and 18 (t[14;18][q32;q21]) were conserved. In addition to these abnormalities, we observed t(2;8)(p12;q24) in the patient's lymphoma cells (Figure 2A). A detailed genetic analysis of the t(14;18)(q32;q21) breakpoint was performed to clarify whether the established cell line came from the original lymphoma clone, which existed in the patient in 1993. Previous reports have indicated that t(14;18)(q32;q21) produces a $BCL2/J_H$ fusion gene and that the breakpoint sequence can be amplified via the PCR (Figure 2C) [18,22]. Genomic DNA was prepared from the original lymphoma cells and from RRBL1 cells harvested at a later date, and both DNA preparations were amplified with $BCL2/J_H$-specific primers (Figure 2D). We obtained a single fragment (approximately 800 bp) from all genomic samples (Figure 2D, lanes 1-4) and confirmed by direct sequencing that each fragment was not a nonspecific band. Sequencing revealed that the PCR fragments contained genomic sequences from *BCL2* and J_H, separated by a 7-bp sequence of unknown origin (Figure 2E). These results confirm that RRBL1 cells came from the same clone as that originally obtained from the patient and that the $CD20^-$ transformed DLBCL cells observed in 2004 originated from the same clone as the $CD20^+$ lymphoma cells evident at the initial diagnosis in 1993.

3.2. CD20 Protein in RRBL1 Cells Is Virtually Undetectable by Immunostaining, but CD20 mRNA Is Detectable by RT-PCR

Next, we confirmed that the CD20 protein is expressed in RRBL1 cells. Immunostaining with anti-CD20 monoclonal antibody was performed with a Cytospin specimen. As indicated in Figure 3A, CD20 protein was detected on Raji cells (a positive control), which were derived from a Burkitt lymphoma patient, but not on RRBL1 cells. Whole-cell lysates were obtained from Raji, RRBL1, and NALM6 cells and used for immunoblotting with anti-CD20 antibody. The NALM6 line, which was used as a negative control, has features of precursor-B-cells that are $CD20^-$. CD20 protein was detected by immunoblotting in Raji cells but not in NALM6 or RRBL1 cells (Figure 3B). A longer exposure revealed a faint CD20 band for RRBL1 cells (data not shown).

Furthermore, we studied CD20 mRNA expression by quantitative RT-PCR analysis (Figure 3C). Raji and 293T cells were used as positive and negative controls, respectively. We also used mRNAs derived from $CD20^+$ lymphoma patients as positive controls for this assay. The histologic diagnoses for these patients were DLBCL (6 patients) and Burkitt lymphoma (2 patients). Quantitative RT-PCR analysis revealed that the CD20 mRNA level of RRBL1 cells was almost 100 times lower than that of Raji cells (Figure 3C, lane 3 versus lane 1). No expression was observed in the negative control (293T cells, lane 2). Clinical samples showed almost the same CD20 expression level as Raji cells. These results suggest that the expression level of CD20 protein in RRBL1

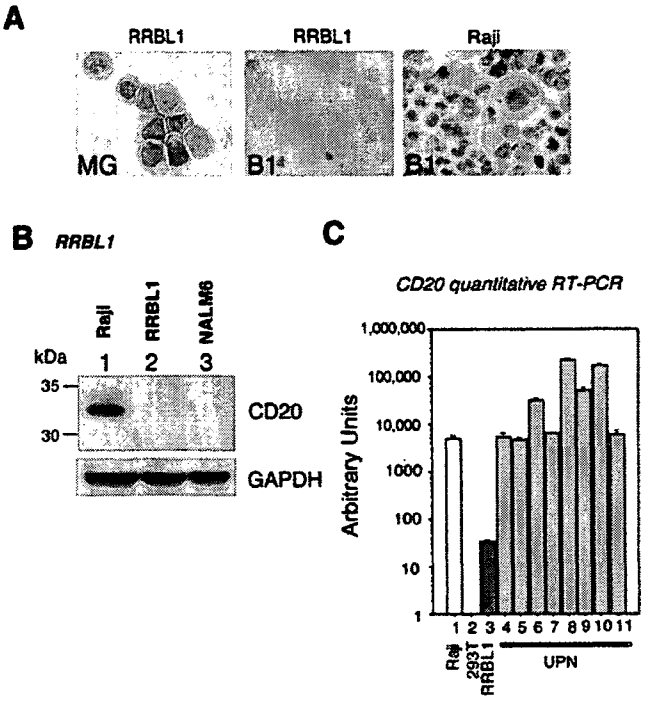

Figure 3. CD20 protein and messenger RNA (mRNA) expression in RRBL1 cells. A, RRBL1 and Raji cells immunostained with anti-CD20 monoclonal antibody B1. Note that RRBL1 cells do not show CD20 protein expression, but Raji cells (positive control) express CD20 at the surface. MG indicates May-Grünwald-Giemsa staining. B, Whole-cell lysates were obtained from Raji, RRBL1, and NALM6 cells, and immunoblotting with anti-CD20 polyclonal antibody was performed. CD20 protein expression was detected in Raji cells but not in RRBL1 or NALM6 cells. Immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with the same membrane as a protein-loading control. C, Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis with a probe specific for CD20 complementary DNA; 293T cells were used as a negative control. The level of CD20 mRNA expression of RRBL1 cells was significantly lower than that of the positive control (Raji cells). mRNAs from $CD20^+$ B-cell lymphoma patients were also analyzed (lanes 4-11). Note that expression of CD20 mRNA in RRBL1 cells was significantly lower than in the patients' lymphoma cells (lane 3 versus lanes 4-11).

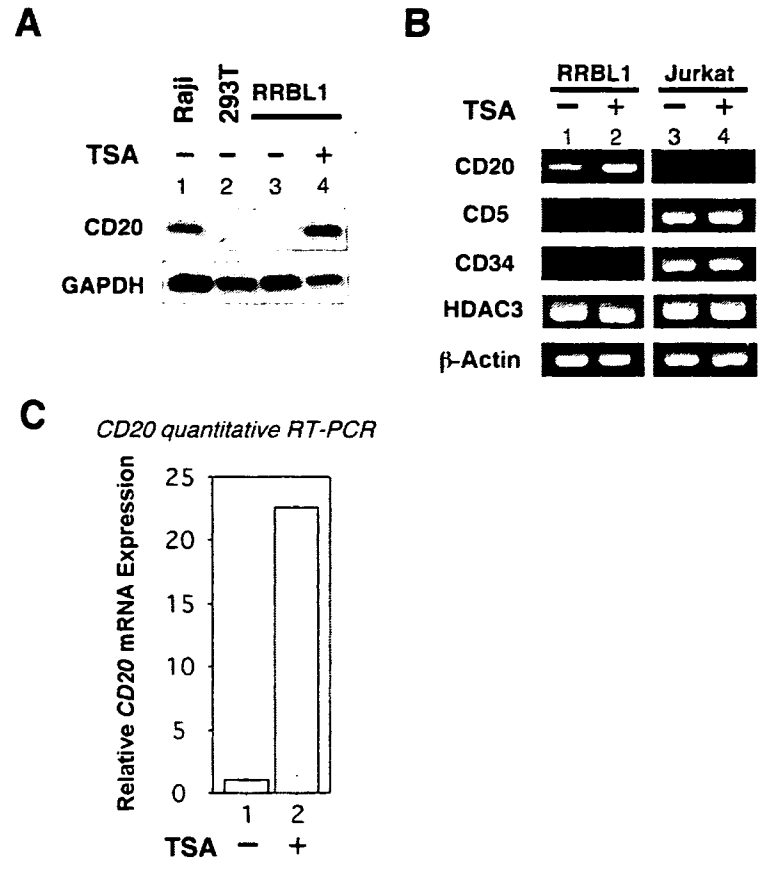

Figure 4. Induction of CD20 protein expression in RRBL1 cells after treatment with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). A, RRBL1 cells were cultured for 48 hours with or without 100 nM TSA. Whole-cell lysates were extracted from Raji, 293T, and RRBL1 cells treated with or without TSA and subjected to immunoblotting with anti-CD20 and GAPDH antibodies. Note that CD20 protein expression was confirmed in RRBL1 cells cultured with TSA (lane 4), as in the positive control (Raji cells, lane 1). B, RNA was also extracted from RRBL1 cells prepared as in (A) and analyzed for CD20, CD5, CD34, HDAC3, and β-actin expression by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay. As a control, Jurkat cells were also incubated with or without TSA under the same conditions as RRBL1 cells and assayed by RT-PCR. Note that CD20 mRNA expression was enhanced only in RRBL1 cells with added TSA. C, Quantitative RT-PCR analysis was carried out with mRNA from RRBL1 cells incubated with or without TSA. The expression level of CD20 mRNA was normalized to the endogenous GAPDH expression level. The level of CD20 mRNA expression in RRBL1 cells cultured with TSA (lane 2) was expressed relative to that of cells cultured without TSA (lane 1).

cells is significantly lower than that in $CD20^+$ cells because of a lower expression level of CD20 mRNA.

3.3. Reexpression of CD20 Protein in RRBL1 Cells after Treatment with the HDAC Inhibitor TSA

We hypothesized that RRBL1 still has the capability to express CD20 protein and that the expression might be regulated by epigenetic mechanisms. To test our hypothesis, we used the epigenetic drug TSA, which can modulate gene expression levels. TSA is known to up-regulate gene expression by modulating histone acetylation through the inhibition of HDAC enzymatic activity [23,24]. RRBL1 cells were incubated in culture medium containing 100 nM TSA for 48 hours, and a whole-cell lysate was prepared and immunoblotted. TSA treatment significantly induced CD20 protein expression (Figure 4A, lane 4). Next, we carried out a RT-PCR analysis by using total RNA from RRBL1 cells treated with or without TSA. Jurkat cells, which are derived from a childhood T-cell acute lymphoblastic leukemia, were also assayed as a control. CD20, CD5, CD34, HDAC3, and β-actin levels were checked by semiquantitative RT-PCR analysis (Figure 4B). We confirmed CD20 expression in RRBL1 cells in the absence of TSA (Figure 4B, lane 1), and adding TSA significantly enhanced CD20 expression (lane 2). In contrast, we detected no CD20 expression in Jurkat cells, with or without TSA. We also assayed for CD5, CD34, and HDAC3 as controls. In the presence of TSA, we detected no induction of gene expression other than for CD20. With the same RNA, we also conducted a quantitative RT-PCR analysis (Figure 4C). In the presence of TSA, we detected a >20-fold increase in CD20 expression in RRBL1 cells. These experiments were repeated, and similar results were obtained. The findings for TSA-treated RRBL1 cells suggest

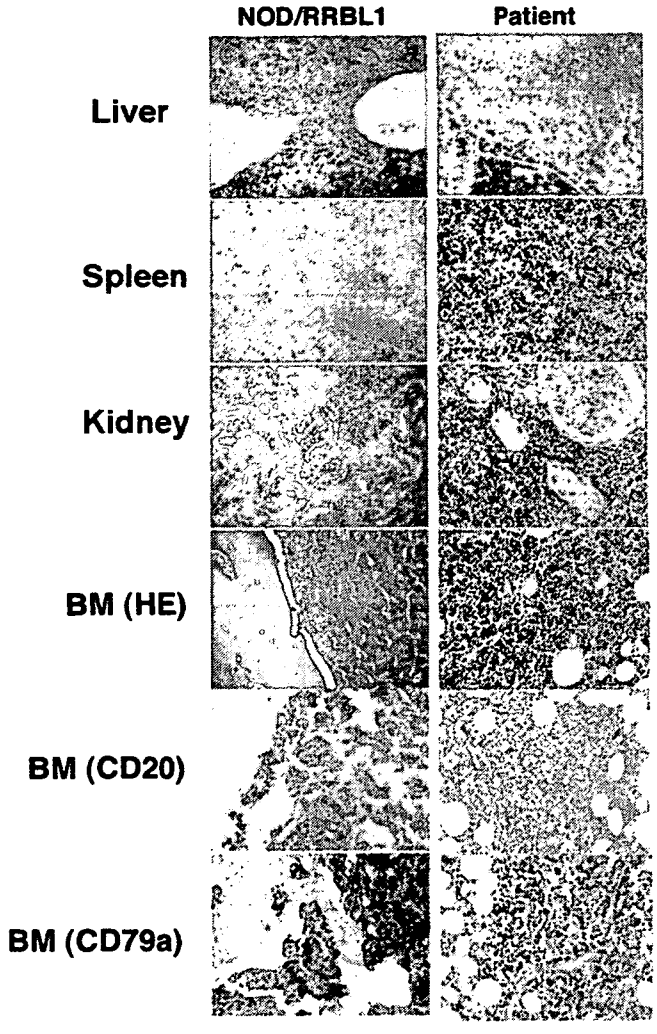

Figure 5. RRBL1 cells (5×10^6) were injected intravenously into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Eight weeks after transplantation, mice were anesthetized and sacrificed, and tissues were prepared for pathologic analyses. Diffuse infiltration of RRBL1 cells into liver, spleen, kidney, and bone marrow (BM) was observed (left column, hematoxylin-eosin [HE] staining). Immunostaining with anti-CD20 and anti-CD79a antibodies was also performed. Note that tumor cells show no CD20 expression (BM [CD20]). The right column presents HE staining and immunostaining results for the patient's original tumor obtained by autopsy. The diffuse infiltration pattern of $CD20^-$ tumor cells closely resembles that of the NOD/SCID-RRBL1 transplantation model.

that *CD20* gene expression is epigenetically repressed, and that this repression produces the $CD20^-$ protein phenotype in RRBL1 cells.

3.4. Xenograft Transplantation of RRBL1 Cells into Immunodeficient NOD/SCID Mice

We speculated that RRBL1 cells could be used as a model of rituximab-resistant $CD20^-$ lymphoma cells and that the cell line would become a more powerful in vivo model if a mouse transplantation model could be established. As indicated in "Materials and Methods," RRBL1 cells were injected subcutaneously into NOD/SCID mice, and tumor growth was observed by measuring tumor size. Thirty days after transplantation, significant tumor expansion was observed in the dorsal region of the mice, and tumor growth continued for 53 days after transplantation (data not shown). This phenomenon indicates that RRBL1 cells can be maintained in NOD/SCID mice. Next, RRBL1 cells were injected intravenously into the tail vein of NOD/SCID mice, and tumor growth was observed. Fifty days after transplantation, organs were removed from the anesthetized mice and subjected to pathologic examinations (Figure 5). Massive infiltration of lymphoid cells was observed in the liver, spleen, kidney, and bone marrow, and the cells did not express CD20 antigen. To determine whether these cells had a B-cell phenotype, we immunostained the same specimens with antibody to CD79a (a known pan–B-cell marker). The results indicated that these infiltrating tumor cells had a human B-cell origin ($CD79a^+$) and maintained the $CD20^-$ phenotype even in vivo. We also compared these pathologic findings with an autopsy specimen obtained from the patient. As indicated in Figure 5 (right column), we found similar tumor invasion of the liver, spleen, kidney, and bone marrow. These experiments indicate that the NOD/SCID-RRBL1 transplantation system mimics the patient's clinical features.

4. Discussion

More than 5 years have passed since rituximab was first used to treat $CD20^+$ B-cell malignancies, including indolent and aggressive lymphomas, in combination with conventional chemotherapies such as the CHOP regimen [25,26]. Recently, considerable numbers of patients with B-cell lymphoma have been recognized to have a rituximab-resistant phenotype [9-12]. Rituximab resistance has been predicted to possibly become a serious problem for the treatment of not only de novo but also refractory B-cell malignancies. From these viewpoints, we think that at least 2 goals should be considered: first, elucidation of the mechanisms behind the $CD20^-$ phenotype; and second, overcoming the resistance to rituximab.

In this report, we describe an RRBL1 cell line, which we derived from a $CD20^-$ DLBCL transformed from an indolent follicular lymphoma after repeated treatment with rituximab. It is interesting that $CD20^-$-transformed lymphoma cells from the patient carried an additional chromosomal abnormality, t(2;8)(p12;q24), in addition to t(14;18). This translocation had previously been reported to most likely involve the *c-Myc* and κ light chain genes and sometimes to coexist with t(14;18) in association with high-grade transformation [27]. There is a possibility that this additional abnormality contributes to the $CD20^-$ transformation and that the transformed $CD20^-$ cells have survived repeated rituximab administrations. Further investigation is needed to test this possibility.

Little CD20 protein was detected in RRBL1 cells by immunohistochemistry and flow cytometry with anti-CD20 antibody. It is interesting that quantitative RT-PCR analysis showed CD20 mRNA expression to be significantly weaker than in $CD20^+$ B-cell lymphoma cells. In addition, immunoblot-

ting with whole-cell lysate from RRBL1 cells showed a faint band for endogenous CD20 expression (data not shown). This lower expression level of CD20 mRNA likely explains the $CD20^-$ phenotype and the resistance to chemotherapy with rituximab originally observed in the patient. Also very interesting was that we successfully induced expression of CD20 mRNA and protein in RRBL1 cells within 48 hours after adding TSA. To explain the low level of CD20 mRNA/protein expression in B-cell lymphoma cells, we suggest investigating the possibility of aberrant transcriptional repression of CD20 mRNA expression.

A number of possibilities for aberrant transcriptional repression can be considered: (1) aberrant transcription regulation by genetic mutation in the CD20 gene promoter region; (2) genetic loss of the CD20 gene; (3) epigenetic changes in CD20 expression, including aberrant CpG methylation [28] in the CD20 promoter region; and (4) abnormalities of transcription factors and/or transcriptional regulators closely associated with CD20 mRNA expression. Mechanisms (1) and (2) are genetic; (3) and (4) are epigenetic. Our findings using TSA strongly suggest that CD20 mRNA expression may be aberrantly down-regulated in RRBL1 cells via some epigenetic mechanisms. Further analyses of CD20 gene expression mechanisms are warranted, especially those that focus on histone-modification status (acetylation and/or methylation), CpG-methylation status of genomic DNA, and the recruitment of specific transcription factors/coregulators in the vicinity of the CD20 promoter region.

Genetic mutations in the CD20-coding sequence have recently been reported as one of the mechanisms of rituximab resistance [10]. Rituximab may not be able to recognize mutated CD20 if the genetic mutation occurs in a specific region, because rituximab recognizes the higher-order protein structure of CD20 [29]. In our case, the CD20 protein in TSA-treated RRBL1 cells was successfully recognized by the anti-CD20 antibody, suggesting that any potential amino acid substitution in the CD20 protein in RRBL1 cells is unrelated to the $CD20^-$ phenotype (as detected by immunoblotting and flow cytometry). In fact, we have confirmed that the CD20 coding sequence and its promoter sequence (approximately 1000 bases upstream from the transcription start sites) do not have any genetic mutations (data not shown). These findings strongly suggest that the reduced expression of CD20 mRNA in RRBL1 cells is closely related to down-regulation of gene expression by epigenetic mechanisms.

We also have shown in this report that RRBL1 cells can be transplanted into NOD/SCID mice. Importantly, the pathologic findings revealed that these RRBL1-transplanted mice mimic the clinical features of liver, spleen, and bone marrow infiltration. Thus, RRBL1 cells promise to be not only a useful cell line for analyzing the mechanisms responsible for the $CD20^-$ phenotype in vitro but also a helpful in vivo system for investigating sensitivity to rituximab, with or without the administration of other drugs, including molecular-targeting drugs.

Finally, if the low level of mRNA expression is one of the reasons for the $CD20^-$ phenotype, stimulation of CD20 mRNA expression may be a good strategy for overcoming resistance to rituximab in vivo. Thus, other kinds of epigenetic drugs, such as DNA methyltransferase inhibitors [28] and histone methyltransferase inhibitors [30], may stimulate CD20 expression, and RRBL1 cells may provide a useful screening system for obtaining such epigenetic drugs. Our findings also may provide useful information for developing a new molecular-targeting therapy combined with epigenetic therapy for $CD20^-$ and/or $CD20^{low}$ B-cell lymphomas.

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References

1. Cheson BD. Monoclonal antibody therapy for B-cell malignancies. *Semin Oncol.* 2006;33:S2-S14.
2. Imai K, Takaoka A. Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer.* 2006;6:714-727.
3. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med.* 2002;346:235-242.
4. van Oers MH, Klasa R, Marcus RE, et al. Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin's lymphoma both in patients with and without rituximab during induction: results of a prospective randomized phase III intergroup trial. *Blood.* 2006;108:3295-3301.
5. Habermann TM, Weller EA, Morrison VA, et al. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. *J Clin Oncol.* 2006;24:3121-3127.
6. Pfreundschuh M, Trumper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. *Lancet Oncol.* 2006;7:379-391.
7. Hiddemann W, Kneba M, Dreyling M, et al. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood.* 2005;106:3725-3732.
8. Czuczman MS, Weaver R, Alkuzweny B, Berlfein J, Grillo-Lopez AJ. Prolonged clinical and molecular remission in patients with low-grade or follicular non-Hodgkin's lymphoma treated with rituximab plus CHOP chemotherapy: 9-year follow-up. *J Clin Oncol.* 2004;22:4711-4716.
9. Alvaro-Naranjo T, Jaen-Martinez J, Guma-Padro J, Bosch-Princep R, Salvado-Usach MT. CD20-negative DLBCL transformation after rituximab treatment in follicular lymphoma: a new case report and review of the literature. *Ann Hematol.* 2003; 82:585-588.
10. Terui Y, Sakurai T, Mishima Y, et al. Identification of CD20 mutations in malignant lymphoma: can they be predictors of

response to rituximab? [abstract]. *Blood.* 2005;106:88a. Abstract 288.
11. Davis TA, Czerwinski DK, Levy R. Therapy of B-cell lymphoma with anti-CD20 antibodies can result in the loss of CD20 antigen expression. *Clin Cancer Res.* 1999;5:611-615.
12. Kinoshita T, Nagai H, Murate T, Saito H. CD20-negative relapse in B-cell lymphoma after treatment with rituximab. *J Clin Oncol.* 1998;16:3916.
13. Smith MR. Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. *Oncogene.* 2003;22:7359-7368.
14. Ninomiya M, Abe A, Yokozawa T, et al. Establishment of a myeloid leukemia cell line, TRL-01, with *MLL-ENL* fusion gene. *Cancer Genet Cytogenet.* 2006;169:1-11.
15. Mason DY, Comans-Bitter WM, Cordell JL, Verhoeven MA, van Dongen JJ. Antibody L26 recognizes an intracellular epitope on the B-cell-associated CD20 antigen. *Am J Pathol.* 1990;136:1215-1222.
16. Atsumi A, Tomita A, Kiyoi H, Naoe T. Histone deacetylase 3 (HDAC3) is recruited to target promoters by PML-RARα as a component of the N-CoR co-repressor complex to repress transcription *in vivo. Biochem Biophys Res Commun.* 2006;345:1471-1480.
17. Kiyoi H, Naoe T, Kitamura K, Yamauchi T, Ichihashi T, Ohno R. Disappearance of minimal residual lymphoblastic leukemia cells 6 months after allogeneic bone marrow transplantation without GVHD. *Bone Marrow Transplant.* 1991;8:59-62.
18. Gribben JG, Freedman A, Woo SD, et al. All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and after treatment. *Blood.* 1991;78:3275-3280.
19. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin mu locus: characterization of embryonic and rearranged J and D genes. *Cell.* 1981;27:583-591.
20. Tomita A, Buchholz DR, Shi YB. Recruitment of N-CoR/SMRT-TBLR1 corepressor complex by unliganded thyroid hormone receptor for gene repression during frog development. *Mol Cell Biol.* 2004;24:3337-3346.
21. Tomita A, Buchholz DR, Obata K, Shi YB. Fusion protein of retinoic acid receptor α with promyelocytic leukemia protein or promyelocytic leukemia zinc finger protein recruits N-CoR-TBLR1 corepressor complex to repress transcription *in vivo. J Biol Chem.* 2003;278:30788-30795.
22. Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ. Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. *Proc Natl Acad Sci U S A.* 1988;85:4869-4873.
23. Kosugi H, Towatari M, Hatano S, et al. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. *Leukemia.* 1999;13:1316-1324.
24. Hsia SC, Tomita A, Obata K, Paul B, Buchholz D, Shi YB. Role of chromatin disruption and histone acetylation in thyroid hormone receptor action: implications in the regulation of HIV-1 LTR. *Histol Histopathol.* 2003;18:323-331.
25. Maloney DG, Grillo-Lopez AJ, White CA, et al. IDEC-C2B8 (rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood.* 1997;90:2188-2195.
26. Maloney DG, Grillo-Lopez AJ, Bodkin DJ, et al. IDEC-C2B8: results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma. *J Clin Oncol.* 1997;15:3266-3274.
27. Pegoraro L, Palumbo A, Erikson J, et al. A 14;18 and an 8;14 chromosome translocation in a cell line derived from an acute B-cell leukemia. *Proc Natl Acad Sci U S A.* 1984;81:7166-7170.
28. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature.* 2004;429:457-463.
29. Binder M, Otto F, Mertelsmann R, Veelken H, Trepel M. The epitope recognized by rituximab. *Blood.* 2006;108:1975-1978.
30. Greiner D, Bonaldi T, Eskeland R, Roemer E, Imhof A. Identification of a specific inhibitor of the histone methyltransferase SU(VAR)3-9. *Nat Chem Biol.* 2005;1:143-145.
31. Kiyoi H, Naoe T. Immunoglobulin variable region structure and B-cell malignancies. *Int J Hematol.* 2001;73:47-53.

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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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

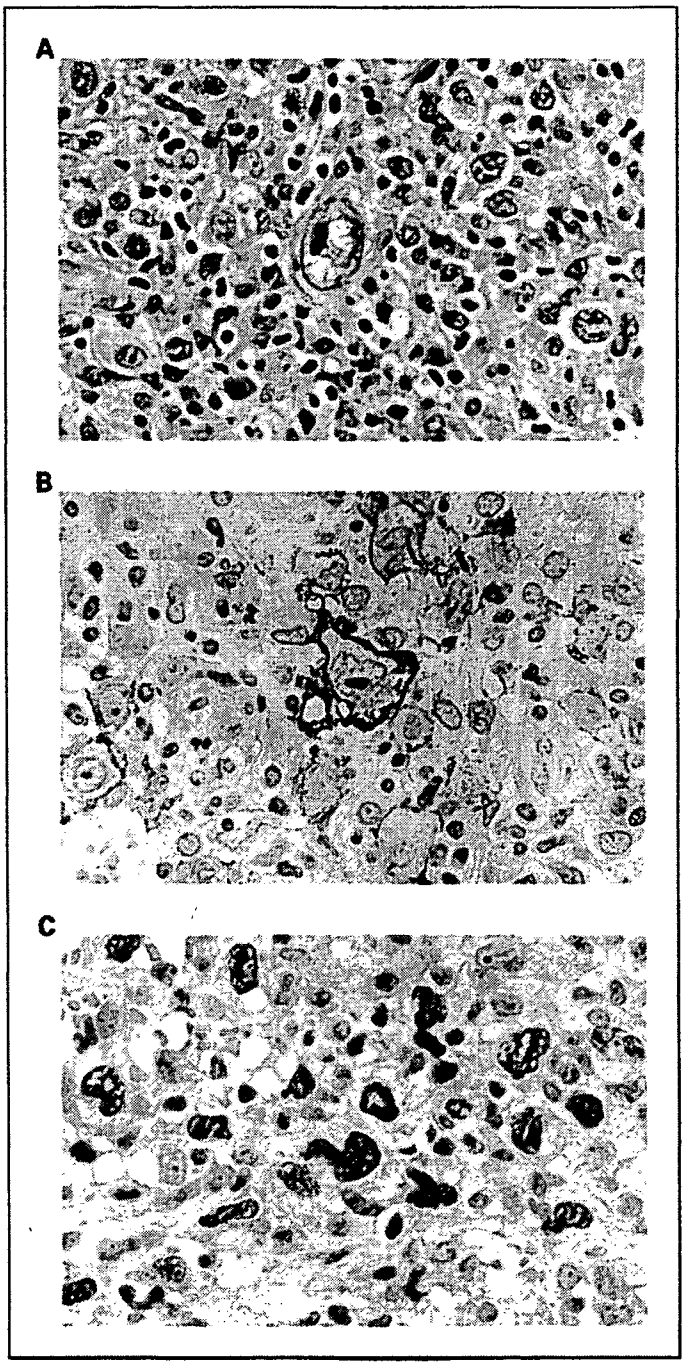

Fig. 1. Senile EBV-associated B-cell LPD, polymorphic subtype, arising in a 62-year-old male. The lesion reveals scattered distribution of Hodgkin and Reed-Sternberg–like giant cells (*A*, ×150), which are positive for CD20 (*B*, ×125). These large cells showed the expression of EBNA2 (*C*, ×125) in 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;

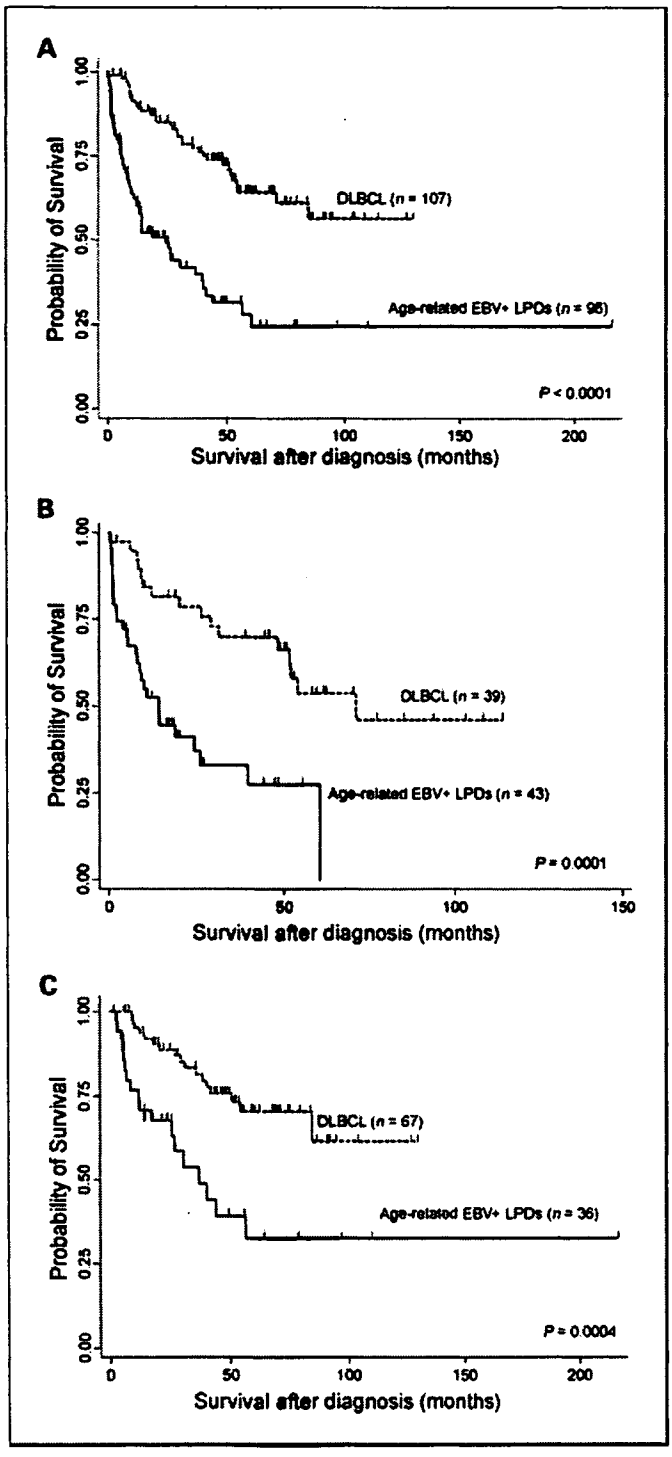

Fig. 2. Overall survival for patients with age-related EBV+ B-cell LPDs and EBV-negative DLBCLs. Age-related EBV+ B-cell LPDs (n = 96) show significantly worse survival than DLBCLs (n = 107) in all patients (A), patients with high-intermediate and high IPI risk (n = 43 and n = 39, respectively; B), and 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 ≤ 60, 60 < age ≤ 75, or age > 75) by performing the stratified log-rank test (P < 0.0001). Overall survival curves according to IPI are shown in Fig. 2B 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. 3A).

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

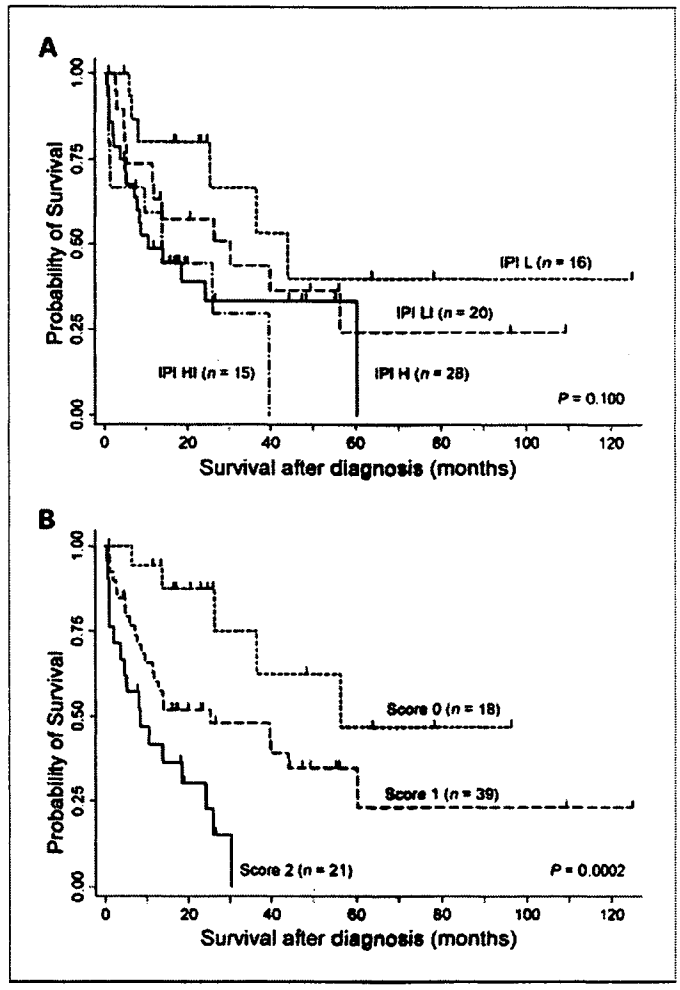

Fig. 3. Overall survival according to IPI (A) and prognostic model based on two simple clinical variables of age older than 70 y and the presence of B symptoms (B) in age-related EBV+ B-cell LPDs. This prognostic model is able to efficiently identify three groups of patients with different outcomes; 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.

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References

1. Fisher R, MillerT, O'Connor O. Diffuse aggressive lymphoma. In: Broudy V, Berliner N, Larson R, Leung L, editors. Hematology 2004 (Am Soc Hematol Educ Program Book). American Society of Hematology: 2004; p. 221–36.
2. Yamaguchi M, Seto M, Okamoto M, et al. *De novo* CD5+ diffuse large B-cell lymphoma: a clinicopathologic study of 109 patients. Blood 2002;99:815–21.
3. Murase T, Nakamura S, Tashiro K, et al. Malignant histiocytosis-like B-cell lymphoma, a distinct pathologic variant of intravascular lymphomatosis: a report of five cases and review of the literature. Br J Haematol 1997;99:656–64.
4. Said W, Chien K, Takeuchi S, et al. Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) in primary effusion lymphoma: ultrastructural demonstration of herpesvirus in lymphoma cells. Blood 1996;87:4937–43.
5. Nakatsuka S, Yao M, Hoshida Y, Yamamoto S, Iuchi K, Aozasa K. Pyothorax-associated lymphoma: a review of 106 cases. J Clin Oncol 2002;20:4255–60.
6. Oyama T, Ichimura K, Suzuki R, et al. Senile EBV+ B-cell lymphoproliferative disorders: a clinicopathologic study of 22 patients. Am J Surg Pathol 2003;27:16–26.
7. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med 2004;350:1328–37.
8. Cohen JI. Epstein-Barr virus infection. N Engl J Med 2000;343:481–92.
9. Rooney CM, Rowe M, Wallace LE, Rickinson AB. Epstein-Barr virus-positive Burkitt's lymphoma cells not recognized by virus-specific T-cell surveillance. Nature 1985;317:629–31.
10. Kelly G, Bell A, Rickinson A. Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. Nat Med 2002;8:1098–104.
11. Swinnen LJ. Overview of posttransplant B-cell lymphoproliferative disorders. Semin Oncol 1999;26:21–5.
12. Kuze T, Nakamura N, Hashimoto Y, Sasaki Y, Abe M. The characteristics of Epstein-Barr virus (EBV)–positive diffuse large B-cell lymphoma: comparison between EBV(+) and EBV(-) cases in Japanese population. Jpn J Cancer Res 2000;91:1233–40.
13. Hamilton-Dutoit SJ, Raphael M, Audouin J, et al. *In situ* demonstration of Epstein-Barr virus small RNAs (EBER 1) in acquired immunodeficiency syndrome-related lymphomas: correlation with tumor morphology and primary site. Blood 1993;82:619–24.
14. Kuzushima K, Kimura H, Hoshino Y, et al. Longitudinal dynamics of Epstein-Barr virus-specific cytotoxic T lymphocytes during posttransplant lymphoproliferative disorder. J Infect Dis 2000;182:937–40.
15. Harris NL, Jaffe ES, Diebold J, et al. The World Health Organization classification of hematological malignancies report of the Clinical Advisory Committee Meeting, Airlie House, Virginia, November 1997. Mod Pathol 2000;13:193–207.
16. Knowles DM. Immunodeficiency-associated lymphoproliferative disorders. Mod Pathol 1999;12:200–17.
17. Kimura H, Hoshino Y, Kanegane H, et al. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. Blood 2001;98:280–6.
18. Siegert W, Nerl C, Agthe A, et al. Angioimmunoblastic lymphadenopathy (AILD)-type T-cell lymphoma: prognostic impact of clinical observations and laboratory findings at presentation. The Kiel Lymphoma Study Group. Ann Oncol 1995;6:659–64.
19. Quintanilla-Martinez L, Fend F, Moguel LR, et al. Peripheral T-cell lymphoma with Reed-Sternberg-like cells of B-cell phenotype and genotype associated with Epstein-Barr virus infection. Am J Surg Pathol 1999;23:1233–40.
20. Carbone A, Gloghini A, Zagonel V, Tirelli U. Expression of Epstein-Barr virus-encoded latent membrane protein 1 in nonendemic Burkitt's lymphomas. Blood 1996;87:1202–4.
21. Tsai DE, Hardy CL, Tomaszewski JE, et al. Reduction in immunosuppression as initial therapy for posttransplant lymphoproliferative disorder: analysis of prognostic variables and long-term follow-up of 42 adult patients. Transplantation 2001;71:1076–88.
22. Project TIN-HsLPF. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med 1993;329:987–94.
23. Project TN-HsLC. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. Blood 1997;89:3909–18.
24. Ouyang Q, Wagner WM, Walter S, et al. An age-related increase in the number of CD8+ T cells carrying receptors for an immunodominant Epstein-Barr virus (EBV) epitope is counteracted by a decreased frequency of their antigen-specific responsiveness. Mech Ageing Dev 2003;124:477–85.
25. Cen H, Williams PA, McWilliams HP, Breinig MC, Ho M, McKnight JL. Evidence for restricted Epstein-Barr virus latent gene expression and anti-EBNA antibody response in solid organ transplant recipients with posttransplant lymphoproliferative disorders. Blood 1993;81:1393–403.
26. Yasunaga J, Sakai T, Nosaka K, et al. Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: its implications in the immunodeficient state. Blood 2001;97:3177–83.
27. Harada S, Kamata Y, Ishii Y, et al. Maintenance of serum immunoglobulin G antibodies to Epstein-Barr virus (EBV) nuclear antigen 2 in healthy individuals from different age groups in a Japanese population with a high childhood incidence of asymptomatic primary EBV infection. Clin Diagn Lab Immunol 2004;11:123–30.
28. Guinee D, Jr., Jaffe E, Kingma D, et al. Pulmonary lymphomatoid granulomatosis. Evidence for a proliferation of Epstein-Barr virus infected B-lymphocytes with a prominent T-cell component and vasculitis. Am J Surg Pathol 1994;18:753–64.
29. Delecluse HJ, Anagnostopoulos I, Dallenbach F, et al. Plasmablastic lymphomas of the oral cavity: a new entity associated with the human immunodeficiency virus infection. Blood 1997;89:1413–20.
30. Weiss LM, Movahed LA, Warnke RA, Sklar J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. N Engl J Med 1989;320:502–6.
31. Hjalgrim H, Askling J, Rostgaard K, et al. Characteristics of Hodgkin's lymphoma after infectious mononucleosis. N Engl J Med 2003;349:1324–32.
32. Clarke CA, Glaser SL, Dorfman RF, et al. Epstein-Barr virus and survival after Hodgkin disease in a population-based series of women. Cancer 2001;91:1579–87.
33. Stark GL, Wood KM, Jack F, Angus B, Proctor SJ, Taylor PR. Hodgkin's disease in the elderly: a population-based study. Br J Haematol 2002;119:432–40.
34. Jarrett RF, Stark GL, White J, et al. Impact of tumor Epstein-Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma: a population-based study. Blood 2005;106:2444–51.
35. Murray PG, Billingham LJ, Hassan HT, et al. Effect of Epstein-Barr virus infection on response to chemotherapy and survival in Hodgkin's disease. Blood 1999;94:442–7.
36. Flavell KJ, Billingham LJ, Biddulph JP, et al. The effect of Epstein-Barr virus status on outcome in age- and sex-defined subgroups of patients with advanced Hodgkin's disease. Ann Oncol 2003;14:282–90.
37. Gandhi MK, Tellam JT, Khanna R. Epstein-Barr