Jpn. J. Cancer Res. **91**, 1233–1240, December 2000

The Characteristics of Epstein-Barr Virus (EBV)-positive Diffuse Large B-Cell Lymphoma: Comparison between EBV$^+$ and EBV$^-$ Cases in Japanese Population

Tetuo Kuze,[1, 2, 3] Naoya Nakamura,[1] Yuko Hashimoto,[1] Yoshikazu Sasaki[1] and Masafumi Abe[1]

[1]First Department of Pathology, Fukushima Medical University, 1 Hikari-ga-oka, Fukushima 960-1295 and [2]Safety Research Section, Research Department, TOAEIYO Ltd., 1 Tanaka, Iizaka, Fukushima 960-0280

We have investigated 114 cases with diffuse large B-cell lymphoma (DLBCL) to clarify the characteristics of DLBCL with Epstein-Barr virus (EBV) infection. Thirteen cases (11.4%) showed EBV-encoded RNA 1 (EBER1) signals by RNA *in situ* hybridization. EBV-encoded latent membrane protein 1 (LMP1) and EBV-encoded nuclear antigen 2 (EBNA2) were expressed in 11 and 4 cases, respectively. Expression of CD30, Bcl-6 and immunoglobulin (Ig) was found in 92%, 31% and 23% with EBV$^+$ DLBCL, and in 15%, 79% and 82% with EBV$^-$ DLBCL, respectively. The sequence of rearranged Ig heavy chain (IgH) variable (V) region gene was analyzed in 5 cases with EBV$^+$ DLBCL and 61 cases with EBV$^-$ DLBCL. Somatic mutation was found in all cases except one with EBV$^-$ DLBCL. Average mutation frequency was 9.6% in EBV$^+$ DLBCL vs. 11.5% in EBV$^-$ DLBCL. The rates of replacement mutation vs. silent mutation (R/S values) in complementarity determining region II and framework region III were 2.7 and 1.5 in EBV$^+$ DLBCL, 2.6 and 1.4 in EBV$^-$ DLBCL. Crippling mutation generating a stop codon was found in 2 of 5 cases (40%) with EBV$^+$ DLBCL, but none of 61 cases (0%) with EBV$^-$ DLBCL. These findings suggest that EBV$^+$ DLBCL and EBV$^-$ DLBCL were both derived from germinal center (GC) or post-GC B cells, and EBV$^+$ DLBCL frequently have a non-functional IgH gene owing to crippling mutation.

Key words: Epstein-Barr virus — Diffuse large B-cell lymphoma — Immunoglobulin heavy chain genes — Somatic mutation

In a Revised European-American Classification of Lymphoid Neoplasms (REAL),[1)] diffuse large B-cell lymphoma (DLBCL) is a morphologically heterogeneous group of non-Hodgkin lymphomas (NHLs) characterized by a diffuse proliferation of large neoplastic lymphoid cells with B-cell phenotype. DLBCL is the most common lymphoma type, and comprises 30–40% of adult NHLs. Based on morphologic features, DLBCL is subgrouped into centroblastic lymphoma (CB), immunoblastic lymphoma (IB), anaplastic large cell lymphoma of B type (B-ALCL) and T cell/histiocyte-rich B-cell lymphoma (TCR-BCL).

Infection with Epstein-Barr virus (EBV), a DNA virus of the herpes virus family, occurs asymptomatically in most cases and results in a life-long immunity. The EBV was first detected in a cell culture derived from an African Burkitt's lymphoma (BL).[2)] Subsequently, EBV has been found to be associated with nasopharyngeal carcinoma, endemic BL, a subset of cases of Hodgkin's disease (HD), and lymphomas developing in the setting of immunosuppression and immunodeficiency.[3)] The EBV genome has been occasionally detected in DLBCL, and Hamilton-Dutouit and Pallesen[4)] reported that EBV-encoded latent membrane protein 1 (LMP1) was detected in 3 of 54 cases of IB and 1 of 4 cases of B-ALCL by immunohistochemistry (IHC). Hummel *et al.*[5)] detected EBV-encoded RNA (EBER) signals in tumor cells in 10 of 83 cases with DLBCL, comprising 3 of 39 cases of CB, 4 of 28 cases of IB and 3 of 16 cases of B-ALCL, by an RNA *in situ* hybridization (RNA-ISH) method. We have reported that EBER1 signals were expressed in virtually all tumor cell nuclei of 6 of 17 cases with B-ALCL, particularly in large pleomorphic cells.[6)] The immunophenotypic and molecular genetic characteristics of DLBCL with EBV infection (EBV$^+$ DLBCL) remain unclear, however, and information about these tumors is limited.

In this study, we have investigated 114 cases of DLBCL in a Japanese population using IHC and molecular techniques. To clarify the characteristics of EBV$^+$ DLBCL, 13 cases of EBV$^+$ DLBCL and 101 cases of EBV$^-$ DLBCL were compared. Most of the EBV$^+$ DLBCL showed LMP1 and CD30 expression, while Bcl-6 and immunoglobulin (Ig) were found in several cases. The analysis of the sequence of rearranged Ig heavy chain (IgH) variable (V) region genes in 5 cases with EBV$^+$ DLBCL and 61 cases with EBV$^-$ DLBCL revealed high frequencies of somatic mutation except in one case with EBV$^-$ DLBCL. Two of 5 cases with EBV$^+$ DLBCL had a non-functional IgH gene owing to crippling mutation, but no case with EBV$^-$ DLBCL displayed crippling mutation.

[3] To whom correspondence should be addressed.
E-mail: kuze.tetsuo@toaeiyo.ne.jp

MATERIALS AND METHODS

Cases One hundred and fourteen cases with DLBCL filed in the First Department of Pathology, Fukushima Medical University for 16 years from 1981 through 1997 were used for the present study. The pathologic diagnosis of DLBCL was established according to the REAL classification.[1)]

The cases consisted of 67 male and 47 female cases, and their ages ranged from 11 to 89 years (mean 64.1 years). Seventy-one cases had local or generalized lymphadenopathy and 43 cases had extranodal lesion. The histological subtypes consisted of 79 cases of CB, 13 cases of IB, 18 cases of B-ALCL and 4 cases of TCRBCL.

IHC Immunohistochemical staining was performed on periodate-lysine-paraformaldehyde-fixed frozen sections or formalin-fixed, paraffin-embedded sections using the avidin-biotin-peroxidase complex method. Monoclonal antibodies used in this study were as follows: CD5 (Leu1, Becton Dickinson, Sunnyvale, CA, or CD5, Novocastra, Newcastle, UK), CD10 (Calla, Becton Dickinson, or CD10, Novocastra), CD30 (Ki-1, Dako, Glostrup, Denmark, or BerH2, Dako), Bcl-6 (Bcl-6, Santa Cruz, Santa Cruz, CA), CD138/syndecan1 (CD138, Serotec, Oxford, UK), Ig (γ, α, δ and μ, Becton Dickinson, or Dako), LMP1 (LMP1, Dako) and EBV-encoded nuclear antigen 2 (EBNA2, Dako).

RNA-ISH procedure for the detection of EBV RNA-ISH on formalin-fixed paraffin-embedded sections were performed using digoxigenin-labeled EBER1 antisense probe, according to a previously described method.[7)] In brief, deparaffinized and dehydrated tissue sections were predigested with pronase and hybridized with the probe at a concentration of 0.25 μg/ml. After washes, the EBER1 signals were detected with a Digoxigenin Nucleic Acid Detection kit (Boehringer Mannheim Biochemica, Mannheim, Germany).

Polymerase chain reaction (PCR) amplification and sequence analysis of IgH V region DNA samples obtained from frozen tissues or formalin-fixed paraffin-embedded tissues were digested with proteinase K, extracted with phenol/chloroform and precipitated by ethanol.

A semi-nested PCR was performed using DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, OH), according to a previously described method.[8)] In brief, the first amplification was performed using an upstream consensus V region primer (FR2A) and a lowstream joining (J) region primer (LJH). For the reamplification, the LJH was replaced by a nested consensus J region primer (VLJH), and the amplified product (1%) of the first round was transferred as a template. An aliquot of the reaction mixture was analyzed on an ethidium bromide-stained 3% agarose or 6% polyacrylamide gel. The PCR products were directly

Table I. Clinical Features, Immunophenotypes and *EBV* Gene and Gene Products Expression in EBV$^+$ DLBCL

Case No.	Age	Sex	Site[a)]	Prognosis (months)	Histological type	Immunophenotypes						*EBV* gene and gene products		
						CD5	CD10	CD30	CD138	Bcl-6	Ig	*EBER1*[b)]	*LMP1*[c)]	*EBNA2*[c)]
1	87	Female	Cervical LN, axillary LN, inguinal LN	ND	Centroblastic	−	−	+	−	−	−	+	−	−
2	54	Male	*Cervical LN*	39, alive	Anaplastic	−	−	+	−	+	−	+	+	−
3	77	Female	*Inguinal LN*, cervical LN, abdominal LN	1, dead	Centroblastic	−	−	+	−	−	−	+	+	−
4	75	Male	*Inguinal LN*, cervical LN, retroperitoneal LN	1, dead	Centroblastic	−	−	+	−	+	M	+	+	−
5	89	Female	*Ilium*, paraaortic LN	1, dead	Anaplastic	−	−	+	−	−	−	+	+	+
6	55	Female	*Tonsil*, cervical LN	24, alive	Centroblastic	−	−	+	−	+	−	+	+	+
7	80	Male	*Salivary gland*, cervical LN, submandibular LN, *inguinal LN*	1, dead	Centroblastic	−	−	+	−	+	−	+	+	−
8	78	Male	*Axillary LN*, inguinal LN	19, alive	Anaplastic	−	−	+	−	−	−	+	+	−
9	82	Female	*Supraclavicular LN*, cervical LN, axillary LN	13, dead	Centroblastic	−	−	+	−	−	−	+	+	−
10	61	Male	*Axillary LN*	139, alive	Anaplastic	−	−	+	−	−	−	+	+	+
11	63	Male	*Cervical LN*	3, dead	Anaplastic	−	−	+	−	−	G	+	+	−
12	63	Male	*Supraclavicular LN*	26, alive	Anaplastic	−	−	+	−	−	D	+	+	+
13	59	Male	*Cervical LN*, axillary LN, inguinal LN, subcutaneous, hepatosplenomegaly	39, dead	TCRBCL	−	−	−	−	−	−	+	−	−

a) The biopsy material is in italics.
b) *EBER1* was detected by RNA *in situ* hybridization.
c) *LMP1* and *EBNA2* were detected by immunohistochemistry.
LN, lymph node; ND, not done.

sequenced by the dye primer method using a Thermo Sequenase Core Sequencing Kit (Thermo Sequenase Core Sequencing Kit with 7-deaza-dGTP; Amersham, Cleveland, OH). Then, oligonucleotide sequences of complementarity determining region II (CDRII) and framework region III (FWIII) were analyzed using a Hitachi SQ-5500 (Tokyo) and compared with the germ line sequences recorded in the GenBank database.

Intraclonal variation of rearranged IgH V region gene was examined according to the previously described method.[9] The PCR products were ligated into the pCR^R 2.1 vector and transformed into TOP10F' cells according to the instruction manual (original TA cloning kit, Invitrogen, Carlsbad, NM). The colony direct PCR assay was used to examine whether colonies included the expected PCR products. Ten or more white colonies were picked up and put into 50 μl of Insert Check ready (Toyobo Co., Ltd., Osaka). PCR conditions consisted of 30 cycles of 95°C for 20 s, 60°C for 5 s and 72°C for 30 s. Then, 9 or 10 samples including the expected PCR products as confirmed by check-electrophoresis for each case were sequenced by the same method as described above.

Statistical methods Statistical analysis was performed using the χ^2 test, Student's *t* test, Kaplan-Meier survival test and Mantel-Haenszel life-table (log-rank test). Tests for comparison were regarded as significant if the two-sided *P* value was less than 0.05 ($P<0.05$).

RESULTS

Clinical features and immunophenotypes The clinical features, immunophenotypes and expression of *EBV* gene and *EBV* gene products in 13 cases with EBV+ DLBCL are summarized in Table I. The age of patients with EBV+ DLBCL ranged from 54 to 89 years (mean 71.0 years), and the male-to-female ratio was 8:5. Three patients had extranodal lesions, and the other patients had local or generalized lymphadenopathy. The histological subtypes comprised 7 cases with CB, 6 cases with B-ALCL and 1 case with TCRBCL.

The clinical data and immunophenotypes of EBV+ DLBCL and EBV− DLBCL are compared in Table II. There was no significant difference in mean age of patients, male-to-female ratio, primary sites (nodal site or extranodal site), or survival time (Fig. 1) between EBV+ DLBCL and EBV− DLBCL. CD5, CD10, CD30, CD138, Bcl-6 and Ig were expressed in 0%, 0%, 92%, 0%, 31% and 23% of EBV+ DLBCL, and 7%, 12%, 15%, 3%, 79% and 82% of EBV− DLBCL, respectively. There was a statistically significant difference in CD30, Bcl-6 and Ig expression between EBV+ DLBCL and EBV− DLBCL ($P<0.05$ by the χ^2 test).

Detection of *EBV* gene (EBER1) and gene products (LMP1 or EBNA2) Thirteen of 114 cases showed EBER1 signals in the nuclei of almost all the tumor cells by RNA-ISH. Among them, LMP1 and EBNA2 were detected in 11 cases and 4 cases, respectively.

Table II. Comparison of Clinical Data between EBV+ DLBCL and EBV− DLBCL

	EBV+ DLBCL (*n*=13)	EBV− DLBCL (*n*=101)
Age		
Mean	71.0	63.3
Range	54–89	11–87
Median age	75	66
(unknown)		(2)
Sex		
Male	8	59
Female	5	42
Site		
Nodal	10	61
Extranodal	3	40
Prognosis		
5-year survival	0.333	0.321
Histological type		
Centroblastic	6	73
Immunoblastic	0	13
Anaplastic	6	12
T-cell/histiocyte rich	1	3
Immunophenotypes		
CD5	0/13 (0%)	6/89 (7%)
CD10	0/13 (0%)	11/89 (12%)
CD30	12/13 (92%)	15/97 (15%)
CD138	0/13 (0%)	2/72 (3%)
Bcl-6	4/13 (31%)	55/70 (79%)
Ig	3/13 (23%)	75/92 (82%)

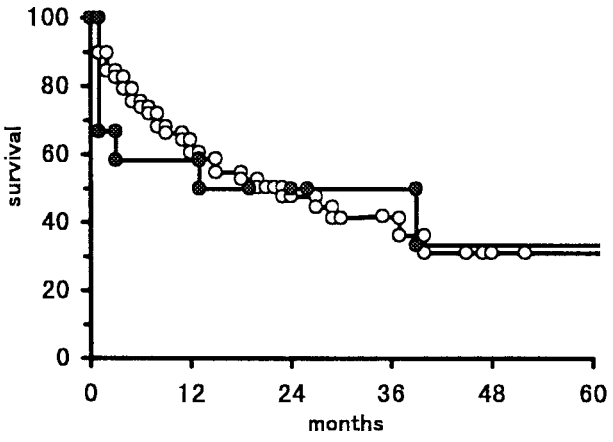

Fig. 1. Survival curves of patients with EBV+ DLBCL (●, *n*=12) and EBV− DLBCL (○, *n*=58).

Table III. Somatic Mutations of Rearranged IgH V Region Genes in EBV$^+$ DLBCL

Case No.	Germline usage[a)]	Homology (%)	No. of nucleotide differences		Replacement/silent		Intraclonal diversity[b)]
			CDRII (%)	FWIII (%)	CDRII	FWIII	
1	VH3/VH3-8	98.6	0 (0.0)	2 (2.1) S	—	1	Yes (2/9)
5	VH4/DP-79	93.8	2 (4.2)	8 (8.3)	∞ (2/0)	0.6	Yes (4/10)
9	VH3/V3-64	76.9	14 (27.5)	20 (20.8)	1.8	2.3	ND
10	VH3/YAC-9	93.5	6 (10.5)	4 (4.2) 1D&S	5	3	No (0/10)
12	VH3/DP-47	89.8	11 (21.6) 9I	4 (4.2)	2.7	1	No (0/10)

a) According to GenBank, using BLASTN for data bank comparison.
b) No. of heterogeneous clones in parenthesis.
Abbreviations: CDR, complementarity determining region; FW, framework region; ND, not done; S, stop codon; D, deletion; I, insertion.
Case 1 had a stop codon in the FWIII region.
Case 10 contained a one base deletion and had a stop codon in the FWIII region.
Case 12 contained a 9-base insertion in the CDRII region.

Table IV. Comparison of the Frequency of Somatic Mutation in the IgH V Region between EBV$^+$ DLBCL and EBV$^-$ DLBCL

	No. of examined cases	No. of cases with IgH V mutations	CDRII			FWIII			Average mutation frequency (%)
			Replacement mutations	Silent mutations	(R/S)	Replacement mutations	Silent mutations	(R/S)	
EBV$^+$ DLBCL	5	5	24	9	(2.7)	23	15	(1.5)	9.6
EBV$^-$ DLBCL	61	60	303	118	(2.6)	336	248	(1.4)	11.5

Sequence analysis of rearranged IgH V region genes in EBV$^+$ DLBCL and EBV$^-$ DLBCL The sequences in 5 of 13 cases with EBV$^+$ DLBCL were identified, but 8 cases could not be sequenced because 6 showed no PCR product (smear band) and 2 gave rise to multiple PCR products in analysis. The sequences obtained were compared with published germline V region sequences. All 5 cases showed somatic mutation of rearranged IgH V region genes (Table III). Crippling mutation generating a stop codon was found in 2 cases. Case 1 with in-frame rearrangement had a stop codon caused by single nucleotide exchange. Case 10 had an out-of-frame rearrangement caused by one base deletion with the result that a new stop codon was formed. Case 12 showed an in-frame rearrangement with 9-base insertion and the other 2 cases (cases 5 and 9) were in-frame.

From 101 cases with EBV$^-$ DLBCL, 61 sequences were obtained and compared with published germline V region sequences. Among them, 60 cases showed somatic mutation of rearranged IgH V region genes. No case displayed out-of-frame or crippling mutation. One case showed in-frame rearrangement with deletion of 15 bases. Forty cases failed to be sequenced because 28 showed a smear band and 12 gave rise to multiple PCR products.

The frequencies of somatic mutation in IgH V region genes in EBV$^+$ DLBCL and EBV$^-$ DLBCL are compared in Table IV. Average values of mutation frequency of the IgH V region in 5 cases with EBV$^+$ DLBCL and 60 cases with EBV$^-$ DLBCL were 9.6% (range; 1.4–23.1%) and 11.5% (range; 0.7–32.0%), respectively. The rates of replacement mutation vs. silent mutation (R/S) were 24 vs. 9 (2.7) in CDRII and 23 vs. 15 (1.5) in FWIII with EBV$^+$ DLBCL, and 303 vs. 118 (2.6) in CDRII and 336 vs. 248 (1.4) in FWIII with EBV$^-$ DLBCL.

Intraclonal variation of rearranged IgH V region genes in EBV$^+$ DLBCL Intraclonal heterogeneity was analyzed in 4 cases (cases 1, 5, 10 and 12). The sequences obtained are shown in Fig. 2. In 2 cases (cases 10 and 12), the sequences of all 10 clones were identical, indicating the mutation process was switched off. Sequence variations were observed in 2 cases. In the case 1, sequence variations were found in 2 of 9 clones, but none of these mutations occurred in more than one clone. In case 5, sequence variations were found in 4 of 10 clones, and one mutation was shared in 2 clones.

DISCUSSION

Although Asians generally have a high incidence of various EBV-associated tumors as compared to Europeans and Americans, some findings on EBV-association in Japanese seem to resemble those in Europeans. For example, the rate of EBV positivity in T cell lymphoma of Japanese cases (11% by Southern blotting)[10] was similar to that of

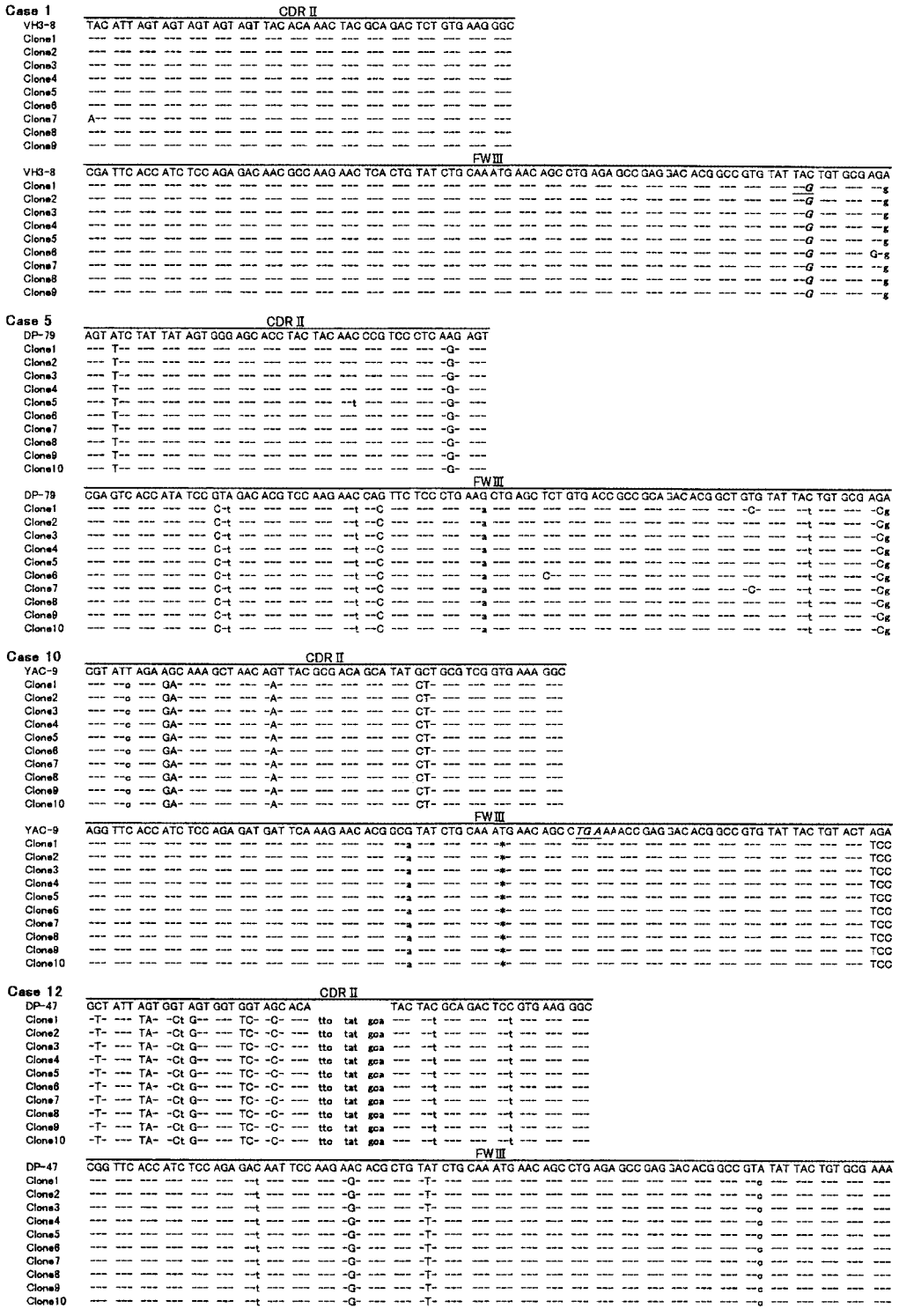

Fig. 2. Intraclonal sequence heterogeneity in four patients with EBV+ DLBCL (cases 1, 5, 10 and 12). The first line displays the published Ig H V region germline genes most homologous to our sequencing data. The second and following lines show our sequencing data. Identity with the germline sequence is shown by dashes, replacement mutation by upper case letters, silent mutation and insertion by lower case letters, and deletion by an asterisk (*). Underlined codons are stop codons originating from mutation. CDR, complementarity determining region: FW, framework region.

European cases (10% by IHC),[4] and was less than that of Taiwanese cases (20% by Southern blotting)[11] or Chinese cases (62% by RNA-ISH).[12] In DLBCL, Hummel *et al.*[5] reported that EBER signals were expressed in tumor cells in 10 of 83 European cases (12%) by RNA-ISH. We found a similar frequency in Japanese cases (13 of 114 cases, 11%) by RNA-ISH.

In our 13 EBV^+ cases, there were 2 cases (15%) of type I latency ($EBER^+$, $LMP1^-$, $EBNA2^-$), 7 cases (54%) of type II latency ($EBER^+$, $LMP1^+$, $EBNA2^-$) and 4 cases (31%) of type III latency ($EBER^+$, $LMP1^+$, $EBNA2^+$). Type III latency is usually associated with immunocompromised status. The coexpression of LMP1 and EBNA2, the hallmark of type III latency, has been documented in nonimmunocompromised DLBCL in 3 European cases[4, 13] and one case in Hong Kong,[14] but most cases with EBV^+ DLBCL were negative for EBNA2. The rate of type III latency in our series was higher than that of the previous reports.[4, 5, 13] One possible explanation for this discrepancy is differences between the patient populations, not only race and genetic factors, but also geographic factors.

CD30, an activation-associated antigen, is expressed in EBV-transformed or immortalized cell lines, activated B and T cells and some lymphoproliferative disorders such as adult T-cell leukemia/lymphoma, ALCL and Reed-Sternberg (RS) cells of HD.[3] In this study, all cases with EBV^+ DLBCL except one were positive for CD30, and most cases with EBV^- DLBCL, except B-ALCL, were negative for CD30. B-ALCL accounted for 6 of 13 cases (46%) with EBV^+ DLBCL and 12 of 101 cases (12%) with EBV^- DLBCL. The rates of B-ALCL and CD30 expression in EBV^+ DLBCL were significantly higher than those of EBV^- DLBCL ($P<0.05$ by the χ^2 test). These data indicate that EBV may be associated with anaplastic large cell morphology and CD30 expression in tumor cells.

Bcl-6 is a novel proto-oncogene that is located in the 3q27 region. Bcl-6 protein is constitutionally expressed by the germinal center (GC) B cells and most cases of DLBCL.[15–17] On the other hand, CD138 is expressed in specific subsets of post-GC B cells, including immunoblasts and plasma cells. It is reported that expression of Bcl-6 and CD138 can discriminate between GC ($Bcl\text{-}6^+$ and $CD138^-$) and post-GC ($Bcl\text{-}6^-$ and $CD138^+$) B cells.[18] In our series, expression of Bcl-6 and CD138 was found in 31% and 0% of EBV^+ DLBCL cases and in 79% and 3% of EBV^- DLBCL cases, respectively. Most RS cells in classical HD show no expression of Bcl-6 protein, although the RS cells in classical HD are now recognized to be derived from GC B cells.[19] These data suggest that EBV infection may directly downregulate the level of Bcl-6 protein expression, as in classical HD.

A semi-nested PCR method for the detection of rearranged IgH genes was not always able to amplify monoclonal rearrangements of IgH genes. IgH gene rearrangements were reported in 16 of 24 cases (67%) of B-HD, 6 of 10 cases (60%) of DLBCL and 6 of 12 cases (50%) of follicular lymphoma.[20] In this study, IgH gene rearrangements were detected in 5 of 13 cases (38%) with EBV^+ DLBCL and 61 of 101 cases (60%) with EBV^- DLBCL. Failure to detect IgH gene rearrangements by the PCR method might be attributable to 1) degradation of the DNA in specimens, 2) somatic mutations at sites complementary to the primers, causing the primers to lose their binding capacity or 3) specimens harboring a relatively high fraction of polyclonal, non-malignant B cells that prevent the identification of the tumor clone.

Somatic mutation of rearranged IgH V region genes in B cells occurs in GC during their differentiation and plays a major role in generating antibody diversity.[21, 22] The analysis of the sequence of rearranged IgH V region genes has proved to be suitable for determining the stage of differentiation of normal and tumor B cells.[22–26] Most DLBCL cases showed somatic mutation of IgH V region genes.[27–31] In this study, we have demonstrated somatic mutation of rearranged IgH V region genes in all 5 cases with EBV^+ DLBCL and 60 of 61 cases with EBV^- DLBCL. Average values of mutation frequency of the IgH V region were 9.6% with EBV^+ DLBCL, and 11.5% with EBV^- DLBCL. The R/S values of EBV^+ DLBCL and EBV^- DLBCL were 2.7 and 2.6 in CDRII, and 1.5 and 1.4 in FWIII, respectively. There was no difference in frequency of somatic mutation between EBV^+ DLBCL and EBV^- DLBCL. These findings suggest that both EBV^+ DLBCL and EBV^- DLBCL are derived from GC B cells or post-GC B cells.

Crippling mutation generating a stop codon was found in 2 of 5 cases (40%) with EBV^+ DLBCL. No case with EBV^- DLBCL showed an out-of-frame rearrangement or crippling mutation. An out-of-frame rearrangement rarely occurs in normal B cells.[22] In lymphoid malignancies, most reported cases displayed an in-frame rearrangement of IgH gene except one case of DLBCL[28] and some cases of HD.[32, 33] Furthermore, crippling mutations within in-frame rearrangements were reported in classical HD. The EBV has been detected in more than half of classical HD. These results suggest that EBV^+ DLBCL as well as classical HD frequently has non-functional IgH genes caused by crippling mutation, resulting in a reduction of Ig expression. Crippling mutation is not the cause of oncogenesis in classical HD, but might be a phenomenon of EBV-infected neoplasms.

The consequent intraclonal heterogeneity is commonly found in follicle center lymphoma and might fit with the location of these tumors in the GC, where the mutation mechanism is likely to be activated. A similar pattern has been reported in some cases of BL and mucosa-associated lymphatic tissue lymphomas. However, the majority of B-

cell tumors, including chronic lymphocytic leukemia, lymphoplasmacytoid tumors and multiple myeloma show stable homogeneous IgH gene sequences.[23–26] In DLBCL, intraclonal heterogeneity was observed in some cases,[30] but not in other cases.[27] The resulting microheterogeneity is also present during lymphoma progression and in response to lymphoma treatment and relapse. In this study, intraclonal heterogeneity was analyzed in 4 cases of EBV+ DLBCL: sequence variations were observed in 2 cases and 2 other cases had identical sequences. Further studies will be required to establish whether the mechanism of intraclonal heterogeneity in DLBCL is associated with EBV infection or not.

In conclusion, there was some significant differences between EBV+ DLBCL and EBV− DLBCL. Most of EBV+ DLBCL showed LMP1 and CD30 expression, and reduction or lack of Bcl-6 and Ig proteins in tumor cells. The rearranged IgH V region genes in EBV+ DLBCL revealed somatic mutation, and frequently had a non-functional IgH gene owing to crippling mutation. The immunophenotypic and molecular genetic characteristics of EBV+ DLBCL are similar to those of HD rather than EBV− DLBCL.

ACKNOWLEDGMENTS

The authors thank Mr. Takeo Ito and Miss Michiko Sato for their excellent technical assistance.

(Received June 26, 2000/Revised September 19, 2000/Accepted September 25, 2000)

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