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Detection of Epstein-Barr Virus DNA in Reed-Sternberg Cells of Hodgkin's Disease Using the Polymerase Chain Reaction and *in situ* Hybridization

Hisashi Uhara,[1,3] Yuichi Sato,[1] Kiyoshi Mukai,[1,4] Ichiro Akao,[1] Yoshihiro Matsuno,[1] Shuichiroh Furuya,[1] Tomohide Hoshikawa,[2] Yukio Shimosato[1] and Toshiaki Saida[3]

[1]*Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104,* [2]*Department of Otorhinolaryngology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 213 and* [3]*Department of Dermatology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390*

Thirty-one cases of Hodgkin's disease were examined for the occurrence of Epstein-Barr virus (EBV) genome by using the polymerase chain reaction (PCR) of DNA in formalin-fixed paraffin-embedded tissues and the *in situ* hybridization technique. The cases were subdivided into 17 cases of nodular sclerosis (NS), nine cases of mixed cellularity (MC), four cases of lymphocyte predominance (LP), and one case of lymphocyte depletion (LD). EBV DNA was detected in eight cases including four cases of NS, three cases of MC and one case of LP. The sensitivity of PCR was higher than that of Southern blot hybridization of DNA from fresh frozen tissue, because Southern blot hybridization using the *Bam*HI-W fragment of EBV detected virus DNA only in two of three cases which were positive by PCR. The results of *in situ* hybridization studies confirmed that EBV genome was localized within the nuclei of Reed-Sternberg (RS) cells and their mononuclear variants. Furthermore, double-labeling studies combining *in situ* hybridization and immunocytochemistry using CD30 (BerH2) and CD15 (LeuM1) as markers of RS cells, as well as pan B-marker (L26) and pan T-marker, CD45RO (UCHL1), were performed to demonstrate the phenotype of EBV DNA-positive cells, confirming that EBV DNA was present in RS cells but not in lymphocytes. The results of this study indicate a significant association between EBV and some cases of Hodgkin's disease.

Key words: Epstein-Barr virus — Hodgkin's disease — Polymerase chain reaction — *In situ* hybridization

Hodgkin's disease (HD) is a unique lymphoproliferative disorder characterized by the presence of Reed-Sternberg (RS) cells in a mixed population of inflammatory cells. In spite of extensive immunological and immunogenetic studies, neither the pathogenesis of HD nor the nature of RS cells has been clarified.[1–5] One interesting recent suggestion is the possible participation of Epstein-Barr virus (EBV) in the pathogenesis of HD. Epidemiologic studies have indicated that the disease may result from an infection, in particular with EBV.[6–8] However, the low sensitivity of current assays and the scarcity of malignant cells in the tissue specimens have made it difficult to detect EBV DNA in tissues of HD patients. Recent Southern blot studies using the *Bam*HI-W fragment within Internal Repeat 1 as a probe have demonstrated the presence of EBV DNA in about 20% of patients with HD.[9–12] However, even this type of analysis may not be sensitive enough to detect EBV DNA because of the scarcity of RS cells in tissue specimens, as discussed above. In this study, we attempted to detect EBV DNA in tissues of HD patients using the very sensitive polymerase chain reaction (PCR) of DNA extracted from formalin-fixed paraffin-embedded tissues. In addition, the *in situ* hybridization technique and a double-labeling technique combining *in situ* hybridization and immunocytochemistry were applied for characterization of EBV DNA-positive cells.

MATERIALS AND METHODS

Cases A total of 31 cases of HD were used for the study. The diagnosis was based on both morphological and immunocytochemical findings. Neoplastic cells in all cases were positive for CD15 and/or CD30 antigen. These cases were subdivided into 17 cases of nodular sclerosis (NS) type, nine cases of mixed cellularity (MC) type, four cases of lymphocyte predominance (LP) type, and one case of lymphocyte depletion (LD) type. Among the 31 cases, biopsy specimens taken prior to therapy were available in 21 and the remaining 10 patients had already been treated at the time of biopsy. In addition, frozen tissues were available in nine cases and these were used in Southern blot hybridization. Thirteen cases of non-Hodgkin lymphoma (NHD), nine cases of adult T-cell leukemia/lymphoma (ATLL) and six cases of reactive lymphadenopathy were also studied for comparison. Results of serological examination as to previous

[4] To whom correspondence should be addressed.

and current EBV infection status were not available for any of the cases.

PCR conditions The PCR method was based on the procedure described by Impraim *et al.*,[13] but with modifications. DNA for PCR was extracted from formalin-fixed paraffin-embedded tissues (six10-μm-thick sections) according to the methods described previously.[14] A DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) was used for temperature regulation of PCR, which consisted of denaturation for 1 min at 94°C, annealing ıor 2 min at 55°C, and extension for 3 min at 72°C. One μg each of archival DNA samples was subjected to 25 cycles of PCR amplification using a GeneAmp DNA Amplification Reagent Kit (Perkin Elmer Cetus). The products of PCR were electrophoresed in 3% agarose gel, and transferred to nitrocellulose membrane. The membranes were hybridized with a [^{32}P]dATP 5′ end-labeled oligomer probe for 1 h at 42°C and washed twice in washing buffer (2×SSC, 20 m*M* NaPB, 0.06% NaPPi, 0.05% SDS) for 1 h at 70°C. Autoradiography was carried out for 4 h at −80°C with Hyperfilm-MP (Amersham International, Amersham, Bucks, England). DNA extracted from an EBV-positive cell line, Raji, was used as a positive control, and DNA extracted from an EBV-negative cell line, Ramos, served as a negative control (both cell lines were obtained from the Japanese Cancer Research Resources Bank, Tokyo).

Oligomer primers and a probe illustrated in Fig. 1 were chosen within the Internal Repeat 1[15] of EBV DNA and synthesized on an Applied Biosystems Model 381A (Applied Biosystems Inc., Foster City, CA). They were purified using oligonucleotide purification cartridges (Applied Biosystems) and stored desiccated at −20°C.

***In situ* hybridization** Paraffin sections, 2.5 μm thick, were subjected to *in situ* hybridization as described previously with several modifications.[16] The paraffin sections were dewaxed in xylene, washed in absolute ethanol, immersed in 0.2 *N* HCl for 30 min, then immersed for 15 min at 37°C in 50 m*M* Tris HCl (pH 7.4) containing 100 μg/ml proteinase K, and treated with 10 μg/ml RNase in phosphate-buffered saline (PBS) for 1 h at 37°C. After enzyme treatment, the tissue sections were post-fixed with 4% paraformaldehyde for 5 min at room temperature, washed three times in 2 g/liter glycine in PBS for 3 min, and dehydrated through a graded ethanol series. The pretreated slides were air-dried and layered with 20 μl of hybridization mixture containing the biotinylated EBV probe (ENZO Biochem Inc., New York, NY) or cytomegalovirus probe (ENZO) at a concentration of 2 μg/ml. After being sealed with rubber cement and a cover slip, the slides were subjected to denaturation for 10 min at 92°C and incubated at 42°C for 16 h. After hybridization, the coverslip was gently removed in 2× SSC and the slides were washed for 1 h each in 2×SSC with 50% formamide at 37°C and in 1×SSC with 50% formamide at 37°C, then three times in 1×SSC for 30 min at 37°C. The slides were immersed in 3% bovine serum albumin in Buffer 1 (0.1 *M* Tris HCl, pH 7.5, 0.1 *M* sodium chloride, 2 m*M* magnesium chloride, 0.05% Triton X-100) for 30 min, then covered with 1 μg/ml streptavidin-alkaline phosphatase conjugate (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, MD) in Buffer 1, and incubated at 37°C for 1 h. The slides were washed twice for 10 min in Buffer 1 and twice for 5 min in Buffer 2 (0.1 *M* Tris HCl, pH 9.5, 0.1 *M* sodium chloride, 50 m*M* magnesium chloride), and visualized in 66 μl of 75 mg/ml nitroblue tetrazolium (NBT) in 70% dimethyl formamide and 50 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethyl formamide in 15 ml of Buffer 2 until reaction signals were recognized by microscopy, and then counterstained with methyl green.

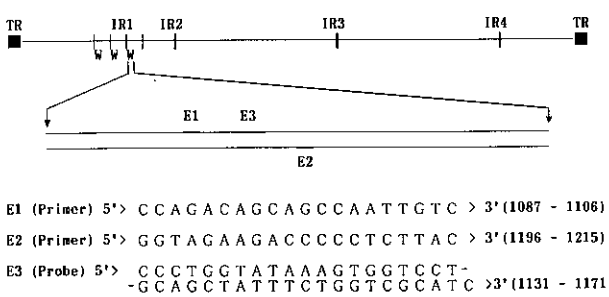

Fig. 1. Oligomer primers and probe used in the polymerase chain reaction. These were chosen from within the Internal Repeat 1 of EBV. The base numbers are from the published sequences.[15]

In the double-labeling study, formalin-fixed paraffin-embedded tissue sections were first stained immunocytochemically for CD15 (LeuM1, Beckton-Dickinson, Rutherford, NJ), CD30 (BerH2, Dakopatts, Glostrup, Denmark), pan T-marker, CD45RO (UCHL-1, DAKO), and pan B-marker (L26, DAKO) by the PAP method and then subjected to EBV DNA *in situ* hybridization as described above. CD15 and CD30 are markers of RS cells, although they are not strictry specific to this cell type.[1,17] 3,3′-Diaminobenzidine (DAB) was used as a chromogen for immunocytochemistry and NBT-BCIP for *in situ* hybridization.

Southern blot and slot-blot analysis of the sample DNA not subjected to PCR amplification In order to compare the sensitivity of PCR with that of Southern and slot blot analyses, Southern blot hybridization was performed on DNA extracted from frozen tissues of nine cases including three cases positive for EBV DNA by PCR. In addition, the results of slot-blot hybridization of DNA

extracted from paraffin-embedded tissues of all 31 cases were compared with those of PCR. Methods of DNA extraction from fresh tissue and formalin-fixed paraffin-embedded tissue were based on those described previously.[14, 18] For Southern blot hybridization, following *Bam*HI digestion of 10 μg of archival DNA, hybridization was performed using ^{32}P-labeled probe (3.0 kb *Bam*HI-W fragment of EBV was kindly provided by Dr. K. Hirai, Department of Virology and Immunology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo). In slot-blot hybridization, the copy number of viral DNA was estimated by comparison with Raji DNA (Raji cell line contains 50 copies of EBV/cell) serially diluted with DNA free of EBV sequence.

RESULTS

Detection of EBV DNA using the PCR method Eight of the specimens from the 31 patients (26%) with HD had detectable EBV genome (Fig. 2). The results are summarized in Table I. Three of the NS specimens and two of the MC specimens were obtained at initial biopsy and the remaining three were from patients who had received therapy prior to biopsy. The EBV genome was not detected in any of nine cases of B cell lymphoma, four cases of T cell lymphoma, six cases of reactive lymphadenopathy, or eight of nine cases of ATLL (Table I).

Sensitivity of PCR, Southern blot hybridization and slot-blot hybridization Viral DNA was detected in two of three cases (cases 4 and 5) positive for EBV by PCR, but the remaining one PCR-positive case (case 15) and six cases negative by PCR did not show any positive band (Fig. 3). A slot-blot hybridization study of DNA extracted from formalin-fixed paraffin-embedded tissue clearly detected at least a single copy of EBV per cell. However, all eight cases which were positive for EBV DNA by PCR were negative by slot-blot hybridization (data not shown). These results indicated that the amount of EBV DNA contained in each PCR-positive specimen was less than a single copy per cell.

***In situ* hybridization** *In situ* hybridization studies were performed on all 31 cases of HD and one EBV-positive

Table I. Cases and Results

	Positive cases/total (%)
Hodgkin's disease	8/31 (26)
Nodular sclerosis	4/17 (23)
Mixed cellularity	3/9 (30)
Lymphocyte predominance	1/4 (25)
Lymphocyte depletion	0/1 (0)
Non-Hodgkin lymphoma	0/13 (0)
Adult T-cell leukemia/lymphoma	1/9 (11)
Reactive lymph node	0/6 (0)

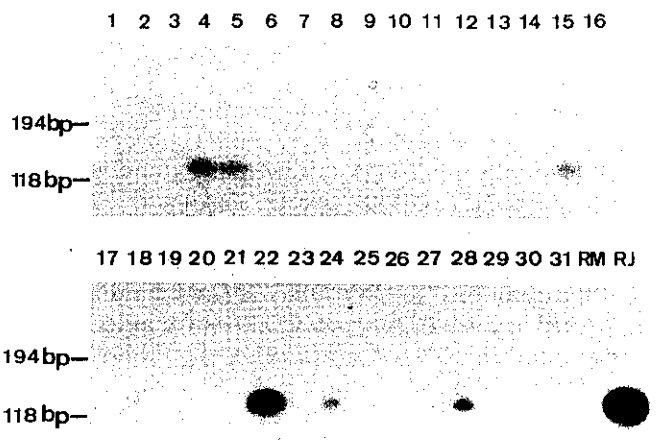

Fig. 2. Autoradiograph of PCR products of DNA extracted from tissues of HD patients. All positive bands migrated at the 129-bp position, characteristic of the target sequences for PCR. Lanes 1–31 are cases of HD, lane Rj is Raji DNA (positive control), and lane Rm is Ramos DNA (negative control). Positive bands are seen in lanes 4 (NS), 5 (NS), 15 (NS), 16 (NS), 18 (LP), 22 (MC), 24 (MC) and 28 (MC).

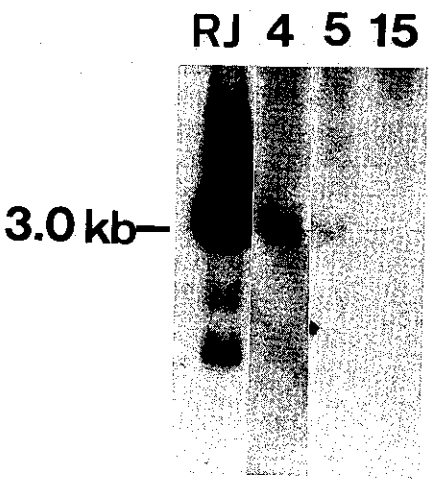

Fig. 3. Southern blot hybridization analysis of EBV DNA in tissues from HD patients. DNA extracted from tissue specimens was digested with *Bam*HI restriction enzyme and hybridized with a radiolabeled 3.0-kb *Bam*HI-W fragment as a probe. Lanes 4, 5 and 15 correspond to the patients in Fig. 2 and Table II. Lane Rj is Raji DNA containing 50 EBV copies per cell. No positive band is seen in lane 15.

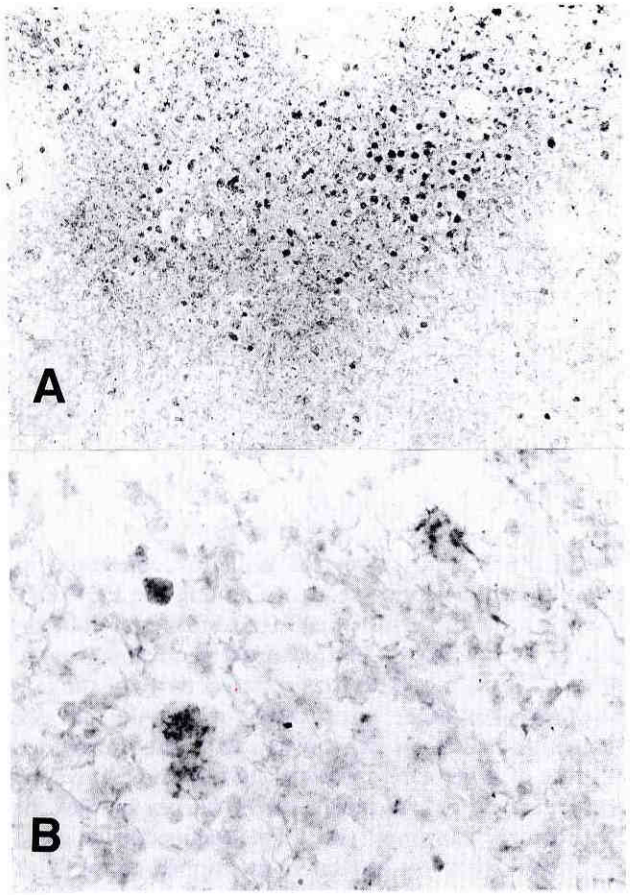

Fig. 4. *In situ* hybridization for EBV DNA in case 5. Strong nuclear labeling for EBV is present in many RS cells and their mononuclear variants (A). Granular reaction products were located in the nuclei of tumor cells (B). (A: ×82, B: ×330)

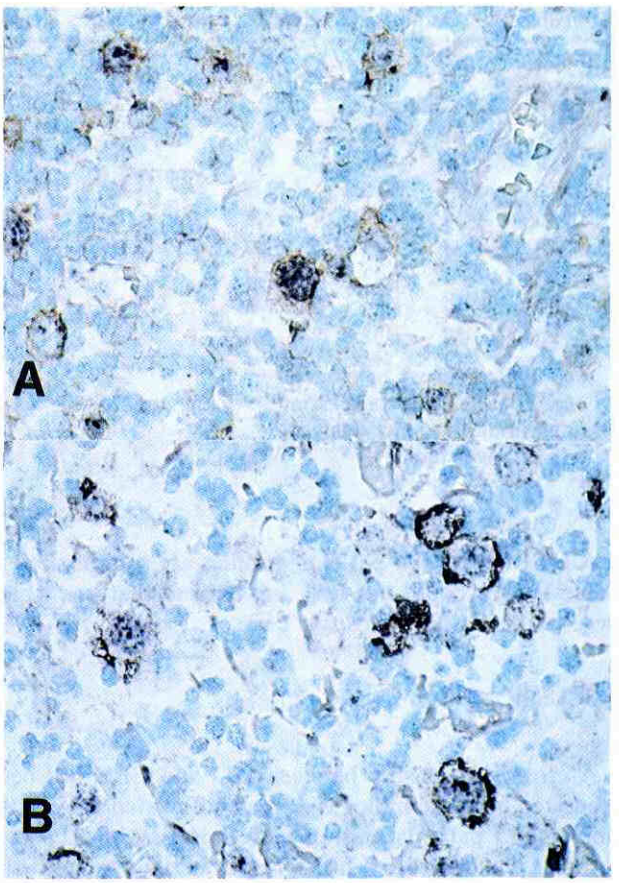

Fig. 5. A double-labeling experiment combining *in situ* hybridization and immunocytochemistry in case 4. RS cells with nuclear labeling for EBV (light violet) show paranuclear and cell surface brown staining for CD30 (A) or pan B-marker (B). (A, B: ×330)

case of ATLL. In all eight cases of HD which were positive by PCR, a positive hybridization reaction for EBV was seen in the nuclei of RS cells and their mononuclear variants. However, one case of EBV-positive ATLL was negative by this method. Cases 4, 5 and 22 showed strong signals and the remaining specimens showed a weaker hybridization reaction in only a minor population of RS cells. In case 5, whose specimen contained central necrosis, a strong hybridization signal was seen in a large number of remaining nuclei within the degenerated or necrotic areas (Fig. 4). Since cytomegalovirus DNA could not be detected in these nuclei, the reaction with the EBV probe was judged to be specific. Of the remaining 26 cases negative by PCR, no case showed a positive reaction for EBV.

To characterize the EBV-positive cells, we used a double-labeling method combining *in situ* hybridization with immunocytochemistry for cell surface markers. A positive staining reaction by immunocytochemistry was recognized as a deposit of light brown reaction product in the paranuclear region and/or cell surface, and that of *in situ* hybridization appeared light violet, thus being differentiated easily from positive immunocytochemical staining. These double-labeling studies were performed in three cases (cases 4, 5 and 22) which showed a strong *in situ* hybridization reaction for EBV. In case 4, EBV DNA was detected in cells positive for CD15, CD30, and pan B-marker, but negative for CD45RO (Fig. 5). In case 5, viral DNA was demonstrated in large cells showing weak positivity only for CD15. In case 22, virus DNA was detected in cells positive for both CD30 and CD15 but negative for pan B- or pan T-markers. Reactive lymphocytes stained by T cell or B cell markers showed no positive hybridization reaction for EBV.

Table II. Details of Positive Cases in Hodgkin's Disease

Case No.	Sex	Age	Diagnosis	P/R[a]	Immunocytochemistry				PCR	ISH[b]	S[c]
					CD15	CD30	Pan-B	CD45RO			
4	M	36	NS	P	+	+	+	−	+	+	+
5	M	51	NS	P	+	−	−	−	+	+	+
15	M	55	NS	R	−	+	−	−	+	+	−
16	M	67	NS	P	+	+	−	−	+	+	nd[d]
18	M	49	LP	R	+	−	−	−	+	+	nd
22	M	33	MC	R	+	+	−	−	+	+	nd
24	M	34	MC	P	−	+	−	−	+	+	nd
28	M	51	MC	P	+	+	−	−	+	+	nd

a) P/R: Primary or recurrence.
b) ISH: *in situ* hybridization.
c) S: Southern blot analysis.
d) nd: not studied.

All the results and patients' profiles are summarized in Table II. We could not find any clinical features which were unique to EBV-positive cases.

DISCUSSION

A close relationship between HD and EBV has been suggested by epidemiologic studies.[7] Elevated EBV titers among HD patients and an increased risk of HD were noted in persons with a history of infectious mononucleosis (IM).[6] Recently, the presence of EBV DNA was directly detected by Southern blot analysis in tissues of some HD patients. Weiss *et al.*,[9] who attempted to detect EBV genome in tissues of HD patients by Southern blot analysis, suggested that EBV genomes might be present in more cases of HD, but were not detected because the copy number of the EBV genome within the tissue was below the sensitivity of Southern blot analysis. Therefore, we tried to detect EBV DNA by using a very sensitive *in vitro* enzymatic amplification of DNA known as the polymerase chain reaction and detected the presence of EBV DNA in 26% of patients with HD. Since PCR uses a short segment of cellular DNA, formalin fixation and paraffin embedding probably did not have a serious effect on the sensitivity of the method. In fact, when sensitivity was compared between PCR using DNA in fixed tissue and Southern blot analysis of DNA from frozen tissue, it became obvious that the sensitivity of the former procedure was superior.

The amplifying target sequence for PCR was chosen within the Internal Repeat 1 (IR1) of EBV-genome, mainly for the following two reasons. Firstly, since this region contains seven to twelve identical sequences (w-fragment),[15] the sensitivity should be several times that obtainable by using a unique single sequence of the viral genome. Secondly, since IR1 is highly conserved as a homologous nucleotide sequence among the EBV DNA, it was expected that false-negative results caused by deletion or heterogeneity of the target sequence would be minimal. With regard to the cross reactivity of the target sequence with other virus DNA, it is true that some nucleotide regions in IR1 contain sequences homologous to those of other DNA viruses, such as the papova virus *ori*-sequence[15] or *a*-sequence of the herpes simplex virus 1[19]; however, the amplifying target sequence did not have any homology to the sequences mentioned above. Therefore, false-positive results due to cross reactivity with other DNA virus were not expected to occur.

The detection rate of EBV DNA in this study was similar to that of recent Southern blot studies carried out in the United States and West Germany.[9-12] Whether there is a significant difference in the positivity rate of EBV DNA in neoplastic tissue of HD patients in various parts of the world needs to be studied further using a larger number of cases. One advantage of our method is that DNA extracted from paraffin blocks can be analyzed with high sensitivity; therefore, retrospective studies such as ours become feasible without compromising the detection rate. Another advantage is that a direct correlation between morphology and the results of DNA analysis may be obtained. Because of its high sensitivity, PCR is an excellent tool for detection of EBV DNA in neoplasms such as HD, in which tumor cells are scarce in tissue specimens, and also when the quantity of biopsied tissue is small.

In addition to PCR, we performed *in situ* hybridization to define which cells contained EBV DNA, and a double-labeling technique combining *in situ* hybridization with immunocytochemistry to characterize the phenotype of EBV-positive cells. EBV DNA was detected in the nuclei of large cells positive for CD30 and/or CD15,

both markers of RS cells, but not in small lymphocytes positive for pan B- or pan T-marker. These results confirmed that the viral genome was localized in the nuclei of RS cells and their mononuclear variants. In contrast, no positive hybridization reaction was seen in one case of ATLL which was positive by PCR. Therefore, it was not clear whether EBV was present in the neoplastic cells in ATLL or in non-neoplastic B-cells. Even when the latter possibility is considered, the negative result of PCR in non-Hodgkin lymphomas and reactive lymph nodes suggests that the occurrence of the viral genome in this ATLL case does not simply reflect usual latent infection. It is likely that the number of viral gene copies in tissue specimens of this ATLL case was higher due to activation of the viral genome by the immunodeficient state of the patient.

In case 4 of this study, EBV DNA was detected in large cells which were positive for CD15, CD30 and pan B-marker. Since EBV receptor has been demonstrated in a fraction of B lymphocytes, it is attractive to speculate that some cases of HD are of B cell origin. However, B cell origin of our case 4 was not confirmed because immunoglobulin gene rearrangement was not detected in this case (unpublished data). Recently a case of T cell lymphoma caused by EBV infection was reported.[20] Therefore, the demonstration of EBV in cases of HD does not necessarily indicate the B-cell origin of HD.

A bimodal age-incidence curve is characteristic of HD, and MacMahon has suggested that HD results from two processes, and specifically that HD in young adults is caused by a biologic agent of low infectivity.[21] Weiss *et al.* reported four patients with EBV-positive HD[10] comprising a 10-year-old Hispanic boy (NS), a 29-year-old white man (NS), a 22-year-old white man (MC), and a 54-year-old Japanese-American woman. Anagnostopoulos *et al.* reported seven cases of EBV-positive HD; four patients were aged under 30 and the remaining three were over 50 years old.[12] In our study, seven patients younger than 30 years old (five NS and two MC, average age 17 years) were included. However, EBV could not be detected in any of these cases. Whether MacMahon's hypothesis is justified and whether there is a geographic difference in the age-specific incidence of EBV-positive HD remain to be investigated.

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