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Short Communication

Epstein–Barr Virus in Adult T-Cell Leukemia/Lymphoma

Masayoshi Tokunaga,* Shosuke Imai,† Yoshiko Uemura,* Takahiro Tokudome,* Toyoro Osato,† and Eiichi Sato‡

From the Department of Pathology, Kagoshima City Hospital, Kagoshima, Japan; Department of Virology,† Cancer Institute, Hokkaido University, School of Medicine, Kita-ku, Sapporo, Japan; and Second Department of Pathology,‡ Kagoshima University, School of Medicine, Kagoshima, Japan*

***Adult T-cell leukemia/lymphoma (ATLL) is a well-known human T-cell lymphotropic virus type-1-related disease. We studied Epstein–Barr virus (EBV) in the tumor cells of ATLL, to investigate the etiological significance of double infection with these viruses. We used polymerase chain reaction and EBV-encoded small RNA-1 in* situ *hybridization to investigate the presence of EBV and immunohistochemistry to detect EBV-related oncoproteins, such as EBV-determined nuclear antigen-2 and latent membrane protein. Polymerase chain reaction performed on DNA of frozen specimens from 96 cases of ATLL revealed that the tumor tissue from 21 cases contained EBV DNA. EBV-encoded small RNA-1 in* situ *hybridization performed on the paraffin sections of the polymerase chain reaction-positive cases indicated EBV in the nuclei of ATLL tumor cells in 16 cases, nine of which were in the pleomorphic nuclei. Latent membrane protein was also detected in the cytoplasm of ATLL tumor cells in 15 cases, and EBV nuclear antigen-2 was observed in the nuclei of ATLL tumor cells in 11 cases. We conclude that EBV was present within tumor cells in about 17% of cases with ATLL and expressed EBV oncoprotein in the tumor cells. It is hypothesized that EBV and human T-cell lymphotropic virus-1 may infect the same T cells in early life and may play a role in the oncogenesis of ATLL.** (Am J Pathol 1993, 143:1263–1269)*

There are two major viruses associated with lymphoid malignancies: human T-cell lymphotropic virus type-1 (HTLV-1) and Epstein–Barr virus (EBV). HTLV-1 is etiologically associated with adult T-cell leukemia/lymphoma (ATLL), which was first reported by Uchiyama et al[1] in Japan as a specific T-cell leukemia with unusual clinical, pathological, and epidemiological features. HTLV-1 has been isolated from T-cell lines established from ATLL patients in the USA[2] and Japan,[3] and its relationship to ATLL is well recognized.[4] ATLL occurs endemically in some restricted areas of the world, including Kagoshima, in Japan, where this study was performed. EBV was initially detected in African Burkitt's lymphoma[5] and has since been detected in lymphoproliferative disorders in immunodeficient individuals[6] and in Hodgkin's disease.[7] Recently, EBV-related T-cell lymphomas have been reported,[8–16] but the association of EBV with ATLL has not been reported previously. We investigated the presence of EBV in tumor cells of ATLL using polymerase chain reaction (PCR), EBV-encoded small RNA-1 (EBER-1) *in situ* hybridization (ISH), and immunohistochemistry of EBV oncoproteins.

Materials and Methods

We studied 96 consecutive cases of ATLL that were diagnosed at the Department of Pathology of Kagoshima City Hospital between January 1989 and December 1991. Diagnosis of ATLL was made by the immunohistochemical demonstration of T-cell

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Address reprint requests to Dr. Masayoshi Tokunaga, Chief, Department of Pathology, Kagoshima City Hospital, Kajiya-cho 20-17, Kagoshima, 892, Japan.

marker expression (CD2, CD3, CD4, T-cell receptor βF1) on the tumor cells of frozen sections in cases with pathological findings of malignant lymphoma and clinical findings of acute or lymphoma type of ATLL. All of these cases were seropositive for anti-HTLV-1 antibody at the initial diagnosis. We studied the initial biopsy specimens that had been stored either as frozen blocks at −80 C or paraffin blocks.

Polymerase Chain Reaction

DNA was extracted from a single 10-μ frozen section. PCR was performed with primers (TC60:5′-CCA-GAG-GAT-AGT-GGA-CTT-3′, and TC61:5′-GAC-CGG-TGC-CTT-CTT-AGG-3′) specific for the EBV *Bam*HI W region. Temperatures during 35 amplification cycles were 94 C, 50 C, and 72 C for 1 minute for each step. Positive controls, consisting of EBV-positive cell line (KUB-1013) and DNA taken from lymphadenitis with infectious mononucelosis, and negative controls of EBV-negative lymphadenitis were included in each experiment. The PCR products were electrophoresed in 3% agarose gel, and cases with distinct electrophoresis band were estimated to be PCR-positive.

In Situ *Hybridization*

In situ hybridization (ISH) was performed on formalin-fixed, paraffin-embedded tissue for PCR-positive cases with modifications of the method described by Chang et al.[17] Tissue sections 3-μ thick were deparaffinized with xylene and rehydrated using graded ethanol into 2× standard saline citrate. The slides were subjected to pronase digestion (protease type xxv; Sigma Chemical Co., St. Louis, MO) at a concentration of 1 mg/ml in 50 mmol/L Tris-hydrochloride (pH 7.4) and 5 mmol/L ethylenediaminetetraacetic acid for 10 minutes in 37 C, washed in glycine (2 mg/ml) in 0.1 mol/L Tris-hydrochloride (pH 7.5) and 0.1 mol/L sodium chloride, followed by dehydration through a graded ethanol series and air drying.

Then 100-μl hybridization mixture was placed on the slides and covered by silicon-treated coverglass, and hybridized overnight at 37 C. The hybridization mixture contained 50% (v/v) formamide, 10% (w/v) dextran sulfate, 20 mmol/L sodium phosphate (pH 7.4), 3× standard saline citrate, 1× Denhardt's solution, 100 μg/ml salmon sperm DNA, 125 μg/ml yeast transfer RNA, and 0.5 ng/μl digoxigenin-labeled oligonucleotide probe. The oligonucleotide probes of sense and anti-sense for EBER-1 were made as described[21] and labeled by digoxigenin labeling kit (Boehringer Mannheim) as suggested by the manufacturer. After removal of the coverglasses, slides were washed twice in 0.5× standard saline citrate for 15 minutes at room temperature. Hybridization was treated by an anti-digoxigenin antibody-alkaline phosphate conjugate (Boehringer Mannheim) for 1 hour at room temperature. The alkaline phosphate was visualized using the substrates nitrobluetetrazolium and 5-bromo-4-chloro-3-indolylphosphate (nucleic acid detection kit, Boehringer Mannheim) to produce a purple-black precipitate at the site of hybridization.

These sections were immunostained, using the antigens for pan T-cell makers (CD3 DAKO) and B-cell marker (L-26 DAKO) and detected by a standard avidin-biotin complex technique with a horseradish peroxidase-conjugated ABC kit (Bectastain). They were then visualized by diamiinobenzidine. The sections were finally counterstained using 2% methylgreen, air-dried, and mounted through xylene on Eukitt (O. Kindle, Germany). We used paraffin sections of two cases of infectious mononucleosis lymphadenitis for a positive control and the sense probe and paraffin sections of 50 cases with EBV non-related lymphadenitis for the negative controls.

Immunohistochemistry

Frozen sections containing lymphoma tissue from each case were studied for lymphocyte characterization using the monoclonal antibodies CD1 (OKT6), CD2 (OKT11), CD5 (OKT1), CD8 (OKT8) (200×–500× Ortho) CD3 (Leu4), CD4 (Leu3a), CD7 (Leu9), CD14 (Leu M3), CD15 (Leu M1), TCR-βF1 (10×–500× Becton-Dickinson, Mountainview, CA), CD19, CD21, CD30 (30×–500×, DAKO), CD20 (B1), CD25 (IL-2R) (Coulter clone, Hialeah, FL; 50× and 400×). Immunohistochemistry was completed by the alkaline phosphatase-labeled avidin technique.

For the EBV *Bam*W PCR-positive cases, monoclonal antibodies for EBV-related antigens were applied by the same immunohistochemical method described above. For the detection of latent membrane protein (LMP) and EBV nuclear antigen-2 (EBNA-2) tissue culture supernatant containing mouse monoclonal antibodies (CS1-4 and PE2) were used as previously described.[10]

Results

In the controls, the results of PCR corresponded very well to those obtained by ISH. ISH showed intense signals in the nuclei of lymphocytes that had

either T- or B-cell phenotype in cases of positive PCR products detected by the agarose gel electrophoresis. Twenty-one out of 96 cases with ATLL showed detectable EBV DNA in the tumor tissue by the PCR. ISH confirmed EBV-positive cells from all the positive cases proved by PCR. In five cases, hybridization was observed only in a few background non-neoplastic lymphocytes (Figure 1A). In 16 cases, unequivocal neoplastic T cells showed intense hybridization. In nine of these 16 cases, hybridization was seen in pleomorphic nuclei (Figure 1B), and these ISH-positive cells tended to form clusters. As for the histological subtypes, 10 cases were of pleomorphic type, six cases large cell type, four cases medium-sized cell type, and one mixed cell type, according to the Japanese lymphoma

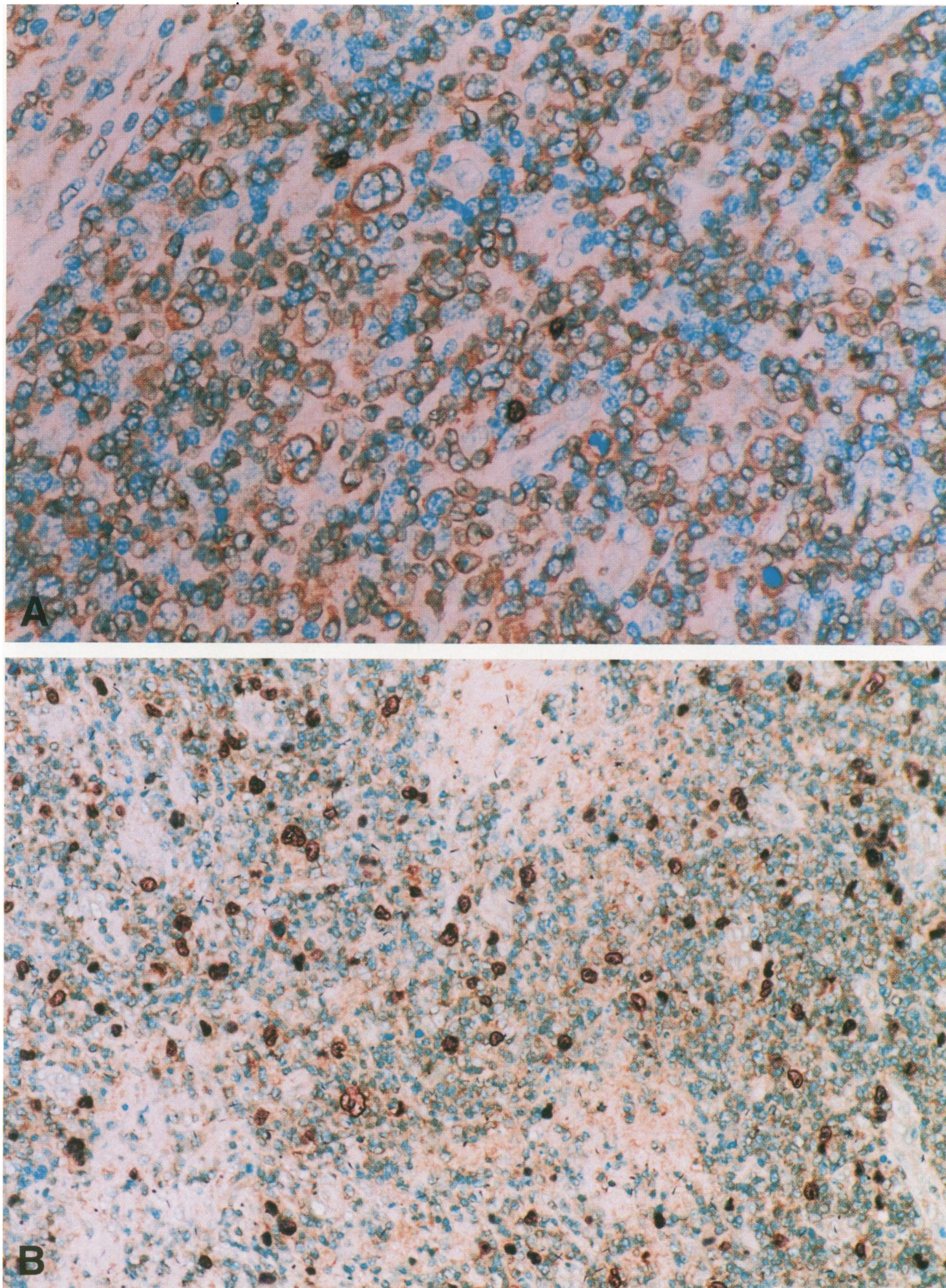

Figure 1. *Double staining of EBER-1 ISH and CD3 immunohistochemistry in a case of large cell type* (**A**), *and pleomorphic type* (**B**) *of ATLL. There are two ISH-positive (black) small lymphocytes in the background of CD3 positive (brown) large lymphocytes* (**A**), *and many ISH-positive pleomorphic cells* (**B**).

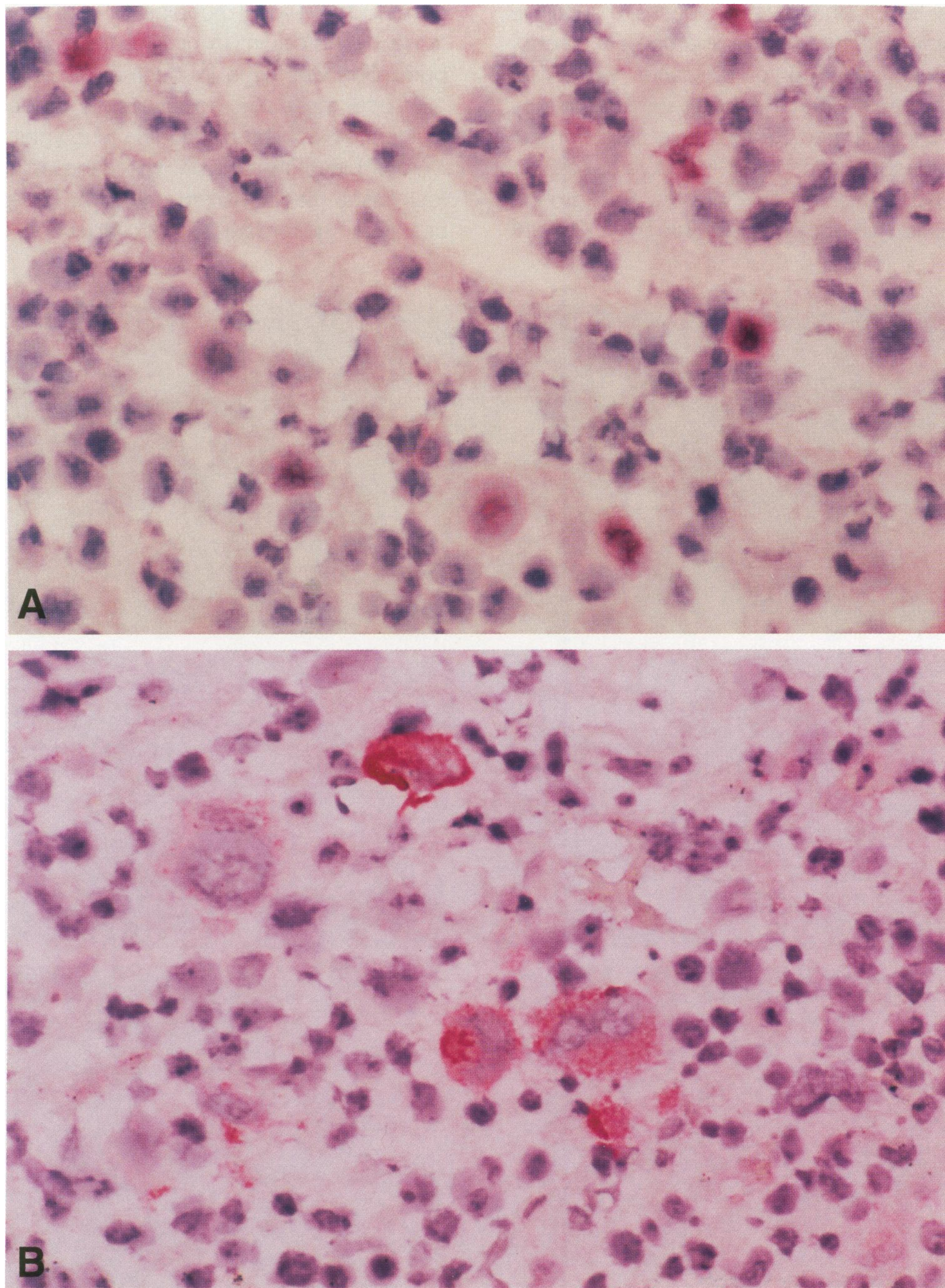

Figure 2. *Immunohistochemical demonstration of EBNA-2 antigen in the nuclei* (**A**), *and LMP in the cytoplasm* (**B**) *of some tumor cells of pleomorphic type ATLL.*

study group classification.[18] The most common combination of phenotypic expression, which was seen in nine cases, was CD2, CD3, CD4, and CD25 with or without CD5 and CD7. Seven cases were double positive for CD4 and CD8. Three cases showed CD3 and CD4 without CD25. Two cases were CD30-positive in addition to T-cell markers. There was no case expressing CD1, CD14, CD15, CD19, CD20 or CD21 in the tumor cells.

Double staining with EBER-1 ISH and pan T-cell marker (DAKO CD3) on the paraffin sections revealed that the hybridized pleomorphic cells expressed cytoplasmic and/or membranous T-cell marker. The immunohistochemistry for EBNA-2 antigen showed clear nuclear staining in pleomorphic tumor cells in nine of 21 EBV DNA-positive cases (Figure 2A). Immunohistochemistry of the LMP antigen revealed strong membranous staining in 15 of

21 EBV DNA-positive cases proved by PCR method. These LMP antigen-positive cells also showed pleomorphic nuclei, peculiar to ATLL (Figure 2B).

Discussion

This is the first report to demonstrate the presence of EBV DNA by PCR, EBER-1 by ISH, and EBV-related oncoproteins LMP and EBNA-2 by immunohistochemistry in tumor cells of substantial numbers of cases with ATLL. The results indicate a coinfection of these two viruses in tumor cells playing a pathogenic role in some cases with ATLL. EBV has been found predominantly in B-cell lymphomas including Burkitt's lymphoma, sinusoidal and Waldeyer's ring lymphoma, and Hodgkin's disease as well as in lymphoproliferations occurring in individuals of immunosuppression.[13,14,16]

ATLL is a unique peripheral T-cell lymphoma closely related to HTLV-1. Almost all cases with ATLL are seropositive for anti-HTLV-1 antibody, and the tumor cells possess proviral DNA of HTLV-1. At first, ATLL was characterized by lymphadenopathy, hepatosplenomegaly, skin rash, leukemic change, and hypercalcemia with aggressive clinical course. Cytopathologically, it was characterized by the pleomorphic tumor cells with helper T-cell type surface markers. Our findings of EBV in ATLL tumor cells may focus new attention on the significance of EBV and HTLV-1. The age distribution, sex ratio, and histological types of these EBV-related ATLL did not differ essentially from that of usual ATLL cases, except for a slightly higher frequency of cases with CD8 expression.[19]

EBV has been implicated as a pathogenic virus by the transformation effects on B cells *in vitro*,[20] and EBV DNA has been detected in the tumor cells of Burkitt's lymphoma, nasopharyngeal carcinoma, lymphoepitheliomalike carcinoma in several organs, Hodgkin's disease, and B-cell lymphoma in patients in a immunocompromised state.[5–7] EBV has been thought to play an important role in carcinogenesis, particularly lymphomas in transplant recipients and acquired immunodeficiency syndrome patients.[6] Ten EBV-specific gene expressions have been clarified in EBV-transformed B cells. Among these, the expression of EBNA-2 and LMP is thought to play an important role in EBV-induced transformation. There are two types of EBV oncoprotein expression in EBV-related neoplasia. In the cells of immunodeficient lymphoma, nasal T-cell lymphoma, infectious mononucleosis, and lymphoblastoid cell lines, the antigen expression of both EBNA-2 and LMP has been observed.[10,21] Tumor cells of Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease express LMP and EBNA-1 without expression of EBNA-2.[22–24] Our study indicates that EBV-related ATLL cells express both EBNA-2 and LMP antigens, as well as EBER-1 RNA expression. These findings are suggestive of similar mechanisms of EBV in ATLL tumor cells and in immunodeficient lymphomas.

Previously, EBV was thought to be not associated with T-cell lymphoproliferation until Kikuta et al[8] first demonstrated the presence of EBV genomes in the T cells of a boy with chronic EBV infection. EBV-related peripheral T-cell lymphoma has also been confirmed in $CD8^+$ T-cell lymphoma with aggressive clinical behavior[15] and in nasal T-cell lymphoma in patients with lethal midline granuloma.[10] On the other hand, a lymphoblastic lymphoma, which is thought to be a central T-cell lymphoma, has been reported to be EBV-negative.[15] These reported findings and our observation on the ATLL suggest that the EBV is closely associated with a spectrum of peripheral T-cell lymphoma as well as B-cell lymphoma and Hodgkin's disease.

We have found many transformed T lymphocytes that are positive for EBER-1 ISH in lymphadenitis with infectious mononucleosis as well as B lymphocytes.[25] These findings suggest that EBV may infect and proliferate in T cells on the usual initial infection pathway with or without symptoms of infectious mononucleosis. HTLV-1 is also known to infect T cells early in life, mainly through mother-to-child transmission.[26] Dual infection of HTLV-1 and EBV has been observed in cultured lymphocytes resulting in chromosomal rearrangement and immortalization.[27] There is also a report to detect the C3d receptor expression on the cell membrane of adult T-cell leukemia.[28]

It is hypothesized that both of these two viruses may infect the same T cells in early life and may play possible roles in the oncogenesis of ATLL. From the standpoint of multistep oncogenesis of ATLL,[29] EBV may play one of the key roles in the development of ATLL following HTLV-1 infection in T cells. It is also supported by our recent findings of clonal proliferation of EBV-infected cells in the ATLL cases (in preparation).

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