NK-cell hosts.[45] It is plausible that killer cells in close contact with EBV-infected B cells may acquire EBV infection directly and then proliferate with clonality.

In the present study, we evaluated prognostic factors among patients with EBV$^+$ T/NK-LPDs. Multivariate analysis showed that age at onset of disease ($\geq$ 8 years) and liver dysfunction were independent risk factors for mortality, and that patients receiving transplantations had a better prognosis. We found previously that older onset age ($\geq$ 8 years) was associated with mortality in patients with CAEBV.[29] Furthermore, a recent report demonstrated that adult patients with CAEBV had progressive and more aggressive courses than those of childhood onset cases.[46] Interestingly, patients with CD4$^+$ T-cell infection had shorter survival rates than those with NK infection, whereas clinical categories were not correlated with survival rates. Onset age of patients with CD4$^+$ T-cell infection was high (median, 14.5 years). These results suggest that adult patients with CD4$^+$ T-cell infection may have more aggressive features and are likely to develop multiple organ failure. Although the reason is unclear, we should be cautious about rapid progression in patients with CD4$^+$ T-cell infection.

We surveyed administered therapies based on physician questionnaire responses. A potential limitation of this study design was the use of retrospective questionnaires; therefore, we should be cautious about the evaluation of treatment efficacy. Nevertheless, it seems that only HSCT induced CR in patients with EBV-associated T/NK-LPDs except for HLH. Some EBV-associated HLH patients responded well to chemotherapy and immunomodulating therapies,[47] but patients with CAEBV were generally refractory to chemotherapy. Similar findings were reported in patients with CAEBV in the United States.[20] Furthermore, Kaplan-Meier estimates indicated that shorter time from onset to HSCT (< 30 months) and inactive disease at HSCT resulted in long survival times, suggesting that earlier HSCT in patients in good condition is preferred. Patients with CAEBV have a higher risk of transplantation-related complications.[41,48] Recently, Kawa et al reported excellent outcome of HSCT with reduced-intensity conditioning.[40] Although the superiority of reduced-intensity conditioning over myeloablative conditioning did not reach statistical significance in that study, it appears that a reduced-intensity regimen is sufficient to prevent transplantation-related deaths.[40,49]

The concept of EBV$^+$ T/NK-LPD was initially proposed by Kawa et al, and then examined by other researchers.[27,44] This umbrella term encompasses specific clinical diseases of the CAEBV T/NK–cell type, EBV-associated HLH, severe mosquito bite allergy, and hydroa vacciniforme, the distinction of which are differentiated based on clinical manifestations. However, if the clinical data are absent regarding the prodromal phase of expansion of EBV$^+$ T/NK–cells with variable clonality, we cannot discriminate systemic diseases such as ANKL and extranasal ENKL from EBV$^+$ NK-LPDs, because EBV$^+$ proliferating cells are indistinguishable in morphology and phenotype. Recently, this issue was highlighted by Takahashi et al.[50] Interestingly, 4 patients of the present series developed ANKL in their clinical course, 2 of whom had only skin symptoms categorized as severe mosquito bite allergy at the time of the diagnosis. In addition, 6 patients who were clinically categorized as CAEBV NK-cell type (4 cases) and T-cell type (2 cases) developed ENKL; the major clinical difference from de novo ENKL was its early onset (median age, 8.5 years). Three patients had hypersensitivity to mosquito bites. There were no differences in pathologic features between these patients and de novo ENKL patients.[50] Furthermore, new development of chromosomal aberrations was seen in 6 patients during follow-up. In this study, most of the patients with EBV$^+$ T/NK-LPDs had clonality of EBV-infected cells. These results indicate that patients with clonally expanding EBV-infected T or NK cells in EBV$^+$ T/NK-LPD eventually develop overt leukemia and lymphoma, the clinicopathologic findings of which are in keeping with those well documented in extranasal ENKL, ANKL, and PTCL, with additional mutations in cancer genes or tumor-suppressor genes.

In 2008, an international meeting was organized at the National Institute of Health to better define the pathogenesis, classification, and treatment of EBV-associated LPDs in nonimmunocompromised hosts.[39] At that meeting, acute and chronic EBV syndromes of T cells and NK cells were clarified to have a broad spectrum, in which hydroa vacciniforme, hydroa vacciniforme–like lymphoma, severe mosquito bite allergy, and systemic EBV$^+$ T-LPD of childhood were listed as EBV$^+$ T/NK-LPDs under an umbrella term of CAEBV of T/NK–cell type.[39] In the present study, EBV$^+$ T/NK-LPD is characterized by the systemic distribution of EBV$^+$ clones beyond the clinical categorization currently proposed as CAEBV, HLH, severe mosquito bite allergy, and hydroa vacciniforme. Furthermore, we also shed light on the clinicopathologic distinctiveness of patients with NK-cell infection, which has not been well addressed in the past even though these patients comprise approximately 40% of EBV$^+$ T/NK-LPD cases. This phenotype was more closely associated with hypersensitivity to mosquito bite and a relatively indolent clinical course, the biologic significance of which should be clarified in the future.

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Authorship

Contribution: H.K. designed the study, followed the patients, analyzed the data, and wrote the manuscript; Y.I. contributed to the study design, followed the patients, and helped to edit the manuscript; S. Kawabe, K.G., and S.E. performed the experiments; Y.T., S. Kojima, and T.N. followed the patients, collected the clinical data, and helped to edit the manuscript; A.K., A.S., and K.K. followed the patients and collected the clinical data; K.O. performed the experiments and helped to edit the manuscript; and S.N. contributed to the study design, performed the experiments, and wrote the manuscript.

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Application of flow cytometric *in situ* hybridization assay to Epstein–Barr virus-associated T/natural killer cell lymphoproliferative diseases

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Epstein–Barr virus (EBV) infects various types of lymphocytes and is associated with not only B cell-origin lymphoma, but also T or natural killer cell lymphoproliferative diseases (T/NK LPD). Recently, we established a novel assay to identify EBV-infected cells using FISH. Using this assay, dual staining with antibodies to both surface antigens and an EBV-encoded small RNA (EBER) probe can be performed. In the present study, we applied this recently developed FISH assay to EBV-associated T/NK LPD to confirm its diagnostic utility. Using FISH, we prospectively analyzed peripheral blood from patients with suspected EBV-associated T/NK LPD. The results were compared with those obtained using immunobead sorting followed by quantitative PCR. In all, 26 patients were included study. Using FISH, 0.15–67.0% of peripheral blood lymphocytes were found to be positive for EBER. Dual staining was used to determine EBER-positive cell phenotypes in 23 of 26 subjects (88.5%). In five of seven patients with hydroa vacciniforme-like lymphoma (an EBV-positive cutaneous T cell lymphoma), EBER-positive cells were identified as $CD3^+CD4^-CD8^-TCR\gamma\delta^+$ T cells. Furthermore, in a 25-year-old male patient with systemic EBV-positive T cell LPD, two lymphocyte lineages were positive for EBER: $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. Thus, we confirmed that our newly developed assay is useful for quantifying and characterizing EBV-infected lymphocytes in EBV-associated T/NK LPD and that it can be used not only to complement the pathological diagnosis, but also to clarify the pathogenesis and to expand the spectrum of EBV-associated diseases. (*Cancer Sci* 2012; 103: 1481–1488)

Epstein–Barr virus (EBV) is ubiquitous and infects not only B cells, but also T and natural killer (NK) cells. There are a number of EBV-associated T/NK lymphoproliferative diseases (LPD) and lymphoma/leukemia, such as EBV-associated hemophagocytic lymphohistiocytosis (HLH), systemic EBV-positive T cell lymphoproliferative disease of childhood (systemic EBV^+ T-LPD), hydroa vacciniforme (HV)-like lymphoma, extranodal NK/T-cell lymphoma, nasal type (ENKL), and aggressive NK cell leukemia (ANKL).[1–5] Severe chronic active EBV disease (SCAEBV), which is seen mainly in East Asia, is now considered to be an LPD caused by clonal expansion of EBV-infected T or NK cells.[6–9] However, the definition of each EBV-associated T/NK LPD is unclear and there is significant overlap between them.[5,9–13] Therefore, diagnosis of EBV-associated T/NK LPD can be problematic.

Because EBV is ubiquitous and latently infects various lymphocytes, detection of EBV alone is insufficient for diagnosis of EBV-associated diseases[14] To diagnose EBV-associated diseases and to explore their pathogenesis, EBV load must be determined; however, the EBV-infected cells must also be identified. *In situ* hybridization (ISH) using the EBV-encoded small RNA (EBER) is widely used to detect EBV-infected cells in tissue specimens.[15–17] However, biopsies are invasive and cannot always be obtained. To overcome these problems, we recently established a novel assay to simultaneously quantify and identify EBV-infected cells using FISH.[18] Both nuclear EBER and surface lymphocyte antigens can be stained using a fluorescein-conjugated probe that specifically hybridizes to EBER. This assay is a more convenient and less invasive procedure than EBER ISH and can be performed on peripheral blood. Using this assay, we determined the phenotype of EBV-infected B cells in patients with EBV infection after stem cell/liver transplantation.[19]

In the present study, we applied the FISH assay to peripheral blood from 26 patients with EBV-associated T/NK LPD to confirm its utility for the diagnosis of EBV-associated T/NK LPD and to further elucidate the pathogenesis of this disease. The results of the FISH assay were validated by comparison with EBV DNA loads determined by quantitative PCR. Furthermore, lymphocyte phenotypes were compared with those determined by immunobead sorting followed by quantitative PCR.

Materials and Methods

Patients and samples. From January 2009 to July 2010, patients who fulfilled the following criteria were prospectively enrolled in the present study: (i) EBV-associated T/NK LPD was suspected or diagnosed based on clinical and histopathological findings, and determination of EBV-infected cell phenotypes was requested from Nagoya University Graduate School of Medicine; (ii) high EBV DNA levels ($\geq 10^{2.5}$ copies/μg DNA) in PBMCs, as determined by quantitative PCR[7,20,21]; and (iii) both the FISH assay and immunobead sorting followed by quantitative PCR could be performed and results compared. Exclusion criteria were as follows: (i) patients with diseases involving infection of B cells, such as infectious mononucleosis and immunodeficiency-associated LPD; (ii) cases of congenital immunodeficiency; (iii) human immunodeficiency virus-positive cases; and (iv) patients who had received either hematopoietic or organ transplantation prior to enrolment.

In all, 28 patients were initially enrolled in the study. However, two subjects, who were initially suspected of having EBV-associated HLH, were excluded from the study because

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Table 1. Determination of Epstein–Barr virus-infected cell phenotypes using FISH and immunobead sorting/quantitative polymerase chain reaction

Pateint no.	Sex	Age (years)	Disease	EBV clonality	TCR gene rearrangement	FISH			EBV DNA (copies/μg DNA)							
						EBER$^+$ cells (%)	EBER$^+$ cell phenotypes	EBV-infected cells	PBMC	CD3$^+$	CD4$^+$	CD8$^+$	CD19$^+$	CD56$^+$	TCRαβ	TCRγδ
1	M	10	SCAEBV	Monoclonal	β	1.0	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	8300	**18 000**	1900	**9900**	5700	5400	ND	ND
2	F	22	SCAEBV	Negative	None	0.31	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	310 000‡	280 000	ND	ND	110 000	190 000	ND	ND
3	M	15	SCAEBV	Monoclonal	None	0.54	CD3$^+$CD4$^+$TCRαβ$^+$	CD4$^+$T	7200	1700	ND	ND	3600	2300	ND	ND
4	M	36	SCAEBV	Monoclonal	None	5.7	CD3$^+$CD56$^+$	CD56$^+$ T	44 000	3400	3900	**47 000**	39 000	**480 000**	ND	ND
5	M	8	SCAEBV	Monoclonal	β	29.9	CD3-CD56$^+$ CD3$^+$CD4$^+$TCRαβ$^+$	NK 82% CD4$^+$T 8%	240 000	17 000	27 000	21 000	90 000	**3 900 000**	ND	ND
6	F	11	SCAEBV	ND	γ	5.3	CD16$^+$CD56$^+$	NK	57 000	17 000	ND	ND	18 000	**93 000**	ND	ND
7	M	14	SCAEBV	Monoclonal	None	49.0	CD56$^+$	NK	600 000‡	1000	ND	ND	ND	**2 000 000**	1200	17 000
8	M	34	SCAEBV	Negative	None	0.32	CD56$^+$	NK	1500	0	0	0	0	**28 000**	ND	ND
9	F	13	SCAEBV	Negative	None	0.15	Not identified	Untypable	830	**14 000**	**19 300**	**3700**	140	810	ND	ND
10	F	6	HV-like lymphoma	Oligoclonal	β,γ,δ	9.0	CD3$^+$TCRγδ$^+$	γδT	170 000	**170 000**	150 000	49 000	**270 000**	130 000	ND	**330 000**
11†	M	6	HV-like lymphoma	Monoclonal	δ	25.9	CD3$^+$TCRγδ$^+$	γδT	42 000‡	**47 000**	ND	ND	9100	**49 000**	6400	**190 000**
12†	M	11	HV-like lymphoma	Monoclonal	γ,δ	4.8	CD3$^+$TCRγδ$^+$	γδT	10 000	**13 000**	1100	1300	5900	**19 000**	210	**87 000**
13	M	12	HV-like lymphoma	Monoclonal	β	36.8	CD3$^+$TCRγδ$^+$	γδT	920 000	ND	60 000	94 000	52 000	**1 500 000**	ND	ND
14†	M	16	HV-like lymphoma	Monoclonal	γ,δ	1.7	CD3$^+$TCRγδ$^+$	γδT	6100‡	**16 000**	ND	ND	2300	4400	8300	**100 000**
15	F	22	HV-like lymphoma	ND	β	13.0	CD3$^+$CD56$^+$	CD56$^+$ T	240 000	**420 000**	ND	ND	140 000	**2 000 000**	ND	ND
16	M	3	HV-like lymphoma	Monoclonal	None	67.0	CD16$^+$CD56$^+$	NK	1 200 000	240 000	110 000	500 000	310 000	**15 000 000**	ND	ND
17	F	1	HLH	ND	None	0.20	CD3$^+$CD4$^+$TCRαβ$^+$	CD4$^+$T	650	**1400**	ND	ND	150	0	ND	ND
18	M	1	HLH	Monoclonal	β	17.5	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	220 000	**760 000**	**360 000**	**1 600 000**	**1 200 000**	**1 600 000**	ND	ND
19	M	1	HLH	Negative	β	0.15	Not identified	Untypable	430	0	20	**510**	120	**1500**	ND	ND
20	F	25	HLH	Polyclonal	None	0.19	Not identified	Untypable	310	**700**	150	**3200**	**8900**	120	ND	ND
21	M	56	ENKL	ND	None	0.32	CD56$^+$	NK	2400	140	0	0	**20 000**	**11 000**	ND	ND
22	F	57	ENKL	ND	None	2.0	CD56$^+$	NK	24 000	12 000	8700	7600	**27 000**	**540 000**	ND	ND
23	M	26	Systemic EBV$^+$ T-LPD	Monoclonal	β, γ	4.5	CD3$^+$CD8$^+$ CD3$^+$CD4$^+$	CD8$^+$T 52% CD4$^+$T 39%	57 000	**110 000**	**110 000**	**130 000**	37 000	**88 000**	ND	ND
24	F	46	Systemic EBV$^+$ T-LPD	Monoclonal	γ	31.3	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	940 000	700 000	53 000	**1 410 000**	170 000	160 000	ND	ND
25	M	14	ANKL	Monoclonal	None	31.0	CD56$^+$	NK	310 000	ND	6500	24 000	5800	**2 000 000**	ND	ND
26	M	56	PTCL	Monoclonal	β	0.55	CD3$^+$CD4$^+$TCRαβ$^+$	CD4$^+$T	3300	**6300**	**6800**	1000	4100	3500	ND	ND

Bold letters indicate that Epstein–Barr virus (EBV) DNA was concentrated in the fraction. †These cases have been reported previously.[18] ‡Samples were obtained on different days when FISH was performed. ANKL, aggressive NK cell leukemia, nasal type; ENKL, extranodal natural killer (NK)/T cell lymphoma, nasal type; HLH, hemophagocytic lymphohistiocytosis; HV-like lymphoma, hydroa vacciniforme-like lymphoma; ND, not done; PTCL, peripheral T cell lymphoma; SCAEBV, severe chronic active EBV disease; systemic EBV$^+$ T-LPD, systemic EBV-positive T lymphoproliferative disease of childhood; TCR, T cell receptor.

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they were shown to have severe infectious mononucleosis and had only B cell infection, leaving 26 patients in the study: nine cases of SCAEBV, seven of HV-like lymphoma, four of HLH, two of systemic EBV$^+$ T-LPD, two of ENKL, one of ANKL, and one of peripheral T cell lymphoma (PTCL). Diagnoses of HV-like lymphoma, systemic EBV$^+$ T-LPD, ENKL, ANKL, or PTCL were made based on biopsy or bone marrow findings according to World Health Organization (WHO) criteria.[10,22–24] Diagnoses of HLH were made on the basis of criteria proposed by an international treatment study group,[25] whereas SCAEBV was diagnosed using previously proposed criteria.[7,26] Briefly, for a diagnosis of SCAEBV to be made, patients had to fulfill the following diagnostic criteria: (i) an illness of >6 months duration (an EBV-related illness or symptoms including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, or hypersensitivity to mosquito bites); (ii) increased quantities of EBV in either affected tissues or peripheral blood; and (iii) no evidence of any prior immunologic abnormalities or of any other recent infection that may explain the condition. There were several overlapping cases. For example, in one patient, ANKL developed at the end stage of SCAEBV. In some patients, HLH developed during the course of other EBV-associated T/NK LPD. In such cases, pathological diagnoses (HV-like lymphoma, systemic EBV$^+$ T-LPD, ENKL, ANKL, and PTCL) were used in preference to SCAEBV and HLH. Of the 26 patients in the study, 14 underwent biopsy (skin, $n = 6$; liver, $n = 3$; intestine, $n = 2$; others, $n = 3$), 19 underwent bone marrow examination, and one underwent an autopsy. Seventeen healthy volunteers who were seropositive for EBV were included in the study as negative controls.

Blood was usually taken at the time of diagnosis, although some subjects had already received treatment, such as steroids, cyclosporin A, and chemotherapies. In six subjects, repetitive sampling was performed with or without treatment. Heparinized blood samples were obtained and PBMCs were separated on density gradients. The PBMCs were cryopreserved at −80°C until required.

Informed consent was obtained from all subjects or their guardians, as well as from the healthy controls. The Institutional Review Board of Nagoya University Hospital approved the use of all specimens that were examined in the present study.

Analyses of EBV DNA. After DNA had been extracted from 1×10^6 PBMCs, real-time quantitative PCR was performed as described previously.[7,20] The amount of EBV DNA was calculated as the number of virus copies per μg PBMC DNA. To determine which cell population harbored EBV, the PBMCs were fractionated into CD3$^+$, CD4$^+$, CD8$^+$, CD19$^+$, CD56$^+$, T cell receptor (TCR) αβ$^+$, and TCRγδ$^+$ cells using an immunobead method (IMag Cell Separation System; BD Biosciences, Franklin Lakes, NJ, USA) that resulted in 97–99% purity. Purified cells were analyzed by real-time PCR and compared with PBMCs.[27,28] Southern blotting with a terminal repeat probe was used to assess EBV clonality, as described previously.[29]

Determination of TCR gene rearrangement. Multiplex PCR using the T cell Gene Rearrangement/Clonality assay (InVivoScribe Technologies, La Ciotat, France) was used to evaluate TCR gene; this assay was developed and standardized in a European BIOMED-2 collaborative study.[30,31]

FISH assay. The FISH assay was performed as described previously.[18,19] First, for surface marker staining, 5×10^5 PBMCs were stained with phycoerythrin (PE)-labeled anti-CD3 (clone UCHT1; eBioscience, San Diego, CA, USA), anti-CD8 (clone B9.11; Immunotech, Marseille, France), anti-CD19 (clone HD37; Dako, Glostrup, Denmark), and Vδ2 (clone B6; BD Pharmingen, San Jose, CA, USA) mAbs, and phycoerythrin cyanine 5 (PC5)-labeled anti-CD4 (clone 13B8.2; Immunotech), anti-CD16 (clone 3G8; Immunotech), anti-HLA-DR (clone IMMU357; Immunotech), and anti-TCRγδ (clone IMMU510; Immunotech) mAbs for 1 h at 4°C. In cases of weak fluorescence signals or incomplete cell separation likely due to degradation or detachment under the harsh hybridization conditions,[18] biotin-labeled antibodies (anti-CD3 clone UCHT1, anti-CD19 clone HIB19, anti-CD56 clone CB56, and anti-TCRαβ clone IP26 [eBioscience]; anti-CD122 clone Mik-b3 [BD Biosciences]) were used, followed by application of PE- or PC5-conjugated streptavidin (eBioscience). Isotype-matched monoclonal mouse IgG antibodies were used as controls.

Cells were fixed, permeabilized, and hybridized with EBER PNA Probe/FITC (Y5200; Dako) or Negative Control PNA Probe/FITC (Dako).[18,19] An Alexa Fluor 488 Signal Amplification Kit (Molecular Probes, Eugene, OR, USA) was used to enhance fluorescence and photostability.

Stained cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences). Lymphocytes were gated by standard forward and side scatter profiles.[32] Up to 50 000 events were acquired for each analysis. Based on experiments involving mixing of EBV-positive and -negative cell lines, the detection limit of the FISH assay was considered to be 0.1% and 0.01% for T and B cells, respectively.[18]

Statistical analysis. Statistical analyses were performed using SPSS for Windows version 18.0 (SPSS, Chicago, IL, USA). The FISH and real-time PCR assays were compared by regression analysis. The Mann–Whitney U-test was used to compare the mean percentages of EBER-positive cells in each group. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Quantification of EBV-infected peripheral blood lymphocytes by FISH. We applied the FISH assay to samples from 26 patients with EBV-associated T/NK LPD. Subject characteristics are given in Table 1. Most subjects were monoclonal, as determined by Southern blot hybridization using an EBV terminal repeat probe. The assay for TCR gene rearrangements detected T cell clonality in 15 patients. The FISH assay detected EBER-positive lymphocytes in each of the 26 patients at levels ranging from

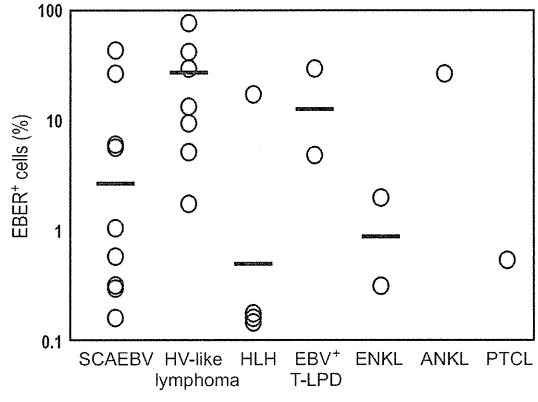

Fig. 1. Quantification of Epstein–Barr virus (EBV)-infected lymphocytes. The FISH assay was used to analyze PBMCs and the percentage of EBV-encoded small RNA (EBER)-positive cells in each disease is shown. Bars indicate the mean for each group. ANKL, aggressive NK cell leukemia; EBV$^+$ T-LPD, systemic EBV-positive T lymphoproliferative disease of childhood; ENKL, extranodal NK/T-cell lymphoma, nasal type; HLH, hemophagocytic lymphohistiocytosis; HV-like lymphoma, hydroa vacciniforme-like lymphoma; PTCL, peripheral T cell lymphoma; SCAEBV, severe chronic active EBV disease.

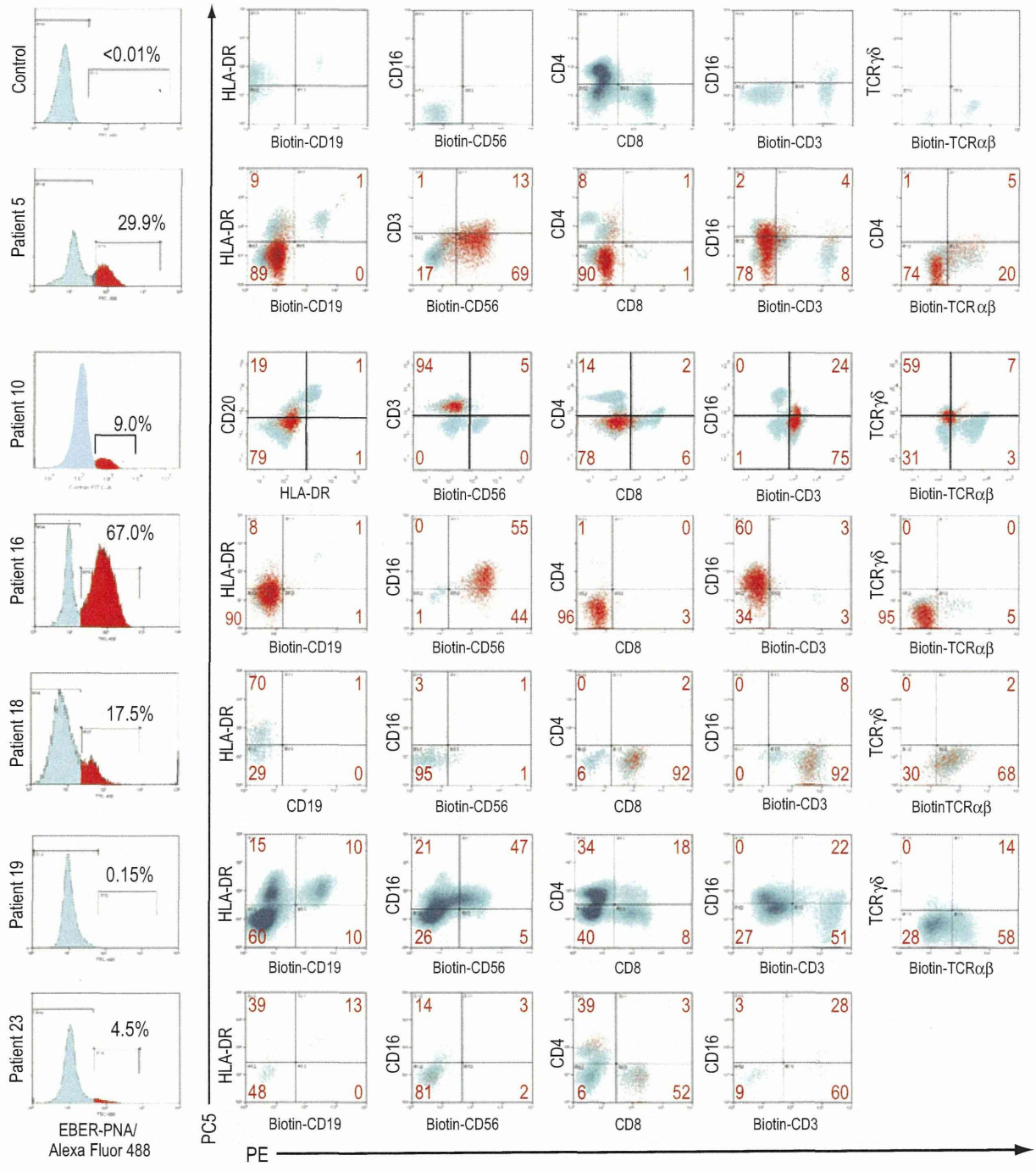

Fig. 2. Characterization of Epstein–Barr virus (EBV)-infected lymphocytes in representative patients. The numbers in each histogram represent the percentage of EBV-encoded small RNA (EBER)-positive lymphocytes. The EBER-positive (red) and EBER-negative (blue) lymphocytes were gated and plotted in quadrants. The numbers in the quadrants indicate the percentage of EBER-positive cells for each surface immunophenotype. Control, a healthy EBV-seropositive volunteer. Patient numbers are the same as given in Table 1. PC5, phycoerythrin cyanine 5; PE, phycoerythrin; PNA, peptide nucleic acid; TCR, T cell receptor.

0.15% to 67.0% (Table 1). The percentage of EBER-positive cells according to disease were as follows: SCAEBV, 2.6 ± 1.8%; HV-like lymphoma, 12.9 ± 1.6%; HLH, 0.6 ± 3.1%; systemic EBV^+ T-LPD, 11.9 ± 2.6%; and ENKL, 0.8 ± 2.6% (Fig. 1). The levels of EBER-positive cells were slightly higher in HV-like lymphoma patients than in patients with SCAEBV or HLH, but the differences did not reach statistical significance ($P = 0.08$ and $P = 0.06$, respectively).

To confirm the specificity of the assay, PBMCs were obtained from 17 healthy volunteers who were seropositive for EBV. However, EBV DNA was detected in the PBMCs of only one volunteer using real-time PCR. The same PBMCs were subjected to the FISH assay and no EBER-positive cells were detected (detection limit >0.1%).

Determination of EBV-infected cell phenotypes by FISH assay. The EBER-positive cell phenotypes were determined by dual staining with antibodies to surface antigens and the EBER probe in 23 of 26 patients (88.5%; Table 1). Representative results of the dual staining are shown in Figure 2. In Patient 5, the EBV-infected cells were predominantly

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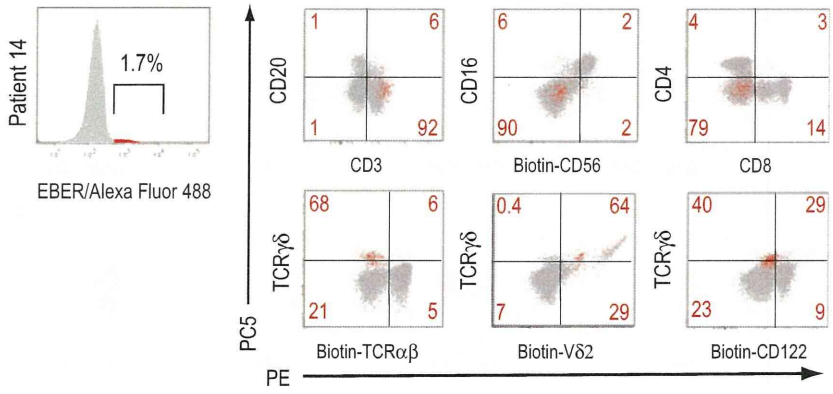

Fig. 3. Characterization of Epstein–Barr virus (EBV)-infected cell phenotypes in a 16-year-old boy with hydroa vacciniforme (HV)-like lymphoma. The EBV-encoded small RNA (EBER)-positive (red) and EBER-negative (gray) lymphocytes were gated and plotted in quadrants. The numbers in the quadrants indicate the percentages of EBER-positive cells for each surface immunophenotype. PC5, phycoerythrin cyanine 5; PE, phycoerythrin; TCR, T cell receptor.

$CD3^-$ $CD56^+$ $TCR\alpha\beta^-$ NK cells; in Patient 10 they were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells; in Patient 16 they were $CD3^-$ $CD16^+$ $CD56^+$ NK cells; and in Patient 18 they were $CD3^+$ $CD4^-$ $CD8^+$ $TCR\alpha\beta^+$ T cells (Fig. 2). We were unable to determine the phenotypes of EBV-infected cells in Patient 19, in whom only 0.15% of cells were EBER positive. Interestingly, in Patient 23, a 26-year-old man with systemic EBV^+ T cell LPD, almost half of the EBER-positive cells were CD4 positive, with the remainder CD8 positive. Thus, two lymphocyte lineages were present in the peripheral blood of this patient. Immunobead sorting followed by quantitative PCR revealed that the quantity of EBV DNA was high in the $CD3^+$, $CD4^+$, and $CD8^+$ fractions (Table 1), supporting the FISH data. Furthermore, TCR gene rearrangement analysis showed two peaks of the rearranged TCR Vγ chain in this patient (data not shown). Similarly, in Patient 5, whose main EBV-infected cells were $CD3^-$ $CD56^+$ $TCR\alpha\beta^-$ NK cells, the $CD3^+$ $CD4^+$ $TCR\alpha\beta^+$ population also included EBER-positive cells (Fig. 2). This observation suggests that the majority of EBV-infected cells in this patient were NK cells, but that there was also a minor population of EBV-infected T cells. In this patient, TCR rearrangement was recognized in the Vβ chain, which would theoretically not be detected in NK cell LPD (Table 1).

Thus, the main EBV-infected cells were identified as NK cells in eight patients, γδ T cells in five patients, $CD8^+$ T cells in five patients, $CD4^+$ T cells in three patients, and $CD56^+$ T cells in two patients (Table 1). These data are mostly in agreement with those generated by immunobead sorting and EBV DNA quantification. For example, in Patient 1 (EBV-infected $CD3^+$ $CD8^+$ $TCR\alpha\beta^+$ T cells), EBV DNA was detected mainly in the $CD3^+$ and $CD8^+$ populations. Conversely, in Patient 6 (EBV-infected NK cells as determined by the FISH assay), EBV DNA was most abundant in the $CD56^+$ population.

In the nine patients with SCAEBV, the main EBV-infected cells were $CD8^+$ T cells in two patients, $CD4^+$ T cells in one patient, and NK cells in five patients; typing was unsuccessful in one patient (Table 1). Thus, the main EBV-infected cells were variable in SCAEBV. Conversely, in five of seven patients with HV-like lymphoma, an EBV-positive cutaneous lymphoma, the EBER-positive cells were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells (Table 1). We further investigated the phenotypes of these $\gamma\delta^+$ T cells, which were positive for Vδ2 but negative for CD122. A representative result (Patient 14) is shown in Figure 3.

We could not identify the EBV-infected cell phenotypes in three patients (Patients 9, 19, and 20), although immunobead sorting and quantitative PCR could identify the predominant population of infected cells. In all three patients, EBER-positive cells accounted for <0.2% of the total population.

Comparison between EBER-positive cells and EBV DNA in peripheral blood. Finally, we compared the FISH assay with real-time quantitative PCR. The number of $EBER^+$ cells determined by the FISH assay was significantly correlated with the EBV DNA load determined by real-time PCR ($P < 0.0001$; Fig. 4a). Patients were divided into NK and T cell infection groups, and the same comparison was performed. A significant correlation was observed and the slope of the correlation was similar in both groups, suggesting that the number of EBV episomes per cell was similar in both groups (Fig. 4b).

We repeated both FISH and real-time PCR on samples from six patients and the resultant longitudinal analyses are shown in Figure 5. In the four patients who had not received any chemotherapy owing to localization of symptoms to the skin or the stability of their condition (Patients 7, 11, 12, and 14), the percentage of EBER-positive cells determined by the FISH assay was stable. However, in the two patients who received hematopoietic stem cell transplantation, the proportion of EBER-positive cells decreased thereafter (Patients 5 and 24).

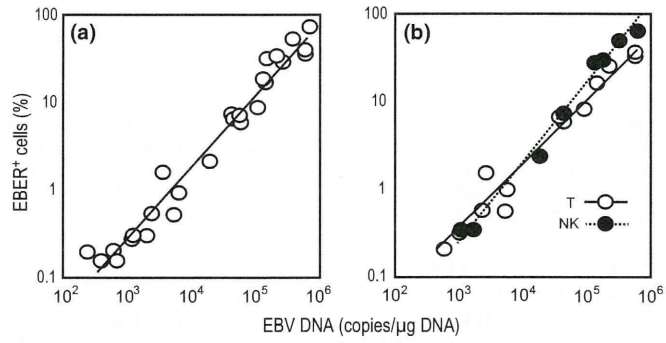

Fig. 4. Correlation between the percentage of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-positive lymphocytes as determined by FISH and the EBV DNA load determined by real-time PCR. (a) All 26 patients with EBV-associated T or natural killer cell lymphoproliferative diseases (T/NK LPD). (b) Patients were divided into T cell (n = 13) and NK cell (n = 8) infection groups, and the correlations were evaluated.

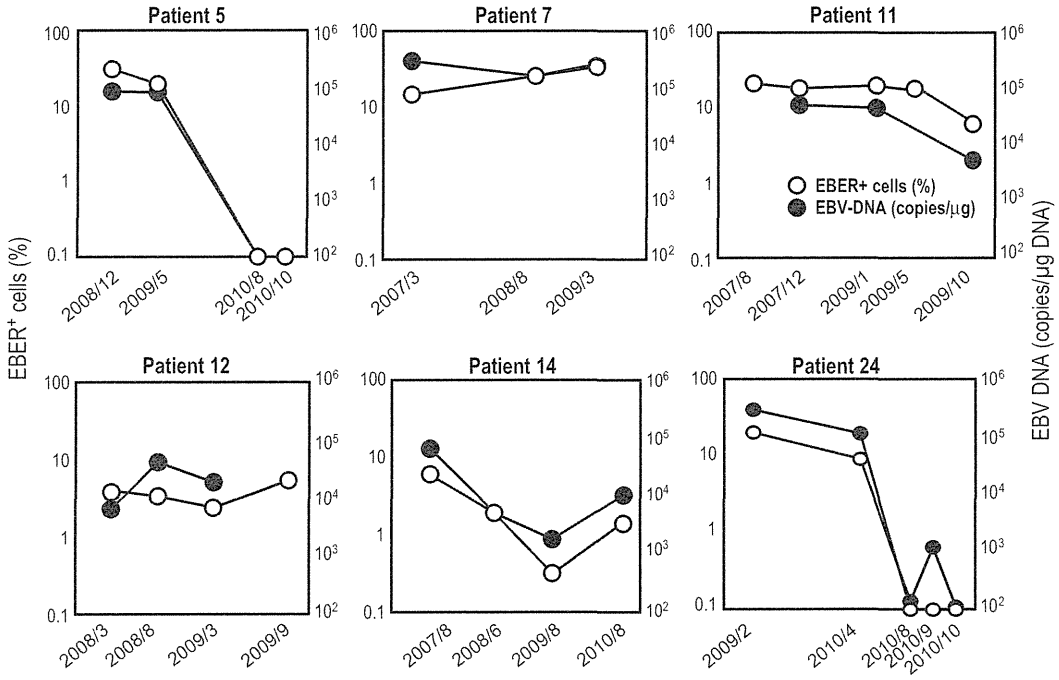

Fig. 5. Longitudinal quantification of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-positive lymphocytes. Samples of PBMCs were obtained repeatedly on the dates indicated and were analyzed by the FISH assay. The results of EBV DNA quantification are also shown for comparison. Patients 7, 11, 12, and 14 did not receive any chemotherapy owing to the stability of their condition. Patients 5 and 24 received hematopoietic stem cell transplantation.

Discussion

Epstein–Barr virus is associated with various types of T/NK LPD. Some are well defined and listed in the revised WHO *Classification of Tumours of Haematopoietic and Lymphoid Tissues*, whereas others are not.[10,13] One of the reasons why these entities are not well defined is that they are relatively rare, especially in the West. Most EBV-associated T/NK LPD are more prevalent in East Asia and Latin America.[8,10] In addition, the diagnosis of such conditions is often problematic. When possible, staining of virus-associated antigens using specific antibodies is the most direct and easiest method of detecting and characterizing EBV-infected cells. Epstein–Barr virus infection of T/NK cells is "latency type II", in which only a few viral antigens (Epstein–Barr virus nuclear antigen-1, latent membrane protein (LMP-1, and LMP-2) are expressed[1,3,33]; however, there are no antibodies available that can stain their extracellular domains. This, together with their low expression levels and weak antigenicity, makes it difficult to staining EBV-infected cells with antibodies against these antigens.

Using the FISH assay, 0.15–67.0% of peripheral blood lymphocytes were positive for EBER in patients with EBV-associated T/NK LPD. The number of EBER-positive cells was correlated with the EBV DNA load determined by quantitative PCR. These results indicate that the FISH assay is useful for the detection and quantification of EBV-infected lymphocytes in patients with EBV-associated T/NK LPD. Furthermore, this assay is applicable for follow-up and evaluation of responses to therapy, as demonstrated in the present study. Because B-LPD, which is also associated with EBV, sometimes develops after stem cell transplantation, differential diagnosis between relapse of T/NK LPD and B-LPD is needed. Our assay is useful for diagnosing not only EBV-associated T/NK LPD, but also B-LPD,[19] and can help to select mAb-based therapy, such as anti-CD20 (rituximab), anti-CD52 (campath-1), or other humanized mAbs targeting lymphocyte surface antigens.

In the present study, using the FISH assay, two different cell lineages were demonstrated in two patients with EBV-associated T/NK LPD. This is particularly interesting in terms of the pathogenesis of EBV-associated T/NK LPD. Biphasic expansion of EBV-infected lymphocytes has been demonstrated in some patients with SCAEBV.[34–37] A recent study using an immuno-FISH assay, which is similar to the FISH assay used in the present study and can characterize EBV-infected cell phenotypes, revealed that not only T/NK cells, but also monocytes were infected with EBV in patients with EBV-associated LPD.[38] There are several possible explanations as to why multiple cell lineages were infected with EBV. First, these patients may have unknown genetic abnormalities, which are associated with the functions of virus-specific or non-specific lymphocytes and allow for infection of T or NK cells or expansion of EBV-infected cells. Second, EBV may infect hematopoietic stem cells that differentiate to multiple cell lineages. Third, EBV-infected lymphocytes may be capable of differentiation, as suggested recently by Ohga *et al.*[37] Further studies are necessary to clarify the mechanism by which EBV infects multiple lineages.

One possible disadvantage of our assay is its relatively low sensitivity. Preliminary studies using cell lines indicated that the assay could detect the phenotype of EBV-infected cells when they comprised at least 0.1% of the total population.[18] However, when human samples were used, cell phenotypes could not be determined when they accounted for <0.2% of the total. Therefore, this assay would not be suitable for patients with low peripheral blood viral loads.

Hydroa vacciniforme-like lymphoma is a recently defined EBV-positive cutaneous malignancy associated with photosensitivity.[10] It is characterized by a papulovesicular eruption that generally proceeds to ulceration and scarring. In some cases, systemic symptoms, including fever, wasting, lymphadenopathy, and hepatosplenomegaly, may be present.[39–42] In HV-like eruptions, both T and NK cells infiltrate the superficial dermis and

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the subcutaneous tissue.[10] Recently, we reported three cases of HV-like lymphoma with EBV-infected γδ T cells using the FISH assay.[18] In five of seven patients in the present study (the three cases in the previous report were included), the EBER-positive cells were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells. The other two cases were of NK and possible NK T cell infection, respectively. These results indicate that γδ T cells play a central role in the formation of HV-like eruptions, although other types of cells can also be involved. This observation accords with other recent reports.[43,44] The γδ T cells are the major T cell population in the skin and mucosal epithelium. The γδ T cells secrete various cytokines and have cytolytic properties.[45] In the present study, EBER-positive γδ T cells were positive for Vδ2, suggesting that they were Vγ9Vδ2 T cells. The Vγ9Vδ2 T cells are the predominant γδ T cell subtype in human peripheral blood.[46] The γδ T cells sense not only infection, but also cellular stress. In patients with HV-like lymphoma, circulating EBV-positive Vγ9Vδ2 T cells may sense and react to cells damaged by ultraviolet radiation. Furthermore, EBER-positive γδ T cells were negative for CD122. A recent study showed that $CD122^-$ $\gamma\delta^+$ T cells produce interleukin (IL)-17.[47] Thus, EBER-positive γδ T cells may produce IL-17 and then induce and activate neutrophils and the epithelium, resulting in the formation of papulovesicular eruptions.

In conclusion, we applied the FISH assay to peripheral blood from 26 patients with EBV-associated T/NK LPD and confirmed that this assay was useful for the diagnosis of this condition. Furthermore, we found that two lymphocyte lineages were present in some patients with EBV-associated T/NK LPD. We showed that γδ T cells were present in peripheral blood from most cases of HV-like lymphoma. Thus, this assay is a direct and reliable method for quantifying and characterizing EBV-infected lymphocytes and can be used not only to complement pathological diagnosis, but also to clarify the pathogenesis of EBV-associated diseases and expand the spectrum of conditions known to be associated with this virus.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Clinical Cancer Research

Pretreatment EBV-DNA Copy Number Is Predictive of Response and Toxicities to SMILE Chemotherapy for Extranodal NK/T-cell Lymphoma, Nasal Type

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Abstract

Purpose: Extranodal NK/T-cell lymphoma, nasal type (ENKL) is an Epstein–Barr virus (EBV)–associated lymphoma for which a new chemotherapeutic regimen called SMILE (steroid, methotrexate, ifosfamide, L-asparaginase, and etoposide) recently showed promising results.

Experimental Design: The amount of EBV-DNA was prospectively measured in whole-blood and plasma samples by real-time quantitative PCR from 26 patients registered in the SMILE phase II study.

Results: Before treatment, the EBV-DNA was detected in 22 samples of whole blood with a median number of 3,691 copies/mL (range: 0–1.14 $\times$ 10^7), but 15 samples of plasma with a median of 867 copies/mL (range: 0–1.27 $\times$ 10^7). Results of these 2 measurements of EBV-DNA well correlated ($R^2 = 0.994$, $P < 0.001$). The overall response rate to SMILE was significantly higher in patients with less than 10^5 copies/mL of EBV-DNA in whole blood at enrollment (90% vs. 20%, $P = 0.007$) and in patients with less than 10^4 copies/mL of EBV-DNA in plasma (95% vs. 29%, $P = 0.002$). The incidence of grade 4 toxicity of SMILE other than leukopenia/neutropenia was significantly higher in patients with 10^5 copies/mL of EBV-DNA or more in whole blood (100% vs. 29%, $P = 0.007$) than that of others and in patients with 10^4 copies/mL or more in plasma (86% vs. 26%, $P = 0.002$).

Conclusions: These findings suggest that whole blood is more sensitive for clinical use than plasma. The EBV-DNA amount in whole blood was useful for predicting tumor response, toxicity, and prognosis after SMILE chemotherapy for ENKL. *Clin Cancer Res; 18(15); 4183–90. ©2012 AACR.*

Introduction

Epstein–Barr virus (EBV) causes a variety of benign and neoplastic diseases, including infectious mononucleosis, posttransplantation lymphoproliferative disorder (PTLD) and EBV-associated malignancies such as lymphomas, including extranodal NK/T-cell lymphoma, nasal type (ENKL), Hodgkin lymphoma, Burkitt lymphoma, age-associated large B-cell lymphoma, and several other T-cell lymphomas (1–3). ENKL is a rare subtype of non-Hodgkin lymphoma, mainly occurs in the nasal/paranasal area, skin, or gastrointestinal tract and is much more common in Asia and Latin America than in Western countries (4–6). The prognosis of ENKL was poor under conventional radiotherapy and/or chemotherapy (4, 7) but has recently improved by concurrent chemoradiotherapy (8, 9) or newly developed SMILE chemotherapy, comprising the steroid dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide (10, 11).

This type of lymphoma is invariably associated with episomal infection of EBV in the tumor cells, which implies its tumorigenic role. The presence of EBV-DNA in peripheral blood has been used as a surrogate marker for estimating tumor amount in several EBV-associated malignancies

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Translational Relevance

Peripheral blood of patients with extranodal NK-cell lymphoma, nasal type (ENKL) contains fragmented Epstein–Barr virus (EBV)-DNA. The amount of EBV-DNA can be a good marker for estimating the tumor burden and prognosis of ENKL patients. We recently developed a novel chemotherapeutic regimen, SMILE, comprising steroid, methotrexate, ifosfamide, L-asparaginase, and etoposide. The tumor response rate and survival rate was dramatically improved. However, it is known that the prognostic significance of certain factors may vary when the treatment modality changes. Therefore, the significance of EBV-DNA was analyzed in this study. Consequently, pretreatment whole blood and plasma EBV-DNA were predictive of response and prognosis. Multivariate analysis showed that plasma EBV-DNA was a significant prognostic factor. Furthermore, the EBV-DNA load was also predictive of adverse events by chemotherapy. Prediction of toxicity is particularly important for the SMILE regimen because it is excessively toxic for some patients.

(12–14). In particular, after organ transplantation, increasing loads of EBV in whole blood, lymphocytes, and plasma are associated with corresponding increases in the risk of PTLD (14). For nasopharyngeal carcinoma, plasma EBV-DNA load is known to be useful for monitoring disease activity and predicting the outcome of treatment (15, 16). The disease activity and prognosis of ENKL can also be monitored by measuring circulating EBV-DNA in plasma (17). For patients registered to the SMILE phase II study, we simultaneously conducted a prospective observational study, SMILE-EBV study, in which the amounts of EBV-DNA in whole blood and plasma were evaluated for ENKL.

Materials and Methods

Study design

The aim of this study is to evaluate the copy numbers of EBV-DNA from whole blood and plasma in patients with ENKL who received SMILE chemotherapy. The predictive value of EBV-DNA for tumor response, toxicity, and prognosis was analyzed, as well as the preference of samples from whole blood or plasma. The eligibility criteria, treatment, and response were described in the report of the phase II study (11). EBV-encoded small RNA (EBER) *in situ* hybridization positivity was counted in accordance with our previous study (18). A total of 38 patients were enrolled: 26 from Japan, 6 from Hong Kong, and 6 from South Korea. The amounts of EBV-DNA were measured in whole-blood and plasma samples from patients participating in this phase II study at 3 time points: before the treatment, after 2 courses of SMILE and after a series of treatments. Because of the lack of an international standardized method for quantification of EBV-DNA, the 26 patients from Japan were the subjects for this study. All samples were measured in a central laboratory. Registration onto the study was conducted by facsimile from the participating institutes to the C-SHOT Data Center (Nagoya, Japan), simultaneously with the entry into the SMILE study. The study was approved by both the Protocol Review Committee and the Institutional Review Board of each institution in Japan. Written informed consent was obtained from all of the patients. The study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, UMIN000000741), as an associated but separate study of SMILE phase II (UMIN-CTR number, UMIN000000712).

Response and toxicity criteria

Complete response was defined as the complete disappearance of all objective signs of disease, including enlarged lymph nodes or hepatomegaly and splenomegaly at restaging. Partial response was defined as at least a 50% reduction of tumor volume without the occurrence of new lesions at restaging. Progressive disease was defined as a greater than 25% increase in the sum of tumor lesions or the emergence of one or more new lesions or clinical symptoms that indicate disease progression. No response was defined as any response that did not fall into the categories defined above. If a patient died of any cause before day 42 of the second course of SMILE and could not undergo the defined restaging procedure, the patient's response was recorded as early death. The overall response rate (ORR) was defined as the proportion of all patients who could be evaluated for response who experienced complete or partial response. Toxicity was graded according to the Common Terminology Criteria for Adverse Events v3.0.

Quantification of EBV-DNA

A 5-mL patient peripheral blood was obtained, sent to the central laboratory (Nagoya University Graduate School of Medicine), and divided into whole-blood and plasma samples. DNA was extracted from 200 μL of either whole blood or plasma, using QIAamp DNA blood kits (Qiagen K.K.). A real-time quantitative PCR assay was carried out and the result was expressed as copies per 1 mL of sample, as previously described (19, 20). The minimum detection level was 2 copies per reaction that was equivalent to 100 copies/mL for whole blood or plasma.

Statistical analysis

Regression analysis compared the copy numbers in whole blood and plasma. Fisher exact test was used to compare the responses or toxicities to the SMILE chemotherapy. Mann–Whitney *U* test and Kruskal–Wallis test were used to compare the levels of EBV-DNA between patient groups. Cut-off value of the categorization by EBV-DNA levels were determined by the receiver operating characteristic analysis. Patient survival data were analyzed by the method of Kaplan and Meier and were compared by log-rank test. Univariate and multivariate analyses were carried out using Cox proportional hazard model. Data were analyzed with STATA version 11 and SPSS (SPSS) software.

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Table 1. Baseline patient characteristics (N = 26)

Characteristic	No. of patients (%)
Age, y	
Median	46.5
Range	17–67
Sex	
Male	14 (54)
Female	12 (46)
Disease state	
Newly diagnosed stage IV	12 (46)
First relapse	11 (42)
Refractory to the first-line treatment	3 (12)
"B" symptoms present	11 (42)
Elevated serum LDH	7 (27)
Performance status	
0	16 (61)
1	5 (19)
2	5 (19)
Detection of EBV-DNA in blood samples before treatment	
Whole blood +, Plasma +	14 (54)
Whole blood +, Plasma −	8 (31)
Whole blood −, Plasma +	1 (4)
Whole blood −, Plasma −	3 (12)

Results

Patient characteristics

The baseline characteristics of 26 eligible patients are listed in Table 1. The median age was 46.5 (range: 17–67) years, and the male:female ratio was 14:12. Twelve patients (46%) had newly diagnosed stage IV disease, 11 were in first relapse, and 3 were in the primary refractory status. EBER *in situ* hybridization was positive in all specimens, with a median positivity of 68% (range: 12%–96%) of lymphoma cells.

Amount of EBV-DNA and correlation between whole blood and plasma

EBV-DNA was detected in 22 samples of whole blood (median: 3.7×10^3, range: 0–1.1×10^7 copies/mL) and 15 samples of plasma (median: 8.7×10^2, range: 0–1.3×10^7 copies/mL). The level of EBV-DNA was not different among the 3 disease state (newly diagnosed, relapsed or refractory) groups at enrollment both in whole blood ($P = 0.19$ by Kruskal–Wallis test) and in whole blood ($P = 0.22$). An inconsistent result was seen in 9 patients. EBV-DNA was positive in whole blood but was negative in plasma in 8 patients. Conversely, in another patient, the EBV-DNA was only detected in plasma. EBV-DNA was not detected in either whole blood or plasma in 3 patients (nos. 9, 23, and 25). The concordance rate between whole blood and plasma was 65% (17 of 26). The viral DNA copy numbers were compared between whole blood and plasma before SMILE chemotherapy. A strong correlation was found between the amounts in whole blood and those in plasma ($r = 0.997$, $P < 0.001$, Fig. 1). No differences were found for the EBV-DNA level among patients with newly diagnosed stage IV, relapsed and refractory status ($P = 0.19$ for whole blood and $P = 0.24$ for plasma). No significant correlation was found between EBER positivity and plasma or whole blood EBV-DNA level (Supplementary Fig. S1).

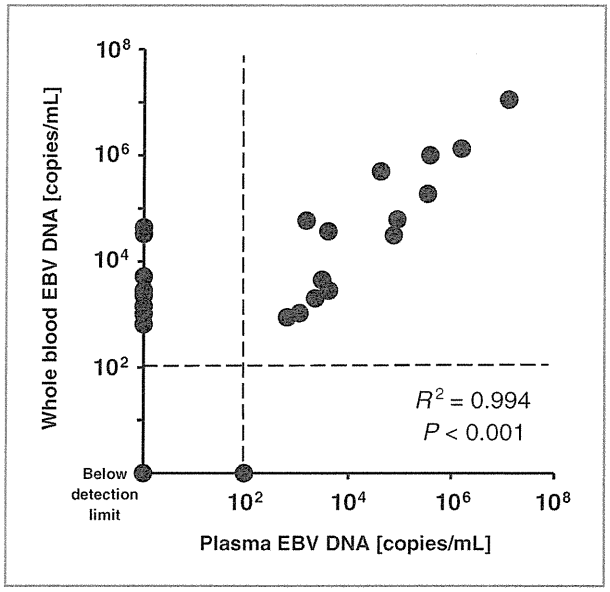

Figure 1. Comparison of EBV-DNA copies between whole blood and plasma in patients with ENKL who received SMILE chemotherapy. The EBV-DNA concentrations in whole blood or in plasma from the patients were measured using real-time PCR assay before SMILE chemotherapy. Dotted lines show the detection limits indicating 100 copies/mL of plasma or whole blood.

Dynamic changes of EBV loads in whole blood and plasma before and after treatment

EBV loads in whole blood or plasma from the 16 patients (8 with complete response, 7 with partial response, and 1 with progressive disease) were measured before the treatment, after 2 courses of SMILE chemotherapy, and after a series of treatments. Viral load declined in most patients with complete response or partial response after 2 courses of SMILE chemotherapy and/or after a series of treatments (Fig. 2). However, 5 patients with complete or partial response did not show the decrease of viral load. Of these, 2 patients experienced disease recurrence, and another patient died of transplant-related mortality in complete response. Other 2 patients maintained response at the time of last follow-up.

Correlation of the amount of EBV-DNA in blood samples and response or toxicities to the therapy

Among the 26 patients, there were 12 patients with complete response, 8 with partial response, 1 with no response, 3 with progressive disease, and 2 with early death (Table 2), and the ORR was 77%. For patients with less than

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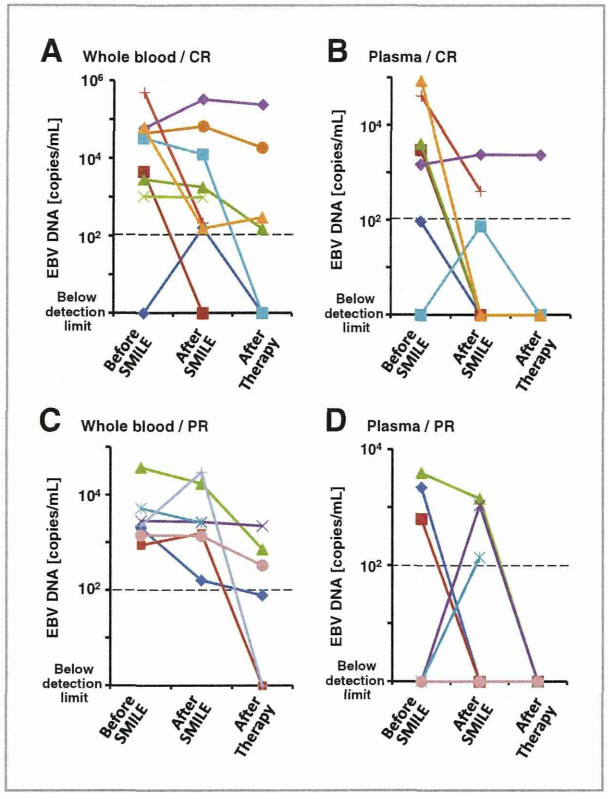

Figure 2. Serial analysis of EBV loads in blood samples from the patients with ENKL. The EBV-DNA concentrations in whole blood or in plasma from the patients were measured using real-time PCR assay before SMILE chemotherapy, after 2 courses of SMILE chemotherapy and after a series of treatments. A, viral loads in whole blood in patients with complete response. B, viral loads in plasma in patients with complete response. C, viral loads in whole blood in patients with partial response. D, viral loads in plasma in patients with partial response. Dotted lines show the detection limits indicating 100 copies/mL of plasma or whole blood. CR, complete response; PR, partial response.

10^5 copies/mL of EBV-DNA in whole blood, the ORR was 90% (19 of 21), but was 20% (1 of 5) in patients with 10^5 copies/mL or more ($P = 0.005$). In addition, the ORR was 95% (18 of 19) in patients with less than 10^4 copies/mL of EBV-DNA in plasma, but was 29% (2 of 7) in patients with 10^5 copies/mL or more ($P = 0.002$). All 3 patients without detectable EBV-DNA in either whole blood or plasma attained complete response. The amounts of EBV-DNA before treatment were not significantly different between patients with complete response and those with partial response (whole blood, $P = 0.82$; plasma, $P = 0.68$).

Grade 4 leukopenia (77%) and neutropenia (88%) were commonly observed. Grade 4 anemia was encountered in one patient and thrombocytopenia was seen in 9 patients. The nonhematologic grade 4 toxicities included infection ($n = 2$), alanine aminotransferase elevation ($n = 1$), and encephalopathy ($n = 1$); 3 patients experienced grade 4 somnolence, which was complicated by a grade 3 infection in one patient and by grade 4 encephalopathy in another patient. One patient experienced grade 2 pancreatitis and had complications from grade 4 hyponatremia, hyperamylasemia, and appetite loss. Grade 4 toxicity other than leukopenia/neutropenia was significantly higher in patients with 10^5 copies/mL of EBV-DNA or more in whole blood (100% vs. 29%, $P = 0.007$). Grade 4 toxicity other than leukopenia/neutropenia was also significantly higher in patients with 10^4 copies/mL of EBV-DNA or more in plasma (86% vs. 26%, $P = 0.002$; Table 2).

Prognostic significance of EBV-DNA

Patients with 10^5 copies/mL of EBV-DNA or more in whole blood showed significantly lower survival than those with less than 10^5 copies/mL (Fig. 3A, $P < 0.0001$). Similarly, the prognosis of patients with 10^4 copies/mL of EBV-DNA or more in plasma was significantly worse than that in those with less than 10^4 copies/mL (Fig. 3B, $P < 0.0001$). EBER positivity of more than 75% was also a factor

Table 2. Correlation of the levels of EBV-DNA and response/adverse events to SMILE chemotherapy for newly diagnosed stage IV, relapsed or refractory ENKL

	Whole blood EBV-DNA			Plasma EBV-DNA (copies/mL)		
	≥10^5 copies/mL	<10^5 copies/mL	*P*	≥10^4 copies/mL	<10^4 copies/mL	*P*
Response						
CR	1	11	0.005	2	10	0.002
PR	0	8		0	8	
NR	0	1		1	0	
PD	2	1		2	1	
ED	2	0		2	0	
Adverse event						
Any grade 4[a]	5	6	0.007	6	5	0.002
No grade 4	0	15		1	14	

Abbreviations: CR, complete response; ED, early death; PD, progressive disease; PR, partial response; NR, No response.
[a]Grade 4 adverse events other than leukopenia and neutropenia.

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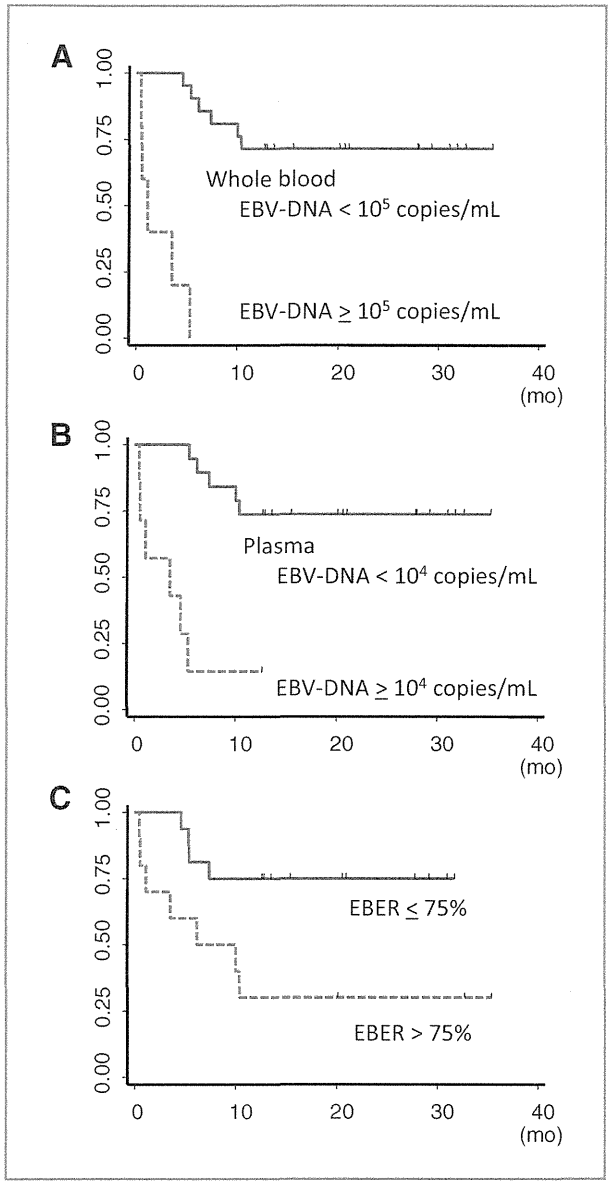

Figure 3. Survival of patients with ENKL who received SMILE chemotherapy by EBV parameters. A, overall survival was significantly lower for patients with a whole blood EBV-DNA level of 10^5 copies/mL or more ($P < 0.0001$). B, overall survival was significantly lower for patients with a plasma EBV-DNA level of 10^4 copies/mL or more ($P < 0.0001$). C, overall survival was significantly lower for patients with EBER positivity of more than 75% ($P = 0.02$).

associated with poor prognosis (Fig. 3C). Plasma and whole blood EBV-DNA before SMILE chemotherapy were significant prognostic factors for overall survival by univariate analysis, as well as serum lactate dehydrogenase (LDH) elevation, B symptom, and EBER positivity (Table 3). Multivariate analysis showed that LDH elevation [HR, 8.5; 95% confidence interval (CI), 1.9–38.0] and pretreatment whole blood EBV-DNA (HR, 65.5; 95% CI, 5.3–813.7) were significant prognostic factors. Plasma EBV-DNA was not prognostic (HR, 3.90; 95% CI, 0.70–21.8) if adjusted by LDH elevation using multivariate analysis. EBER positivity showed marginal significance (HR, 3.3; 95% CI, 0.95–11.8) if included in the model with LDH elevation.

Discussion

For EBV-associated malignancies, the significance placed on EBV-DNA in peripheral blood as a biomarker has increased in recent decades. Previous studies have reported that the level of EBV-DNA in a peripheral blood compartment is a useful biomarker in EBV-associated malignancies (14, 21). Lei and colleagues found a significant reduction of plasma EBV-DNA in patients with EBV-associated lymphoid malignancies (Hodgkin lymphoma, nasal NK/T cell lymphoma, PTLD, and Burkitt lymphoma) during the course of effective therapy (21). In addition, disease progression was associated with a rapid increase in plasma EBV-DNA levels in patients with ineffective therapy. Gandhi and colleagues showed that EBV-DNA is specifically detected in plasma of EBV-positive Hodgkin lymphoma patients before treatment (22). Viral DNA was undetectable following therapy in responsive patients and patients with long-term remission. Patients who experienced relapse had a significantly higher plasma EBV-DNA concentration before treatment. The plasma DNA concentration was persistently low or undetectable in patients with complete clinical remission. Overall survival and relapse-free survival were significantly higher for patients with a pretreatment plasma EBV-DNA level of less than 1,500 copies/mL. Au and colleagues reported that plasma EBV-DNA level is valuable as a tumor biomarker and for prognostication in EBV-positive lymphoma (17). EBV-DNA in plasma became undetectable for patients in remission but was elevated for those with refractory disease. A high level of EBV-DNA was significantly associated with inferior overall survival by multivariate analysis. Subgroup analysis of NK cell lymphoma showed that the level of EBV-DNA was also correlated with disease stage. Presentation of a high level of EBV-DNA was also significantly associated with inferior overall survival by multivariate analysis in their cohort. Prognostic factors of lymphoma may change when the treatment modality changes (23). In this study, however, EBV-DNA copy number in plasma or whole blood was also predictive of response and survival of ENKL patients who received SMILE chemotherapy, in agreement with other observations in the literature.

Another novel finding is that severe adverse events of the chemotherapy were also predictable using the EBV-DNA amount, which has not been identified by other studies in the literature. This analysis is only possible by examining patients who receive exactly the same treatment, ideally subjects of prospective studies. Because the level of EBV-DNA was not different by the 3 disease status groups (newly diagnosed, relapsed, or refractory), we examined the patients together in this study. As an interpretation of this finding, patients with higher tumor burden may experience more severe toxicity because of poor general condition or tissue damage by the tumor. Another hypothesis is that the

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Table 3. Prognostic factors affecting overall survival

		Univariate		Multivariate[a]	
Variables	**Unfavorable factors**	**Hazard ratio (CI)**	***P***	**Hazard ratio (CI)**	***P***
Age	>50 years	0.5 (0.2–1.9)	0.33	—	
LDH level	Elevated	8.6 (2.4–30.4)	0.001	8.5 (1.9–38.1)	0.005
B symptom	Present	5.0 (1.3–19.0)	0.02	—	
WB EBV-DNA	$\geq 10^5$ copies/mL	53.2 (5.9–482.0)	<0.001	65.5 (5.3–813.7)	0.001
Plasma EBV-DNA	$\geq 10^4$ copies/mL	10.3 (2.9–36.3)	<0.001	—	
EBER	>75%	4.0 (1.2–13.7)	0.03	—	

[a]Final model.

toxicity by chemotherapy is mediated by certain toxic substances in tumor cells. Because NK cells possess cytotoxic activities, almost all ENKL have cytotoxic molecules such as perforin or granzymes. In several EBV-associated malignancies, the high viral load may be explained by the tumor releasing viral components (24, 25). Toxic substances that are released from tumor cells degraded by chemotherapy such as SMILE, although they may not be cytotoxic molecules, may contribute to the high rate of adverse reactions after chemotherapy. Whatever the reason, measurement of EBV-DNA may be helpful for patient stratification to avoid excessive toxicity because the myelosuppressive adverse reaction of SMILE is rather profound for a part of patients.

Plasma is used as samples in most studies for evaluating EBV-DNA as a biomarker in EBV-associated disease (13–17, 22). However, controversies exist as to which blood compartment should be used for measuring EBV because several compartments of blood, whole blood, peripheral blood mononuclear cells, plasma, and serum can be used in the studies. Our previous study compared the usefulness of plasma and mononuclear cells for detecting EBV-DNA in ENKL patients, although the treatment was not unified (16). For the diagnosis of EBV-associated PTLD, earlier studies used peripheral blood mononuclear cells because EBV infection occurs in this cell compartment (26, 27). Plasma or serum samples are readily obtained and widely used for diagnosing EBV-associated PTLD; however, the sensitivity seemed to be low (28, 29). Several reports have revealed that whole blood, containing both cellular and humoral compartments, is better than plasma/serum when testing patients with PTLD (20, 30, 31). Recently, Spacek and colleagues reported that plasma is better than whole blood for the monitoring and estimation of prognosis for Hodgkin lymphoma (32). Plasma samples may be recommended as a biomarker of disease activity rather than peripheral blood mononuclear cells in patients with Hodgkin lymphoma, as shown in another study (20). However, comparison among each blood compartment has not been well investigated. Useful compartments may differ among diseases (14). In this study, the levels of EBV-DNA in plasma were compared with those in whole blood. Although strong correlation was detected between the viral copy numbers in whole blood and those in plasma, EBV-DNA was more frequently detected in whole-blood samples before treatment. Notably, EBV-DNA was only detected in whole blood in 8 patients, whereas it was only positive in plasma in one patient. This suggests that whole blood is more suitable than plasma to examine the EBV-DNA for ENKL. The reason for the phenomenon that EBV-DNA was only detected in whole blood remains undetermined. Among such 8 patients in this study, only 4 patients showed bone marrow involvement, and none accompanied leukemic presentation. The only possible explanation is that EBV-DNA might be lost or degraded in the fractionation procedure. Another point of interest is that EBV-DNA was not detected in either whole blood or plasma in 3 patients, although EBER was positive in tissue samples. Therefore, EBV-DNA detection in peripheral blood cannot be used as an alternative to the histologic detection of EBV or the diagnosis of ENKL. Moreover, the levels of 10^5 copies/mL of EBV-DNA in whole blood and 10^4 copies/mL of EBV-DNA in plasma seem to be cut-off values: the patients with copy numbers lower than these showed significantly better outcome. These 2 copy numbers also showed clinical value to predict severe adverse events.

In conclusion, our study indicates that the level of EBV-DNA in plasma or whole blood can predict response and adverse events of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory ENKL. Whole-blood samples were more suitable for this purpose, although plasma was preferable for other purposes such as diagnosis of EBV infection.

Disclosure of Potential Conflicts of Interest

R. Suzuki received honoraria from Kyowa-Hakko Kirin Company. K. Oshimi is currently an employee of Eisai Pharmaceutical Co., Ltd. (Tokyo, Japan). No potential conflicts of interest were disclosed by the other authors.

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Pin1 Interacts with the Epstein-Barr Virus DNA Polymerase Catalytic Subunit and Regulates Viral DNA Replication

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Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) protein is known as a regulator which recognizes phosphorylated Ser/Thr-Pro motifs and increases the rate of *cis* and *trans* amide isomer interconversion, thereby altering the conformation of its substrates. We found that Pin1 knockdown using short hairpin RNA (shRNA) technology resulted in strong suppression of productive Epstein-Barr virus (EBV) DNA replication. We further identified the EBV DNA polymerase catalytic subunit, BALF5, as a Pin1 substrate in glutathione *S*-transferase (GST) pulldown and immunoprecipitation assays. Lambda protein phosphatase treatment abolished the binding of BALF5 to Pin1, and mutation analysis of BALF5 revealed that replacement of the Thr178 residue by Ala (BALF5 T178A) disrupted the interaction with Pin1. To further test the effects of Pin1 in the context of virus infection, we constructed a BALF5-deficient recombinant virus. Exogenous supply of wild-type BALF5 in HEK293 cells with knockout recombinant EBV allowed efficient synthesis of viral genome DNA, but BALF5 T178A could not provide support as efficiently as wild-type BALF5. In conclusion, we found that EBV DNA polymerase BALF5 subunit interacts with Pin1 through BALF5 Thr178 in a phosphorylation-dependent manner. Pin1 might modulate EBV DNA polymerase conformation for efficient, productive viral DNA replication.

The Epstein-Barr virus (EBV) is a human gammaherpesvirus that mainly infects and establishes latent infection in B lymphocytes, but it also can infect other types of cells, such as NK, T, and epithelial cells.

EBV has both a latent state and a lytic replicative cycle in the nuclei of EBV-infected cells (1). During the latent phase of the EBV life cycle, the EBV genome is maintained as a circular plasmid molecule, which is amplified once in S phase by cellular DNA replication machinery. However, a small percentage of infected cells switch from the latent stage into the lytic cycle, which is triggered by the expression of an immediate-early protein, BZLF1, to produce progeny viruses. This type of activation contributes to the development and maintenance of human cancers (2, 3), suggesting that the EBV switching mechanism is also a key determinant of EBV pathogenesis. After induction of productive viral replication, the EBV genome is amplified 100- to 1,000-fold by viral replication machinery composed of BALF5 DNA polymerase (Pol), BMRF1 polymerase processivity factor, BALF2 single-stranded DNA-binding protein, and BBLF4-BSLF1-BBLF2/BBLF3 (BBLF2/3) helicase-primase complex via a rolling-circle mechanism in discrete sites in nuclei, called replication compartments (4, 5). BALF5 possesses intrinsic DNA polymerase and 3′-to-5′ exonuclease activities (6) and forms a complex with the BMRF1 polymerase accessory protein to exhibit high polymerase processivity (7). The DNA polymerase and exonuclease domains are highly conserved among a variety of DNA polymerases (8, 9). Unlike the case of the eukaryotic chromosomal replication apparatus, the EBV DNA Pol holoenzyme is used in the synthesis of both leading and lagging strands at the replication fork (6).

The peptidyl-prolyl bond has a low rate of spontaneous *cis-trans* isomerization. This is frequently a limiting step for protein folding and usually requires an isomerase to catalyze the process. Phosphorylation on a serine or threonine residue preceding proline (pSer/Thr-Pro) is a key regulatory mechanism, and the conformation of certain phosphorylated Ser/Thr-Pro bonds is regulated specifically by the prolyl isomerase Pin1 (10). The WW domain of Pin1 binds only to specific pSer/Thr-Pro motifs, which are isomerized by the peptidyl-prolyl isomerase (PPIase) domain to induce conformational changes in proteins (11). In this way Pin1 regulates various protein functions, including protein stability, catalytic activity, phosphorylation status, protein-protein interactions, and/or subcellular localization (11–14). Pin1 works in concert with protein kinases that phosphorylate Ser/Thr-Pro motifs, and protein phosphatases, in turn, can also be regulators of the process (15). Pin1 has a pivotal role in a variety of biological processes such as cell cycle control (16), and its deregulation contributes to various pathological conditions, most notably cancer (11, 12, 17, 18). Pin1 is overexpressed in various human cancers, contributing to centrosome amplification, chromosome instability, and tumor development *in vitro* and *in vivo* and correlating with poor clinical outcomes (10, 19–22). In contrast, inhibition of Pin1 suppresses tumorigenesis *in vitro* (23) and prevents cancer development induced by overexpression of oncogenes such as Neu or Ras (24) or by knockout of tumor suppressors such as p53 (25) in mice.

Thus, Pin1 has key roles in control of cellular functions. However, its significance for EBV replication has yet to be clarified in detail. In this study, we show that Pin1 interacts with EBV DNA polymerase BALF5 and modulates productive viral DNA replica-

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