With regard to the safety of SMILE, myelosuppression and infection should be carefully monitored during and after SMILE chemotherapy. To avoid severe AEs, the use of granulocyte colony-stimulating factor is considered mandatory, starting on day 6 and continuing until recovery beyond the nadir. In addition, full-dose administration of SMILE should be avoided for patients who are in poor condition, including those with lymphopenia less than 500/μL or large tumor burden. A lymphocyte count was added to the eligibility criteria because all three of the patients who died of neutropenic infection in the phase I and phase II SMILE studies had low lymphocyte counts before treatment. Decreased-dose SMILE[21] and less-intensive L-asparaginase chemotherapies[22-24] are candidate strategies for those patients with poor pretreatment conditions.

L-asparaginase–based chemotherapy has been highlighted as a promising treatment for ENKL. L-asparaginase was shown to induce apoptosis of ENKL cells in vitro; this result was attributed to low asparagine synthetase expression.[25] In fact, there were several case reports in the early 2000s in which ENKL showed an excellent response to L-asparaginase.[26-30] Recently, a phase II study of L-asparaginase, methotrexate, and dexamethasone (AspaMetDex) for relapsed or refractory ENKL was reported by a French group.[22] Nineteen patients were enrolled, and the CR rate was 61%. The median survival time was 12.2 months, and the 1-year OS was 45%. The AspaMetDex therapy is also promising, but there are several differences from the SMILE study. First, 53% of patients in our SMILE study had newly diagnosed stage IV ENKL which showed poor prognosis with conventional chemotherapy.[6] In contrast, the GELA (Groupe d'Etude des Lymphomes de l'Adulte)/GOELAMS (Groupe Ouest-Est des Leucémies et des Autres Maladies du Sang) study included only patients with relapsed/refractory disease. This resulted in a different ratio of patients with advanced-stage disease between the SMILE study (27 of 38 patients, 71%) and the AspaMetDex study (seven of 19 patients, 37%). Second, 17 of the 19 patients were initially treated with anthracycline-based chemotherapy in the French study. In contrast, 81% of the patients who had prior therapy in our study received platinum-based chemotherapy before SMILE, which suggests that different patient groups were selected in the two studies. Currently, these SMILE and AspaMetDex regimens are both promising for relapsed/refractory ENKL. A comparative study is required for a conclusion, but is not realistic for this type of rare lymphoma.

The optimal course of SMILE chemotherapy and the most appropriate timing of HSCT for patients after two courses of SMILE remain undetermined. In addition, the optimal treatment strategy for patients who cannot undergo HSCT needs further clinical evaluation. It has been speculated that the SMILE regimen may also be effective for T-cell lymphomas because ENKL and mature T-cell lymphomas share several clinical and pathologic features, such as extranodal predilection and expression of cytotoxic molecules. This speculation should be confirmed in further clinical studies.

In conclusion, the results of this phase II study demonstrate that two cycles of SMILE is an effective chemotherapy regimen for patients with newly diagnosed stage IV, relapsed, or refractory ENKL. However, the SMILE regimen is potentially toxic, and careful patient monitoring is needed.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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CORRESPONDENCE

Elevated expression of activation-induced cytidine deaminase in T and NK cells from patients with chronic active Epstein-Barr virus infection

Chronic active Epstein-Barr virus (EBV) infection (CAEBV) is a systemic EBV-positive lymphoproliferative disorder characterized by persistent or recurrent infectious mononucleosis-like symptoms [1]. Clonally expanding EBV-infected T cells or natural killer (NK) cells play a pathogenetic role in patients with CAEBV, particularly in East Asia, and may induce skin manifestations represented by hydroa vacciniforme-like eruptions or hypersensitivity to mosquito bites (HMB) [2]. The patients can be haematologically classified into two groups based on the predominantly infected cell types, T cells or NK cells [3]. The detailed pathomechanism of CAEBV and its progression to malignant conditions remain unknown.

Activation-induced cytidine deaminase (AID), a member of the cytidine deaminase family, is essential for somatic hypermutation and class-switch recombination in immunoglobulin genes [4]. Recently, evidence has shown that inappropriately expressed AID acts as a genomic mutator which contributes to tumorigenesis such as gastric carcinoma and adult T cell leukemia/lymphoma (ATLL) [5, 6]. By using a skin specimen and peripheral blood mononuclear cells (PBMCs) from a patient with CAEBV, and EBV-infected T and NK cell lines, we investigated the role of AID in CAEBV.

A 25-year-old male (case 23 in *table 1*) was referred to our hospital with a 2-year-history of high fever, erythema and lymph node swelling after mosquito bites. On examination, he showed exaggerated vesicular skin lesions with central necrosis at the mosquito bite sites *(figure 1A)*. Laboratory analysis demonstrated increased lymphocytes (44.5%)

Table 1. Details of the 23 CAEBV patients enrolled in this study. Patients with severe symptoms were defined as having a clinically active disease; patients with no symptoms or with only skin symptoms were defined as having an inactive disease.

Patient	Age (years)	Gender	Cell type infected	Disease type	HSCT	Outcome	*AID/GAPDH* ($x10^{-5}$)
1	15	F	T	Inactive	-	Alive	1.96
2	10	M	T	Inactive	-	Alive	1.25
3	13	M	T	Inactive	+	Dead	N.D.
4	18	F	T	Active	+	Alive	N.D.
5	45	F	T	Inactive	-	Alive	4.99
6	13	F	T	Active	+	Alive	0.387
7	6	M	T	Active	+	Alive	0.0115
8	4	F	T	Active	+	Alive	3.86
9	23	F	T	Inactive	+	Dead	0.0915
10	24	F	T	Active	-	Dead	N.D.
11	37	M	T	Active	+	Alive	2.35
12	14	M	NK	Inactive	+	Alive	1.05
13	5	F	NK	Active	+	Alive	0.0812
14	12	M	NK	Inactive	-	Alive	N.D.
15	11	F	NK	Inactive	+	Daed	N.D.
16	14	F	NK	Inactive	-	Dead	4.47
17	16	M	NK	Inactive	+	Dead	0.758
18	9	M	NK	Inactive	-	Alive	0.841
19	20	F	NK	Active	-	Alive	N.D.
20	14	F	NK	Inactive	+	Dead	N.D.
21	11	F	NK	Inactive	+	Alive	3.48
22	26	M	NK	Inactive	+	Alive	0.432
23	25	M	NK	Inactive	-	Alive	0.166

HSCT: haematopoietic stem cell transplantation; N.D.: not detected.

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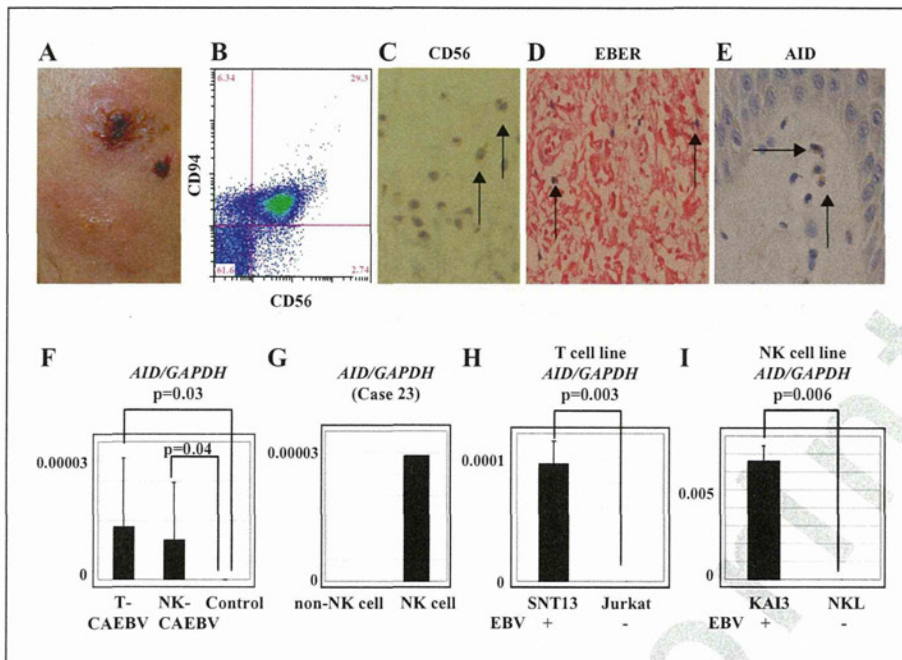

Figure 1. A) Erythema and vesicles with central necrosis on the mosquito biting cheek.
B) Flow cytometry analysis showed an increased frequency of $CD56^{+}CD94^{+}$ cells in PBMCs.
C, D, E) CD56 (**C**), EBER (**D**), AID (**E**) positive cells in infiltrating cells in the dermis of a hand skin biopsy specimen (arrows).
F) AID mRNA expression of PBMCs was significantly higher in T-cell CAEBV and NK-cell CAEBV than normal controls.
G) *AID* mRNA was dominantly expressed by NK-cells in the case 23
H, I) *AID* mRNA expression levels were significantly higher in EBV-infected SNT13 (**H**) and KAI3 (**I**) cell lines than non-infected Jurkat or NKL cells, respectively. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

with a normal leukocyte count and high titers of antibodies to viral capsid antigen (VCA) (x640), early antigen (EA) (x20), and EBV nuclear antigen (EBNA) (x320). EBV DNA (PCR) was 7.1×10^4 copies/μg DNA. Flow cytometry analysis showed an increased frequency of $CD56^{+}CD94^{+}$ NK cells in PBMCs *(figure 1B)*, a characteristic feature of HMB [2]. Immunohistochemical examination of a skin biopsy specimen demonstrated that dermal infiltrating cells expressed CD56 *(figure 1C)*. *In situ* hybridization study revealed EBV-encoded small nuclear RNA (EBER) positive cells in infiltrating cells in the dermis *(figure 1D)*. Based on clinical and laboratorial findings, the patient was diagnosed as having HMB, CAEBV and NK cell lymphocytosis. To explore the expression of AID in the skin of this patient, we performed an immunohistochemical study using an anti-human AID antibody (ZYMED, Carlsband, CA). AID was expressed in lymphocytes infiltrating in the dermis *(figure 1E)*.

We analyzed the expression level of AID mRNA in frozen samples from PBMCs of 11 T-cell CAEBV patients, 12 NK-cell CAEBV patients and 7 normal subjects without histories of EBV-related clinical symptoms, using real-time PCR 7300 system (Applied Biosystems, Foster City, CA) [3, 6]. The patients' details are summarized in *Table 1*. *AID* mRNA expression of both T-cell and NK-cell CAEBV was significantly higher than that of normal controls *(figure 1F)*. However, *AID* mRNA expression level was not correlated with patients' age, gender, cell type infected or prognosis *(table 1)*. To ascertain whether *AID* mRNA was expressed by NK cells in our CAEBV patient (case 23 in *table 1*), we separated NK cells from the patient's PBMCs by immunomagnetic sorting with an NK cell isolation kit (Militenyi Biotec, Bergisch Gladbach, Germany). *AID* mRNA was expressed dominantly by NK cells in the patient *(figure 1G)*, suggesting a role of AID in the pathogenesis.

We further compared the expression levels of *AID* mRNA between EBV-infected T (SNT13) and non-infected T-cell (Jurkat) lines and between EBV-infected NK (KAI3) and non-infected NK-cell (NKL) lines [4]. The expression level of *AID* was significantly higher in EBV-infected T and NK cells than non-infected T and NK cell lines, respectively *(Figure 1H, I)*.

Our previous study revealed that AID expression was elevated in human T-cell leukemia virus (HTLV)-I infected ATLL patients, but not in HTLV-I carriers, indicating a role of AID in neoplastic development [6]. Taken together, high AID expression might also play some role in a pathogenesis of another virus-induced lymphocytic disorder, CAEBV. ■

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Clinicopathological analysis of the age-related differences in patients with Epstein–Barr virus (EBV)-associated extranasal natural killer (NK)/T-cell lymphoma with reference to the relationship with aggressive NK cell leukaemia and chronic active EBV infection-associated lymphoproliferative disorders

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Clinicopathological analysis of the age-related differences in patients with Epstein–Barr virus (EBV)-associated extranasal natural killer (NK)/T-cell lymphoma with reference to the relationship with aggressive NK cell leukaemia and chronic active EBV infection-associated lymphoproliferative disorders

Aims: Extranodal natural killer (NK)/T-cell lymphoma (NKTL), comprising nasal NKTL and extranasal NKTL (ENKTL), is associated with Epstein–Barr virus (EBV). A bimodal age distribution was noted in NKTL patients. We examined the clinicopathological differences between two age groups of ENKTL patients ($n = 23$) and compared the findings with those of aggressive NK cell leukaemia (ANKL; $n = 10$) and monoclonal chronic active EBV infection-associated T/NK-cell lymphoproliferative disorders [chronic active EBV infection/TNK-lymphoproliferative disorders (CAEBV/TNK-LPD)] of NK-cell type ($n = 45$).
Methods and results: Distinct differences existed between elderly (>50 years; $n = 13$) and younger (≤50 years; $n = 10$) ENKTL patients; the latter showed a higher disease stage ($P = 0.0286$), worse performance status ($P = 0.0244$), more frequent B symptoms ($P = 0.0286$) and more frequent liver, spleen and bone marrow involvement ($P = 0.0222$, 0.0005 and 0.0259, respectively). Few clinicopathological differences existed between younger ENKTL and ANKL patients. Patients with monoclonal CAEBV/TNK-LPD of NK-cell type ($n = 45$) showed features similar to those in younger ENKTL/ANKL patients, except a more juvenile onset of CAEBV-related symptoms and better prognosis. However, the onset age of overt leukaemia/lymphoma in CAEBV/TNK-LPD patients and overall survival thereafter were similar to those in younger ENKTL/ANKL patients.
Conclusions: ENKTL (≤50 years) is distinct from that in elderly patients and may encompass ANKL and overlap in the clinicopathological profile with NK-cell type CAEBV/TNK-LPD.

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Keywords: aggressive NK cell leukaemia, chronic active EBV infection, Epstein–Barr virus, extranodal NK/T-cell lymphoma, nasal type, T/NK-cell lymphoproliferative disorders

Abbreviations: ANKL, aggressive NK cell leukaemia; BM, bone marrow; CAEBV, chronic active EBV infection; EBV, Epstein–Barr virus; ENKTL, extranasal NKTL; HMB, hypersensitivity to mosquito bite; LDH, lactate dehydrogenase; LGL, large granular lymphocytosis; LPD, lymphoproliferative disorders; NK, natural killer; NKTL, NK/T-cell lymphoma; PB, peripheral blood; PBSCT, peripheral blood stem cell transplantation; TCR, T cell receptor

Introduction

Epstein–Barr virus (EBV), a member of the Herpesviridae, is associated with a variety of lymphoproliferative disorders (LPD).[1–7] B-cell lymphomas predominate among EBV-associated LPD, which are often associated with immunodeficiency but not with a specific geographical distribution. Conversely, some EBV$^+$ T/natural killer (NK)-cell LPD are more prevalent in Asia and Latin America and have been categorized in the recent World Health Organization (WHO) classification as extranodal NK/T-cell lymphoma (NKTL), nasal type; aggressive NK cell leukaemia (ANKL); and systemic EBV-positive T-cell lymphoproliferative disease of childhood.[8–10] These T/NK-cell LPD commonly share a cytotoxic phenotype, CD56 positivity to varying degrees, a constant association with EBV, a relatively younger onset, a propensity to involve extranodal sites and aggressive clinical behaviour.[1–12]

Extranodal NKTL, nasal type is subclassified into two subtypes: nasal NKTL, which affects the nasopharyngeal region, and extranasal NKTL (ENKTL), which affects other extranodal sites such as the skin, gastrointestinal tract, testis, uterus and soft tissue.[8] Patients with ENKTL present with a significantly greater occurrence of disseminated disease and more aggressive biological features than patients with nasal NKTL. ENKTL is characterized by a higher disease stage, elevated lactate dehydrogenase (LDH) level, more frequent B symptoms, poorer performance status and shorter 5-year overall survival.[11,13] Thus, ENKTL may be a systemic disease distinct from nasal NKTL.

ANKL is also characterized by systemic involvement and aggressive behaviour occurring in the younger population.[9] The clinical distinctiveness of ANKL from ENKTL remains unclear, because they are currently distinguished based only on the degree of bone marrow (BM) and/or peripheral blood (PB) involvement.[11,14]

Systemic EBV-positive T-cell lymphoproliferative disease of childhood, a new category in the 2008 WHO classification, represents a main type of aggressive T/NK-cell LPD in children and young adults.[10] This disease belongs to a category documented originally as chronic active EBV infection (CAEBV)-associated T/NK-cell LPD (CAEBV/TNK-LPD). CAEBV/TNK-LPD occurs almost exclusively in East Asia (particularly Japan and Korea) and Latin America and shows a broad spectrum of severity, ranging from polyclonal T/NK-cell proliferation to monoclonal overt leukaemia/lymphoma.[12,15–20] Ohshima *et al.*[18] graded CAEBV/TNK-LPD into a continuous spectrum of categories, including categories A1 (polymorphic and polyclonal), A2 (polymorphic and monoclonal) and A3 (monomorphic and monoclonal, i.e. NK cell lymphoma/leukaemia or peripheral T-cell lymphoma). In addition, tumour progression from category A1 through category A2 to category A3 (leukaemia/lymphoma) in the clinical course of CAEBV/TNK-LPD patients has also been speculated to occur.[15,18] The histopathology of monoclonal CAEBV/TNK-LPD without overt leukaemia/lymphoma in category A2 and that with overt leukaemia/lymphoma in category A3 is different. The former exhibits a reactive appearance such as mildly atypical lymphocyte infiltration and/or a hyperplastic pattern with a mild increase in transformed lymphocytes, whereas the latter shows diffuse proliferation of monomorphic atypical medium- to large-sized lymphoid cells with irregular nuclei, frequent mitotic figures and necrosis.[18] Furthermore, when the EBV-infected cells show clonality in categories A2 and A3 of CAEBV/TNK-LPD, the clinical features between NK-cell and T-cell types differ significantly.[6,15,21,22] The characteristics of CAEBV/TNK-LPD of NK-cell type include an increased prevalence of hypersensitivity to mosquito bite (HMB), large granular lymphocytosis (LGL), high IgE titres in the serum and association with the development of NK/T-cell leukaemia/lymphoma, whereas those of T-cell type are high fever, high titres of viral capsid antigen immunoglobulin (Ig)G and early antigen IgG, shorter survival times, and association with the development of T-cell leukaemia/lymphoma.[21–23]

A characteristic bimodal age distribution and generally worse prognosis in elderly compared to younger patients has been well documented in EBV-associated

Hodgkin lymphoma[24–26] or diffuse large B-cell lymphoma.[27] Ohshima *et al.*[18] recently reported a bimodal age distribution in patients with extranodal NKTL, nasal type, with a small peak at 16–25 years and a larger peak at 56–65 years. However, clinicopathological differences between these two groups of patients are largely unknown. Moreover, although ENKTL occurring in younger patients may be related to ANKL and/or NK-cell type CAEBV/TNK-LPD, their interrelationship has not been studied extensively.

The purpose of this study was to understand the clinicopathological defferences between younger and elderly patients with ENKTL, to compare them with those in patients with ANKL or NK-cell type monoclonal CAEBV/TNK-LPD, and thus to elucidate the relationship among these disease entities. Cases of polyclonal CAEBV/TNK-LPD in category A1 and T-cell type CAEBV/TNK-LPD in categories A2 and A3 were excluded from our comparative study, as those were apparently distinct from ENKTL.

Materials and methods

PATIENTS

The study included 23 patients with ENKTL, selected from the NK cell Tumor Study Group case files and 10 patients with ANKL examined in our previous study.[28]

Table 1. Diagnostic criteria of ENKTL, ANKL, monoclonal NK-cell type CAEBV/TNK-LPD without and with overt leukaemia/lymphoma

	ENKTL	ANKL	NK-cell type CAEBV/TNK-LPD without overt leukaemia/lymphoma (Ohshima's category A2)	NK-cell type CAEBV/TNK-LPD with overt leukaemia/lymphoma (Ohshima's category A3)
Tumour cells in BM (%)/PB (%)	<30/<30	≥30 and/or ≥30	Any/any	Any/any
CD56	+	+	+	+
Cytotoxic molecules	+	+	+	+
EBV association	+	+	+	+
Clonality	+	+	+	+
Overt lymphoma/leukaemia	+	+	–	+
Fulfilling of diagnostic Criteria of CAEBV	ND	ND	+	+
Increased quantities of EBV*	+	+	+	+
Hypersensitivity to mosquite bite	ND	ND	–/+	+/–

–/+: fewer than 50% of patients show the symptoms; +/–: more than 50% of patients show the symptoms.
*Increased EBV-DNA copies in PB.
Diagnostic critetia of CAEBV/LPD:
(1) Illness of more than 6 months' duration: EBV-related illness or symptoms including fever, persistent hepatitis, extensive lymphadenopathy,
hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme or hypersensitivity to mosquito bites (HMB).
(2) Increased quantities of EBV in either affected tissues or peripheral blood. The amount of EBV was defined as increased when one or more of the following criteria were met:
(a) EBV DNA was detected in tissues or peripheral blood by Southern blot hybridization;
(b) EB-encoded small RNA 1 (EBER1) RNA^+ cells were detected in tissues or peripheral blood; or
(c) more than $10^{2.5}$ copies/μg DNA were detected in peripheral blood mononuclear cells (PBMC).
(3) No evidence of any prior immunological abnormalities or of any other recent infection that might explain the condition.
ENKL, Extranasal natural killer (NK)/T-cell lymphoma; ANKL, aggressive NK cell leukaemia; CAEBV, chronic active Epstein–Barr virus (EBV) infection; LPD, lymphoproliferative disorder; BM, bone marrow; PB, peripheral blood; ND, not well documented in clinical charts.

Forty-five patients with CAEBV/TNK-LPD showing clonal proliferation of EBV$^+$ NK cells were also selected from our previous studies,[20,21] excluding cases of T-cell type CAEBV/TNK-LPD with a T-cell receptor (TCR) rearrangement. Cases of ENKTL and ANKL variously affected extranasal sites and commonly showed CD56 and cytotoxic molecule expression and EBV association in tumour cells; these cases were distinguished by the degree of PB and BM involvement, i.e. tumour cells in excess of 30% of the nucleated cells in ANKL.[28] Patients with NK-cell type CAEBV/TNK-LPD fulfilled the diagnostic criteria according to Kimura *et al.*[22] (Table 1), and were subdivided into 23 cases without overt leukaemia/lymphoma (category A2 or Suzuki *et al.*'s[20] group B, chronic state) and 22 cases with overt leukaemia/lymphoma (category A3 or Suzuki *et al.*'s[20] group C, NK cell leukaemia/lymphoma).

IMMUNOPHENOTYPE, EBV AND MOLECULAR STUDIES

Immunoperoxidase studies were performed on formalin-fixed, paraffin-embedded tissue sections from biopsy specimens of tissue obtained from patients with EBV$^+$ ENKTL, using the avidin–biotin–peroxidase complex method. A panel of monoclonal antibodies against CD3 (F7.2.38, dilution 1:25; Dako, Glostrup, Denmark), CD4 (1F6, dilution 1:40; Novocastra, Newcastle, UK), CD5 (4C7, dilution 1:40; Novocastra), CD8 (C8/144B, dilution 1:25; Dako), CD45RO (UCHL1, dilution 1:80; Dako), CD56 (1B6, dilution 1:50; Novocastra), granzyme B (GrB-7, dilution 1:80; Monosan, Uden, the Netherlands), *T cell intracellular antigen-1* (TIA-1, dilution 1:80; Immunotech, Marseille, France) and perforin (5B10, dilution 1:20; Novocastra) were used. For all antibodies, except CD4, slides were pretreated using a microwave procedure for antigen retrieval. To identify cells infected with EBV in specimens from patients with CAEBV/TNK-LPD, peripheral blood mononuclear cells were fractionated into CD3$^+$, CD4$^+$, CD8$^+$, CD16$^+$, CD19$^+$ and CD56$^+$ cells using the immunobead method.[21] Fractionated cells were analysed either by quantitative polymerase chain reaction or *in situ* hybridization with the EBV-encoded small nuclear early region (EBER) oligonucleotides probe.[21] Patients were defined as having an NK-cell type infection when cells containing EBV were mainly CD16$^+$ or CD56$^+$.[21] A portion of each lymph node was stored at −80°C and the nodes were examined using monoclonal antibodies for T cells (CD2, CD3, CD4 and CD8) and NK cells (CD16, CD56 and CD57).[20] To exclude T-cell type large granular lymphocyte leukaemia and leukaemic infiltration of other T-cell lymphomas, the tumour cells of ANKL patients needed to be negative for surface CD3, as determined by flow cytometry, or germline configurations of T-cell receptor genes by Southern blot analysis.[20,28] The presence of EBV was determined by *in situ* hybridization using EBER oligonucleotides or by Southern blotting using a terminal repeat probe.[20,21,28]

STATISTICAL ANALYSIS

Variables related to clinicopathological findings were compared between the two groups by the chi-squared and Mann–Whitney *U*-tests. Patient survival data were analysed by the Kaplan–Meier method. Differences in survival were tested by the log-rank test. All data were analysed with the aid of the Statview software program (version 5; SAS Institute Inc., Cary, NC, USA). Because stem cell transplantation dramatically influences outcome, patients who had undergone transplantation were excluded from the overall survival analysis.

Results

COMPARISON OF EBV$^+$ ENKTL CASES BETWEEN YOUNGER AND ELDERLY PATIENTS INCLUDING CASES OF EBV$^+$ ANKL

The EBV$^+$ ENKTL group comprised 23 patients (11 males and 12 females) ranging from 3 to 89 years (median, 57 years), and that of EBV$^+$ ANKL comprised 10 patients (three males and seven females) ranging from 12 to 45 years (median, 29 years). The age profile associated with the ENKTL clinical stage is displayed in Figure 1. Clinical stage varied with age and was distinctly different in more advanced (stages III and IV) and localized (stages I and II) clinical stages in the younger and elderly groups, respectively. All patients aged 50 years or younger were characterized by an advanced clinical stage, except

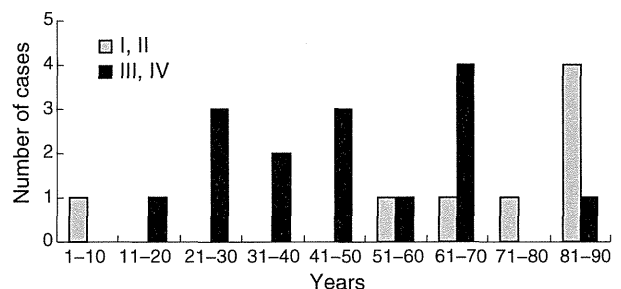

Figure 1. Age distribution associated with clinical stage of Epstein–Barr virus (EBV)$^+$ extranasal natural killer/T-cell lymphoma (ENKTL). Clinical stage varies distinctly with age, showing a distinct difference between more advanced (stages III and IV) and localized (stages I and II) clinical stages in younger and elderly groups, respectively.

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Table 2. Clinical and immunophenotypical features in age-defined subgroups of patients with ENKTL and ANKL

	EBV$^+$ extranasal NK/T-cell lymphoma (≤50)	EBV$^+$ extranasal NK/T-cell lymphoma (>50)	EBV$^+$ ANKL (≤50)	*P**	*P†*
No. of patients	10	13	10		
Sex (male/female)	6/4	5/8	3/7	0.3053	0.1775
Age (years) median (range)	31 (3–48)	69 (53–89)	29 (12–45)	–	0.7133
PS					
0	2/10 (20%)	4/13 (31%)	0/10 (0%)	0.1654	0.5146
1	1/10 (10%)	6/13 (46%)	4/10 (40%)		
2	2/10 (20%)	1/13 (8%)	1/10 (10%)		
3	2/10 (20%)	0/13 (0%)	1/10 (10%)		
4	3/10 (30%)	2/13 (15%)	3/10 (30%)		
PS≥2	7/10 (70%)	3/13 (23%)	5/10 (50%)	0.0244	0.3613
Clinical stage					
I	1/10 (10%)	4/13 (31%)	0/10 (0%)	0.1221	0.3292
II	0/10 (0%)	3/13 (23%)	0/10 (0%)		
III	1/10 (10%)	0/13 (0%)	0/10 (0%)		
IV	8/10 (80%)	6/13 (46%)	10/10 (100%)		
Advanced stages (III, IV)	9/10 (90%)	6/13 (46%)	10/10 (100%)	0.0286	0.3049
B symptoms	9/10 (90%)	6/13 (46%)	10/10 (100%)	0.0286	0.3049
Extranodal site					
Skin	4/10 (40%)	10/13 (77%)	2/10 (20%)	0.0721	0.3291
Liver	7/10 (70%)	3/13 (23%)	8/10 (80%)	0.0222	0.6056
Spleen	8/9 (89%)	2/12 (17%)	8/10 (80%)	0.0005	0.5921
Bone marrow	6/10 (60%)	2/13 (15%)	8/8 (100%)	0.0259	0.0425
Peripheral blood	2/10 (20%)	0/12 (0%)	8/10 (80%)	0.1042	0.0073
Lymph node	4/10 (40%)	5/13 (30%)	4/10 (40%)	0.9403	1
LDH > normal	8/10 (80%)	10/13 (77%)	10/10 (100%)	0.8592	0.136
Immunophenotype					
cyCD3	9/10 (90%)	10/10 (100%)	1/2 (50%)	0.3049	0.1659
CD4	0/8 (0%)	1/9 (11%)	0/10 (0%)	0.331	1
CD8	4/9 (44%)	0/8 (0%)	2/9 (22%)	0.0311	0.3173
CD45RO	4/6 (67%)	3/3 (100%)	–	0.2568	–
EBER (ISH)	10/10 (100%)	13/13 (100%)	10/10 (100%)	1	1

*Epstein–Barr virus (EBV)$^+$ extranasal natural killer (NK)/T-cell lymphoma (≤50) versus EBV$^+$ extranasal NK/T-cell lymphoma (>50).

†EBV$^+$ extranasal NK/T-cell lymphoma (≤50) versus EBV$^+$ ANKL (≤50).

ANKL, Aggressive NK cell leukaemia; PS, performance status; LDH, lactate dehydrogenase; EBER, EBV-encoded early small RNA; ISH, *in situ* hybridization.

one juvenile case of 3 years of age with an unusual lesion restricted to the liver. Therefore, we used 50 years as the cut-off age in the present study and compared clinicopathological features between these two age-related groups. Table 2 summarizes the clinicopathological features of the patients with ENKTL aged 50 years or younger (n = 10) and those older than 50 years (n = 13). Compared with elderly patients, younger patients showed worse performance status (P = 0.0244), higher disease stage (P = 0.0286), more frequent B symptoms (P = 0.0286) and more frequent involvement of the liver, spleen, and bone marrow (P = 0.0222, 0.0005 and 0.0259, respectively). Although not statistically significant, elderly patients were more likely to show skin involvement. Furthermore, the survival curve for younger patients was generally worse than that for elderly patients, but the difference was not statistically significant because of the low number of cases examined (Figure 2). There were no significant differences in immunohistochemical and histological findings except for more frequent CD8 expression (P = 0.0311) in the younger group (Table 2). The sections showed diffuse infiltration of pleomorphic, medium- to large-sized lymphoid cells with variable numbers of smaller cells (Figure 3A). These tumour cells had elongated, angulated or irregularly folded nuclei with granular chromatin, although their cytomorphological findings varied across patients

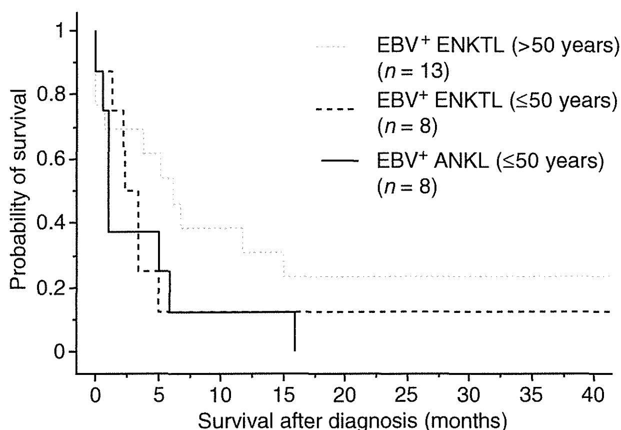

Figure 2. Overall survival curve for age-defined subgroups of patients with Epstein–Barr virus (EBV)$^+$ extranasal natural killer (NK)/T-cell lymphoma (ENKTL) and EBV$^+$ aggressive NK cell lymphoma (ANKL) (patients who received a transplantation are excluded). Younger patients with EBV$^+$ ENKTL (≤50 years) (n = 8) generally show worse survival than elderly patients with EBV$^+$ ENKTL (>50 years) (n = 13) (P = 0.2262), although the result is not statistically significant. No significant difference was observed between EBV$^+$ ENKTL (≤50 years) (n = 8) and EBV$^+$ ANKTL (≤50 years) (n = 8) (P = 0.6239).

(Figure 3A). Angiocentric–angiodestructive growth was observed in some sections. An associated inflammatory infiltrate, consisting of small lymphocytes, plasma cells and histiocytes, was often present. Frequent mitotic figures and varying numbers of apoptotic bodies were also observed. Lymphoma cells showed a characteristic CD56$^+$, CTM$^+$, EBV$^+$ phenotype (Figure 3B–D).

Subsequent analysis of EBV$^+$ ANKL patients aged 50 years or younger (n = 10) compared to EBV$^+$ ENKTL patients aged 50 years or younger (n = 10) showed few clinical and prognostic differences except for more frequent BM and PB involvement in the former group (P = 0.0425 and 0.073, respectively), according to the disease definition (Table 2). No significant difference was observed in overall survival between these two groups (Figure 2). Therefore, in this study, these patients were integrated into a single group of EBV$^+$ ENKTL/ANKL.

COMPARISON OF CASES WITH EBV$^+$ ENKTL/ANKL AND MONOCLONAL CAEBV/TNK-LPD OF NK-CELL TYPE IN YOUNGER PATIENTS

Compared with EBV$^+$ ENKTL/ANKL patients (≤50 years), monoclonal CAEBV/TNK-LPD of NK-cell type patients (n = 45; category A2 and A3) were characterized by their infantile and juvenile onsets of CAEBV-related symptoms as well as the highest peak at ages 1–10 years (P < 0.0001) (Figure 4). These patients had a mean age of 9.9 years (range 2–48 years), whereas patients with EBV$^+$ ENKTL/ANKL had a mean age of 31 years (range 3–48 years) (Table 3). The relative ratio of cases with EBV$^+$ ENKTL/ANKL among all patients increased with age from 4% in the first decade to 100% in the fourth decade (Figure 4). A comparison of clinical parameters among EBV$^+$ ENKTL/ANKL, monoclonal CAEBV/TNK-LPD of NK-cell type without overt leukaemia/lymphoma and NK-cell type CAEBV/TNK-LPD with overt leukaemia/lymphoma demonstrated no significant differences, no sex predominance, a high incidence of B symptoms and involvement of the spleen and liver and elevated LDH for all groups (Table 3). EBV$^+$ ENKTL/ANKL patients were associated with a worse prognosis than monoclonal CAEBV/TNK-LPD of NK-cell type patients (with and without overt leukaemia/lymphoma; categories A2 and A3), with the results showing a clear statistical difference (P < 0.0001) (Figure 5). In contrast, when survival curves in both groups were compared from the onset of overt EBV$^+$ lymphoma/leukaemia (at which time specific treatment became necessary), the statistical difference was reduced (P = 0.0219) (Figure 5). A

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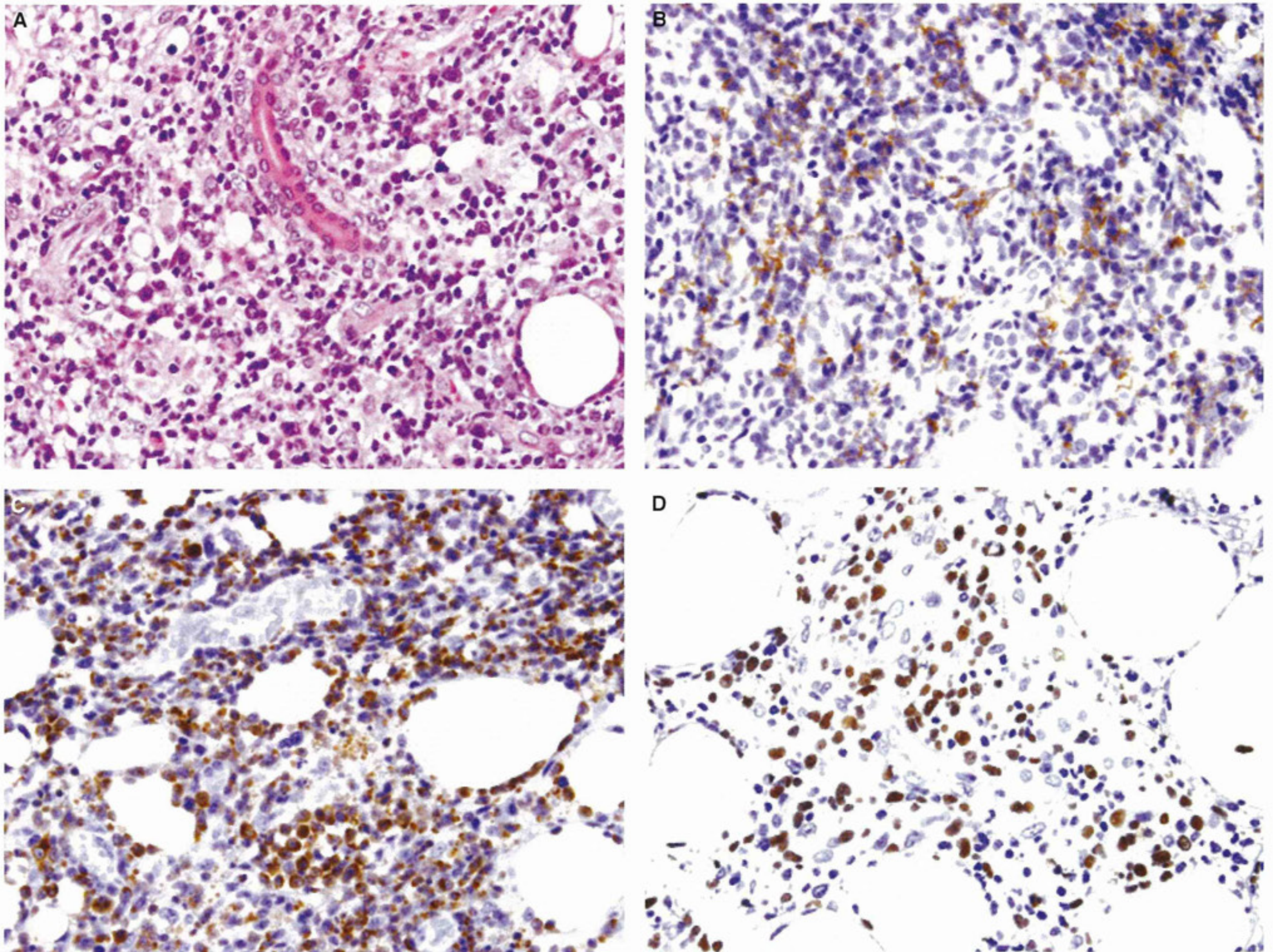

Figure 3. Epstein–Barr virus (EBV)$^+$ extranasal natural killer (NK)/T-cell lymphoma (ENKTL). **A**, Diffuse infiltration of pleomorphic medium- to large-sized cells with irregular nuclei. Mitotic figures and associated inflammatory infiltrate are also seen. **B**, Tumour cells are positive for anti-CD56 antibody with membranous reactivity. **C**, Perforin shows strong granular positivity of tumour cells. **D**, *In situ* hybridization for EBV-encoded early small RNA shows numerous positive cells.

unique manifestation of HMB was noted in 11 of 26 patients (42%) with monoclonal CAEBV/TNK-LPD of NK-cell type. Interestingly, in the retrospective review of clinical records, this unique manifestation was found in three well-documented EBV$^+$ ENKTL/ANKL cases, suggesting a CAEBV background. In addition, the age at detection of overt leukaemia/lymphoma in NK-cell type CAEBV/TNK-LPD was comparable to that for nasal and extranasal NK/T-cell lymphoma reported by Ohshima *et al.*[18] in a younger population, providing a possible explanation for the small peak at 16–25 years (Figure 6A). Age at death due to overt EBV$^+$ lymphoma/leukaemia was also similar between NK-cell type CAEBV/TNK-LPD and EBV$^+$ ENKTL/ANKL for patients younger than 30 years (Figure 6B). Histological and phenotypical features of CAEBV/TNK-LPD with overt leukaemia/lymphoma (EBV$^+$) were considerably similar to those of EBV$^+$ ENKTL/ANKL (Figure 7). Therefore, the diagnosis of CAEBV/TNK-LPD with overt leukaemia/lymphoma is not possible without clinical information indicating whether patients fulfilled the diagnostic criteria of CAEBV/TNK-LPD.

THERAPY

Most patients with ENKTL/ANKL were treated with chemotherapy containing/without anthracycline, but no response was observed in 69% of the cases. The remaining cases with a partial response ($n = 3$) or a complete response ($n = 3$), among which two cases were followed by peripheral blood stem cell transplantation (PBSCT) or bone marrow transplantation, were also accompanied by recurrence a short time after treatment. One case with a complete response, which

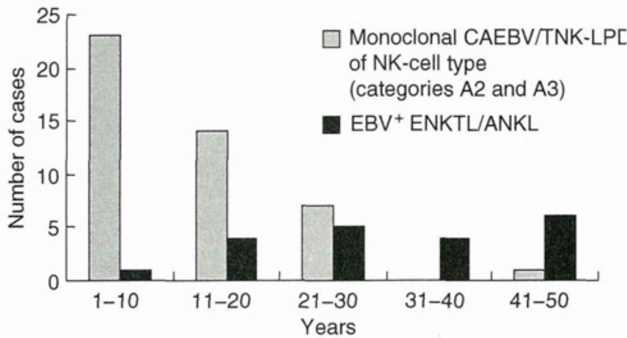

Figure 4. A, Onset age distribution of patients with monoclonal chronic active Epstein–Barr virus (EBV) infection (CAEBV)/TNK-LPD of NK-cell type (categories A2 and A3) and Epstein–Barr virus (EBV)$^+$ extranasal natural killer (NK)/T-cell lymphoma (ENKTL)/aggressive NK cell lymphoma (ANKL). Compared with EBV$^+$ ENKTL/ANKL patients, monoclonal CAEBV/TNK- lymphoproliferative disorders (LPD) of NK-cell type patients (with and without overt leukaemia/lymphoma; categories A2 and A3) are characterized by infantile and juvenile onsets of CAEBV-related symptoms with the highest peak at ages 1–10 years ($P < 0.0001$). The relative ratio of cases with EBV$^+$ ENKTL/ANKL among all cases examined increases with age from 4% in the first decade to 100% in the fourth decade.

was further treated by PBSCT, was alive without the disease. One case had no treatment because of the rapidly deteriorated clinical course.

A vast heterogeneous clinical approach was used for NK-cell type CAEBV/TNK-LPD patients because their initial clinical manifestations varied from case to case, with treatments spanning from antiviral treatment as acyclovir to chemotherapy containing anthracycline, or haematopoietic stem cell transplantation. Approximately 60% of the patients had a fetal clinical course despite treatment with a median follow-up period of 100 months.

CHROMOSOMAL EXAMINATION

The karyotype of lymphoma/leukaemia cells was examined for four cases with ENKTL, nine cases with ANKL and six cases with NK-cell type CAEBV/TNK-LPD patients by standard cytogenetic procedures, as described previously.[22,28] Four cases with ENKTL, two cases with ANKL and six cases with monoclonal CAEBV/TNK-LPD of NK-cell type exhibited aberrant chromosomal patterns. Some patients had multiple, different aberrations. However, there was no specificity in patterns or accumulation. Although del(6)(q21;25) or i(6)(p10) is reported as a common nasal tumour abnormality, these cytological aberrations were not observed in any cases, suggesting that extranasal cases may be distinct in pathogenesis from nasal lymphomas. The karyotype of lymphoma cells could not be determined in two cases with ANKL, and was normal diploid in five cases with ANKL.

Discussion

A bimodal age distribution of patients with nasal and extranasal NKTL, with a small peak at 16–25 years and a larger peak at 56–65 years, has recently been reported.[18] However, implications of this age distribution and clinicopathological differences between younger and elderly patients with NKTL remain largely unclear. It has also been reported that ENKTL is a systemic disease characterized by aggressive biological behavior and may be distinctive from nasal NKTL.[11,13] In our analyses, clinical features of EBV$^+$ ENKTL differed significantly between younger (≤50 years) and elderly patients (>50 years), although morphology and immunophenotype were indistinguishable between these two groups. Lesions in half (54%) the elderly cases were localized and restricted mainly to the skin. In contrast, most cases involving younger patients (nine of 10 cases, 90%) were characterized by an advanced clinical stage with frequent involvement of the liver, spleen and BM. These findings were very similar to those in patients with EBV$^+$ ANKL (≤50 years), which exhibits more frequent involvement of BM and PB. In addition, the overall survival of patients with EBV$^+$ ENKTL (≤50 years) and EBV$^+$ ANKL (≤50 years) was nearly identical. Clinical features of younger patients (≤50 years) with ENKTL showed a dramatic shift towards EBV$^+$ ANKL. These analyses suggested that the two groups might constitute a continuous spectrum between EBV$^+$ ENKTL and EBV$^+$ ANKL, leading to their single categorization as EBV$^+$ ENKTL/ANKL in the present study. EBV$^+$ ENKTL/ANKL in younger patients appears to be regarded generally as a systemic disease, lending further support to the hypothesis of an underlying endogenous systemic and localized immune dysregulation in younger and elderly patients, respectively.

A small peak of NKTL in patients aged 16–25 years prompted us to question the relationship between EBV$^+$ ENKTL/ANKL in younger patients and those with monoclonal CAEBV/TNK-LPD of NK-cell type, a high-risk disease associated with the development of EBV$^+$ NK/T-cell leukaemia/lymphoma. A broad spectrum of clinical findings, as well as tumour progression from category A1 through category A2 to category A3 (leukaemia/lymphoma) in the clinical course of CAEBV/TNK-LPD patients, has been suggested.[15,18] Therefore, clinical manifestations and outcomes might vary from case to case in CAEBV/TNK-LPD, depending on

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Table 3. Clinical features of cases of ENKTL/ANKL, monoclonal CAEBV/TNK-LPD of NK-cell type without overt leukaemia/lymphoma, and NK-cell type CAEBV/TNK-LPD with overt leukaemia/lymphoma

	EBV$^+$ ENKTL/ ANKL (≤50)	Monoclonal CAEBV/LPD of NK-cell type without overt lymphoma/ leukaemia (Ohshima's category A2)	NK-cell type CAEBV/LPD with overt lymphoma/ leukaemia (Ohshima's category A3)	*P**	*P*†	*P*‡
No. of patients	20	23	22			
Age at diagnosis (years) (median, range)	31 (3–48)	6 (2–24)	11 (2–48)	<0.0001	<0.0001	0.1724
Age at the onset of overt Lymphoma/leukaemia (years) (median, range)	31 (3–48)	–	20 (5–52)	–	0.0385	–
Age at death (years) (median, range)§	32 (12–51)	19 (15–25)	21 (9–53)	0.1033	0.136	0.6081
Sex (male/female)	9/11	14/9	12/10	0.298	0.5366	0.6677
B symptoms	19/20 (95%)	7/9 (78%)	12/14 (86%)	0.1589	0.3475	0.6241
Sites of involvement at presentation						
Skin	6/20 (30%)	3/9 (33%)	3/14 (21%)	0.8575	0.5772	0.4652
Liver	15/20 (75%)	7/9 (78%)	9/14 (64%)	0.8715	0.4998	0.4925
Spleen	16/19 (84%)	7/9 (78%)	8/14 (57%)	0.6781	0.0844	0.3106
Lymph node, secondary	8/20 (40%)	6/9 (67%)	7/14 (50%)	0.1837	0.5633	0.4313
LDH > normal	18/20 (90%)	9/14 (64%)	8/11 (72%)	0.068	0.2109	0.6533
Hypersensitivity to mosquite bite	3/12 (25%)	2/8 (25%)	9/18 (50%)	0.2337	0.1709	1

*Epstein–Barr virus (EBV)$^+$ ENKTL/ANKL (≤50) versus monoclonal CAEBV/LPD of NK-cell type without overt leukaemia/lymphoma.
†EBV$^+$ ENKTL/ANKL (≤50) versus NK-cell type CAEBV/LPD with overt leukaemia/lymphoma.
‡Monoclonal CAEBV/LPD of NK-cell type without overt leukaemia/lymphoma versus NK-cell type CAEBV/LPD with overt leukaemia/lymphoma.
§Patients who received a transplantation are excluded.
ENKL, Extranasal natural killer (NK)/T-cell lymphoma; ANKL, aggressive NK cell leukaemia; CAEBV, chronic active EBV infection; LPD, lymphoproliferative disorder; LDH, lactate dehydrogenase.

the stage at which CAEBV-related symptoms are recognized. In our study, there were few differences in the main clinicopathological parameters among the three groups of EBV$^+$ ENKTL/ANKL (≤50 years), monoclonal CAEBV/TNK-/LPD of NK-cell type without overt leukaemia/lymphoma (category A2) and NK-cell type CAEBV/TNK-LPD with overt EBV$^+$ leukaemia/lymphoma (category A3). From the time of detection of overt leukaemia/lymphoma (EBV$^+$), the survival curve of NK-cell type CAEBV/TNK-LPD showed a shift towards EBV$^+$ ENKTL/ANKL (≤50 years). Moreover, long-term survival of both groups appeared to be similar. The more favourable outcome associated with CAEBV/TNK-LPD with overt leukaemia/lymphoma might be because once diagnosed with CAEBV/TNK-LPD, patients appeared to have received a close follow-up for the potential development of overt leukaemia/lymphoma, enabling

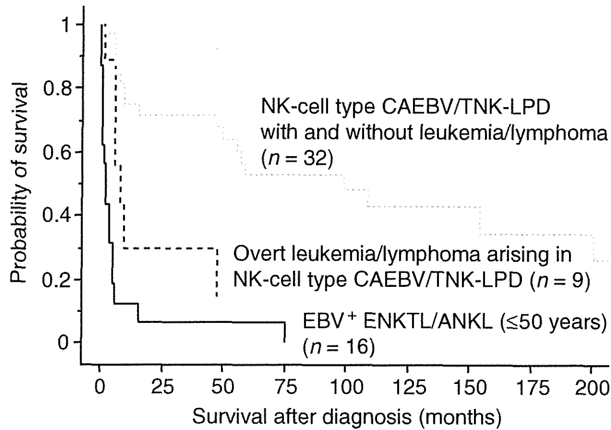

Figure 5. Overall survival from the onset of chronic active Epstein–Barr virus (EBV) infection (CAEBV)-related symptoms or the onset of overt lymphoma/leukaemia (patients who received a transplantation are excluded). Two survival curves of CAEBV/TNK-lymphoproliferative disorders (LPD) of NK-cell type, each calculated from the onset of CAEBV-related symptoms and the detection of overt leukaemia/lymphoma, respectively, are compared with that of EBV$^+$ extranasal NK/T-cell lymphoma (ENKTL)/aggressive NK cell lymphoma (ANKL). Patients with EBV$^+$ ENKTL/ANKL (n = 17) show significantly worse survival than those with monoclonal CAEBV/TNK-LPD of NK-cell type (with and without overt leukaemia/lymphoma; categories A2 and A3) (n = 25) from the onset of CAEBV-related symptoms (P < 0.0001). In contrast, when survival curves in both groups were compared from the onset of overt EBV$^+$ lymphoma/leukaemia, the statistical difference was reduced (P = 0.0219). In addition, long-term survival appears to be similar between the two groups.

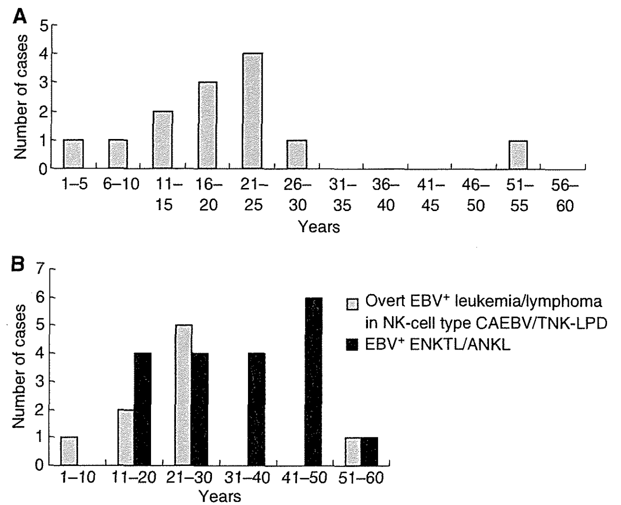

Figure 6. **A**, Age at onset of overt leukaemia/lymphoma in natural killer (NK)-cell type chronic active Epstein–Barr virus (EBV) infection (CAEBV)/TNK-lymphoproliferative disorders (LPD) (category A3). The onset age of overt leukaemia/lymphoma arising in NK-cell type CAEBV/TNK-LPD was comparable with that of nasal and extranasal NK/T-cell lymphoma (ENKTL) indicated by Ohshima *et al.*,[18] both showing a small peak at 16–25 years. **B**, Age at death due to overt EBV$^+$ leukaemia/lymphoma in NK-cell type CAEBV/TNK-LPD (category A3) and EBV$^+$ ENKTL/aggressive NK cell lymphoma (ANKL). Age at death due to EBV$^+$ overt leukaemia/lymphoma is similar between NK-cell type CAEBV/TNK-LPD and ENKTL/ANKL patients younger than 30 years.

prompt initiation of treatment for aggressive diseases based on CABEV/TNK-LPD. Initiating treatment with antiviral agents such as acyclovir or immunochemotherapy with steroids and cyclosporine A before the onset of overt leukaemia/lymphoma might improve the outcomes of patients with CAEBV/TNK-LPD. These analyses indicated that CAEBV/TNK-LPD generally exhibits an indolent course until overt leukaemia/lymphoma arises, exhibiting features similar to EBV$^+$ ENKTL/ANKL (≤50 years), followed by a rapidly deteriorated clinical course and very poor subsequent prognosis.

Interestingly, the age of onset for overt leukaemia/lymphoma (EBV$^+$) arising in NK-cell type CAEBV/TNK-LPD was consistent with that of NK/T-cell lymphoma documented by Ohshima *et al.*[18] in a younger population. In addition, age at death due to overt leukaemia/lymphoma (EBV$^+$) was similar between these two systemic EBV$^+$ diseases in patients younger than 30 years. These analyses indicated that these systemic EBV$^+$ diseases in a younger population are often indistinguishable based on clinical, pathological and immunophenotypical findings alone, and that only the clinical detection of peculiar CAEBV episodes suggests a diagnosis of CAEBV/TNK-LPD. Sufficient studies have not been conducted regarding the presence or absence of CAEBV and its related symptoms in EBV$^+$ ENKTL/ANKL. Indeed, in the present ENKTL/ANKL series, the presence or absence of any history suggesting CAEBV/TNK-LPD, such as HMB in childhood, antibody titres against EBV-related proteins or EBV viral loads in the serum, has not been fully examined. Therefore, the possibility of CAEBV/TNK-LPD should be considered, particularly in younger populations with any type of systemic EBV$^+$ NK cell LPD.

In conclusion, we analysed the relationship among EBV$^+$ ENKTL, EBV$^+$ ANKL and monoclonal CAEBV/TNK-LPD of NK-cell type. Clinical features significantly differed between younger and elderly patients with EBV$^+$ ENKTL. Compared with elderly patients (>50 years), younger patients (≤50 years) had systemic disease characterized by aggressive behaviour. Our study also provided additional evidence that a continuous spectrum of EBV$^+$ ENKTL to EBV$^+$ ANKL exists, particularly in younger populations. To the best of our knowledge, this is the first report analysing the

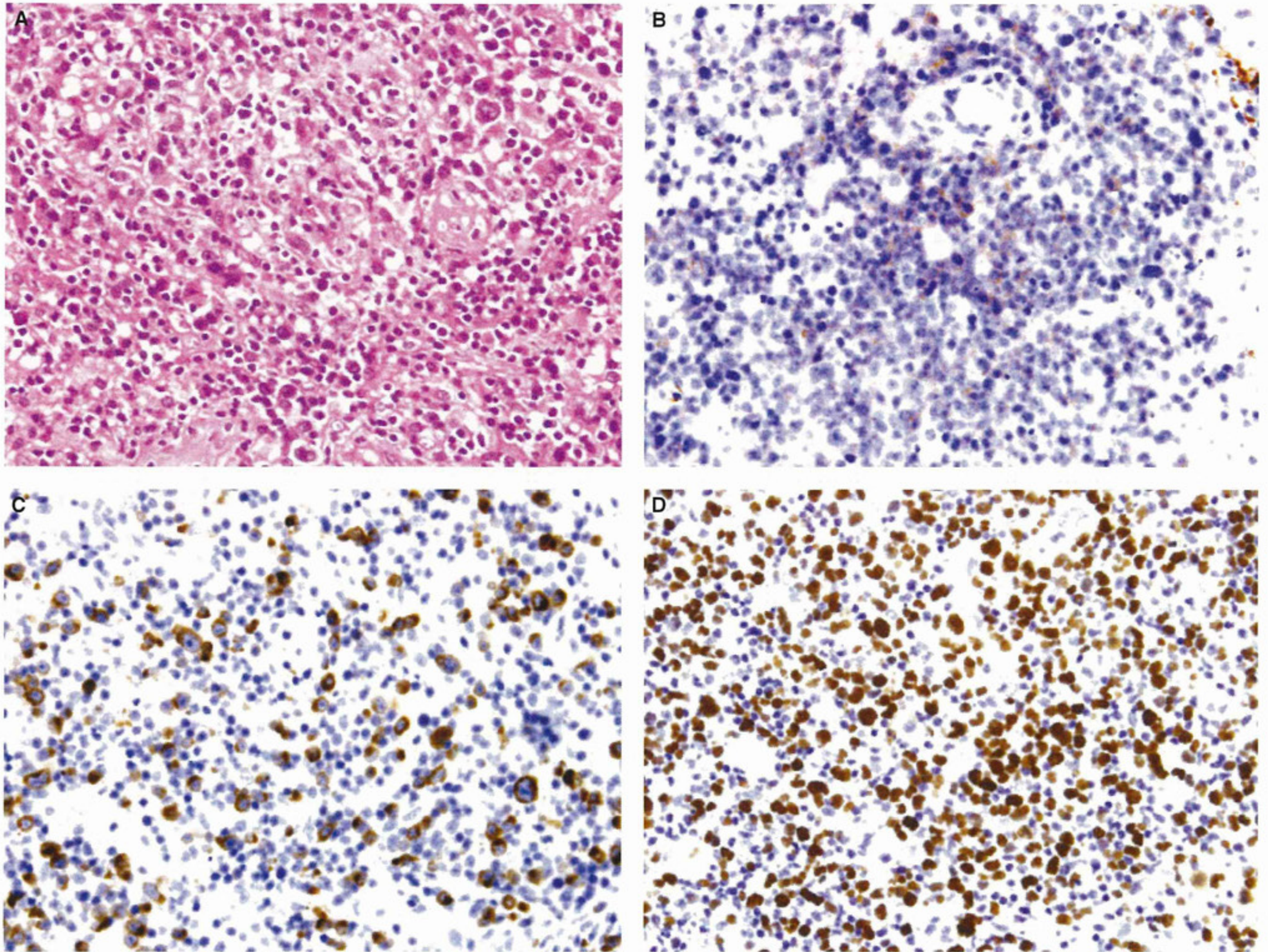

Figure 7. Overt Epstein–Barr virus (EBV)$^+$ leukaemia/lymphoma arising in chronic active EBV infection (CAEBV)/TNK-lymphoproliferative disorders (LPD) (category A3). **A**, Diffuse infiltration of pleomorphic, medium- to large-sized cells with irregular nuclei. **B**, Tumour cells show membrane staining for CD56. **C**, Perforin shows strong granular cytoplasmic staining in tumour cells. **D**, Numerous tumour cells are highlighted by *in situ* hybridization for EBV-encoded RNA (EBER).

relationship among a series of patients with systemic EBV$^+$ NK/T-cell lymphoma/leukaemia consisting of three diseases, EBV$^+$ ENKTL, EBV$^+$ ANKL and monoclonal NK-cell type CAEBV/TNK-LPD with overt EBV$^+$ leukaemia/lymphoma in a younger population. These systemic EBV$^+$ NK/T-cell lymphomas/leukaemia in the younger population shared clinicopathological features and overlapping geographical distributions with or without episodes of CAEBV/TNK-LPD, although their pathogenesis remains to be clarified. It is indicated that not enough attention is being given to detecting the presence of CAEBV/TNK-LPD and its related symptoms in the diagnosis of systemic EBV$^+$ NK T cell lymphoma/leukaemia. The 2008 WHO classification of malignant lymphoma highlighted the recognition of early lesions, age-related disease, and site-specific impact of the disease. The disease spectrum analysed in the present study of EBV$^+$ ENKTL, EBV$^+$ ANKL and monoclonal CAEBV/TNK-LPD of NK-cell type address these issues for systemic EBV$^+$ NK/T-cell lymphomas/leukaemias. Further investigation is necessary to elucidate the relationship among these systemic EBV$^+$ NK/T-cell lymphomas/leukaemias.

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Epigenetic Histone Modification of Epstein-Barr Virus BZLF1 Promoter during Latency and Reactivation in Raji Cells

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The Epstein-Barr virus (EBV) predominantly establishes latent infection in B cells, and the reactivation of the virus from latency is dependent on the expression of the viral BZLF1 protein. The BZLF1 promoter (Zp) normally exhibits only low basal activity but is activated in response to chemical or biological inducers, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophores, or histone deacetylase (HDAC) inhibitors. In some cell lines latently infected with EBV, an HDAC inhibitor alone can induce BZLF1 transcription, while the treatment does not enhance expression in other cell lines, such as B95-8 or Raji cells, suggesting unknown suppressive mechanisms besides histone deacetylation in those cells. Here, we found the epigenetic modification of the BZLF1 promoter in latent Raji cells by histone H3 lysine 27 trimethylation (H3K27me3), H3K9me2/me3, and H4K20me3. Levels of active markers such as histone acetylation and H3K4me3 were low in latent cells but increased upon reactivation. Treatment with 3-deazaneplanocin A (DZNep), an inhibitor of H3K27me3 and H4K20me3, significantly enhanced the BZLF1 transcription in Raji cells when in combination with an HDAC inhibitor, trichostatin A (TSA). The knockdown of Ezh2 or Suv420h1, histone methyltransferases for H3K27me3 or H4K20me3, respectively, further proved the suppression of Zp by the methylations. Taken together, the results indicate that H3K27 methylation and H4K20 methylation are involved, at least partly, in the maintenance of latency, and histone acetylation and H3K4 methylation correlate with the reactivation of the virus in Raji cells.

The Epstein-Barr virus (EBV) is a human gammaherpesvirus that establishes latent infection predominantly in B lymphocytes. Only a small percentage of infected cells switch from the latent stage into the lytic cycle and produce progeny viruses. Although the mechanism of EBV reactivation *in vivo* is not fully understood, it is known to be elicited *in vitro* by the treatment of latently infected B cells with chemical or biological reagents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore, sodium butyrate, and anti-immunoglobulin (Ig). The stimulation of the EBV lytic cascade by these reagents leads to the expression of two presumed viral immediate-early genes, BZLF1 and BRLF1. The BZLF1 protein is a transcriptional activator that shares structural similarities to basic leucine zipper (b-Zip) family transcriptional factors, and BZLF1 expression alone can trigger the entire reactivation cascade (1, 50, 53).

The expression of the BZLF1 gene is tightly controlled at the transcriptional level. The BZLF1 promoter (Zp) normally exhibits low basal activity and is activated in response to TPA or the other reagents described above. The promoter is activated by transcriptional factors, including myocyte enhancer factor 2D (MEF2D) (35) and Sp1/3 (34). Cellular b-Zip-type transcription factors, such as the cyclic AMP response element binding protein (CREB), activating transcription factor (ATF), activator protein 1 (AP-1) (33, 42, 43, 48), or a spliced form of X-box binding protein 1 [XBP-1(s)] (2), also play crucial roles in promoter activation. We previously showed the importance of CREB and its calcineurin-dependent activation by transducer of regulated CREB 2 (TORC2) (43). Once produced, BZLF1 itself can bind to and activate its own promoter (16, 41). Most of the positive factors have been demonstrated or are presumed to upregulate the BZLF1 promoter by recruiting transcriptional coactivators, such as histone acetylases. On the other hand, the activity of Zp is restricted by repressive factors, including Jun dimerization protein 2 (JDP2) (42), Zinc finger E-box binding factor (ZEB) (58), Yin Yang 1 (YY1) (40), and sumoylation of BZLF1 (19, 41), since those factors facilitate the access of repressive transcriptional cofactors, such as histone deacetylase (HDAC), to the promoter and/or block the binding or functions of the transcriptional activators noted above.

The silencing of the BZLF1 promoter in latently infected cells is mediated, at least in some cell lines, such as Akata, by low levels of histone acetylation, since inhibitors of HDAC, like sodium butyrate or trichostatin A (TSA), can reverse the silencing (37, 38). However, treatment with butyrate or TSA alone does not efficiently induce BZLF1 transcription in cell lines like B95-8 or Raji, suggesting that the molecular mechanisms that govern the suppression of BZLF1 transcription in those cells must be more than just the low acetylation levels of the promoter (10, 11, 17). 5′-CG-3′ dinucleotide (CpG) DNA methylation was one possible cause of promoter repression, because inhibitors of DNA methylation, such as 5-aza-2′-deoxycytidine (5-Aza), elicit BZLF1 transcription from the promoter (57). Nevertheless, it is highly likely that CpG methylation is not involved in the process, because the methylation of the Zp has not been detected in any cells, including B95-8, Raji, and Akata (15). In addition, treatment with 5-Aza induces BZLF1 transcription within a very short period of time (15 min or less) (10), although it takes days to bring about the hypomethylation of the CpG DNA, because 5-Aza is an inhibitor of DNA methyltransferase and does not actively reverse or abolish

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methylation without *de novo* DNA amplification. Therefore, as Countryman and others discuss in their report (11), it is likely that 5-Aza activates EBV lytic gene expression by an unknown mechanism that does not require viral DNA replication or DNA demethylation.

Possible epigenetic modifications for silencing the promoter other than CpG methylation include histone modification, such as histone H3 lysine 27 trimethylation (H3K27me3), H4K20me3, or H3K9me2/me3 (28). H3K27me3 is a histone modification that is involved in the suppression of a wide variety of genes (29). The methylation is mediated by enhancer of Zeste 2 (Ezh2), a member of polycomb repressor complex 2 (PRC2) (5). H4K20me3 also is a repressive chromatin marker that is frequently associated with heterochromatin. The H4K20me3 methyltransferase is a member of the SET domain-containing proteins, suppressor of variegation 420 h (Suv420h) (49). H3K9me2, catalyzed by G9a, is a typical repressive marker of facultative heterochromatin, whereas H3K9me3 methylation, predominantly found in constitutive heterochromatin, is mediated by enzymes including Suv39h.

In the present study, we found that the Zp is modified by negative markers, such as H3K27me3 and H4K20me3, in latently infected cells and upon reactivation modification by active markers, such as histone acetylation, and H3K4 methylation is increased. The treatment of cells with TSA and 3-deazaneplanocin A (DZNep), an inhibitor of H3K27me3 and H4K20me3 (39, 51), augmented levels of BZLF1 in Raji cells. The knockdown of Ezh2 or Suv420h1 by RNA interference markedly increased BZLF1 induction when treated with TSA. These results indicate that the Zp promoter in Raji cells is silenced, at least to some extent, by H3K27me3 and H4K20me3 during latency.

MATERIALS AND METHODS

Cell culture and reagents. 293EBV-bacterial artificial chromosome (BAC) epithelial cells (43) were maintained in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum. Akata, Raji, and lymphoblastoid cell line (LCL) EBV-BAC cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. SNK6 cells (44) were cultured in RPMI 1640 medium supplemented with 10% human serum and interleukin-2 (IL-2). Horseradish peroxidase-linked goat antibodies to mouse/rabbit IgG were from Amersham Biosciences. Anti-histone H3 (ab1791) and anti-Suv420H1 (ab49251), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14C10 and 2118), and anti-H3K4me3 (17-614) were purchased from Abcam, Cell Signaling, and Millipore, respectively. Anti-H3K9Ac (39137), anti-H3K9me2 (39375), anti-H3K9me3 (39161), anti-H3K27me3 (39155 and 39535), anti-H4K20me3 (39180), and anti-Ezh2 (39875) antibodies were from Active Motif.

Immunoblotting and ChIP assay. Immunoblotting was carried out as described previously (43). Chromatin IP (ChIP) assays were performed essentially as described previously (43) with formaldehyde cross-linked chromatin from 1×10^6 cells for each reaction. Cells were lysed, and chromatin was sonicated to obtain DNA fragments with an average length of 300 bp. Following centrifugation, the chromatin was diluted 10-fold with ChIP dilution buffer and precleared with protein A agarose beads containing salmon sperm DNA (Upstate). Immune complexes were collected by the addition of protein A agarose beads, and DNA was purified using a QIAquick PCR purification kit (Qiagen) after the uncoupling of the cross-linking and proteinase K digestion. The PCR products were then analyzed by real-time PCR for the quantification of DNA sequences using the following primers and SYBR Premix Ex *Taq* II (TaKaRa). The recovered DNA was amplified by PCR using the following specific primers: for Zp (Zp0), 5′-TAGCCTCGAGGCCATGCATATTTCAACTGG-3′ and 5′-GCCAAGCTTCAAGGTGCAATGTTTAGTGAG-3′; for Zp-3000, 5′-ACCTCACTACACAAACAGAC-3′ and 5′-TTCAACACAGCAGGCCTCTC-3′; for Zp-2000, 5′-CCACTTCGGGATAGTGTTTC-3′ and 5′-TTCCTTGTTGAGGACGTTGC-3′; for Zp-1200, 5′-GACAGAGGAGCTACGTGAG-3′ and 5′-ATGAAACTGTCCGGACTCCG-3′; for Zp-600, 5′-AGGTATGTTCCTGCCAAAGC-3′ and 5′-GTTCATGGACAGGTCCTGTG-3′; for Zp +500, 5′-GGAGAAGCACCTCAACCTG-3′ and 5′-CTCCTTACCGATTCTGGCTG-3′; for the BRLF1 promoter (Rp), 5′-TAAGATCTTGGGGACGATGG-3′ and 5′-ACCATTAAAATCTTTCCTCC-3′; for the origin of lytic DNA replication (oriLyt), 5′-CCGGCTCGCCTTCTTTTATCCTC-3′ and 5′-CCTGGTTCAACCCTATGGAGGGGAC-3′; for the BMRF1 promoter (Mp), 5′-TAAAGCAGTTTCTGGAGGCC-3′ and 5′-GCCCAGAAACCTGAGCAAGT-3′; for the Q promoter of EBNA (Qp), 5′-GGCTCACGAAGCGAGAC-3′ and 5′-GTCGTCACCCAATTTCTGTC-3′; for the dyad symmetry in the origin of latent replication (oriP DS), 5′-GTGACAGCTCATGGGGTGGG-3′ and 5′-GATAAGCGGACCCTCAAGAG-3′; for the C promoter of EBNA (Cp), 5′-AGTTGGTGTAAACACGCCGT-3′ and 5′-TCCACCTCTAAGGTCCCACG-3′; for the β-globin promoter (Globinp), 5′-AGGACAGGTACGGCTGTCATC-3′ and 5′-TTTATGCCCAGCCCTGGCTC-3′; and for the GAPDH promoter (GAPDHp), 5′-CGTGCCCAGTTGAACCAGG-3′ and 5′-AGGAGGAGCAGAGAGCGAAG-3′. Real-time PCR was performed in 10 μl of solution containing 0.2 μM primers, 0.2 μl ROX dye, and the sample DNA in 1× One Step SYBR reverse transcription-PCR (RT-PCR) buffer. The intensity of ROX dye was used to compensate for volume fluctuations among the tubes. PCR included 10 s at 95°C and 40 cycles at 95°C for 5 s, followed by 45 s at 60°C. Immediately after the PCR, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. A standard curve was constructed using serial dilutions of DNA and was used to quantitate the amount of DNA.

siRNA. Duplexes of 21-nucleotide small interfering RNA (siRNA) specific to human Ezh2 or Suv420h1 mRNA, including two nucleotides of deoxythymidine (dTdT) at the 3′ end, were synthesized and annealed (Gene Design, Inc.). The sense and antisense sequences of the duplex were the following: for Ezh2, 5′-CCAUGUUUACAACUAUCAAdTdT-3′ and 5′-UUGAUAGUUGUAAACAUGGdTdT-3′; for Suv420h1, 5′-CCAUGAUUGCAGACCUAAUdTdT-3′ and 5′-AUUAGGUCUGCAAUCAUGGdTdT-3′; and for control siRNA, 5′-GCAGAGCUGGUUUAGUGAAdTdT-3′ and 5′-UUCACUAAACCAGCUCUGCdTdT-3′. Raji cells (1×10^5) were transfected with 50 pmol of the duplex RNA per well of a 24-well plate using a microporator (Digital Bio). Two days after transfection, TPA was added for Raji cells, followed by incubation for another day.

RT-PCR. Total cell RNA was purified using TriPure isolation reagent (Roche) and subjected to real-time RT-PCR using a One Step SYBR PrimeScript RT-PCR kit II (TaKaRa) and real-time PCR system 7300 according to the manufacturer's instructions. PCR was performed in 10 μl of solution containing 0.2 μM primers, 0.2 μl ROX dye, and the sample RNA in 1× One Step SYBR RT-PCR buffer. The intensity of the ROX dye was used to compensate for volume fluctuations among the tubes. PCR included 5 min at 42°C, 10 s at 95°C, and 40 cycles at 95°C for 5 s, followed by 40 s at 60°C. Immediately after RT-PCR, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. An arbitrary RNA value was set to 1.0, and a standard curve was constructed using serial dilutions of RNA from the RNA set to 1.0. The amount of mRNA was quantitated based on the standard curve. Real-time PCR with GAPDH primers was also performed to serve as an internal control for input RNA. Primers used for RT-PCR were the following: for GAPDH mRNA, 5′-TGCACCACCAACTGCTAGC-3′ and 5′-GGCATGGACTGTGGTCATGAG-3′; for BZLF1 mRNA, 5′-AACAGCCAGAATCGCTGGAG-3′ and 5′-GGCACATCTGCTTCAACAGG-3′; and for EBNA2 mRNA, 5′-TTAGAGAGTGGCTGCTACGCATT-3′ and 5′-TCACAAATCACCTGGCTAAG-3′.

RESULTS

Epigenetic histone modification of the BZLF1 promoter during latency and reactivation by chemical inducers. The reactivation

of EBV from latency is tightly blocked at the level of the transcription of the BZLF1 gene. It has been reported that the silencing of the gene is dependent only on low levels of histone acetylation in some cell lines, such as Akata (37, 38). However, because TSA alone does not induce BZLF1 transcription in certain cells, like B95-8 or Raji, it has been estimated that there must be other molecular mechanisms that are responsible for the silencing of BZLF1 transcription (10, 11, 17). To analyze the mechanisms that govern BZLF1 transcription besides histone acetylation in such cell lines, we first examined levels of various epigenetic histone modifications at certain regions of EBV DNA, including −3000 to +500 of Zp, the BRLF1 promoter oriLyt, the BMRF1 promoter (Mp), the Q promoter of EBNA (Qp), the dyad symmetry in the origin of latent replication (oriP DS), and the C promoter of EBNA (Cp). We chose Raji cells for two reasons. First, the cells have a repressive mechanism besides low-level acetylation. Second, as EBV in Raji cells lacks the single-stranded DNA binding protein BALF2 and thus is replication incompetent (20), we can observe epigenetic alterations that affect transcriptions of BZLF1 or other genes without the complication associated with viral genome amplification. To achieve lytic induction, Raji cells were treated with TPA, A23187, and sodium butyrate (T/A/B) (Fig. 1, gray bars), which efficiently induces the lytic cycle in the cell line. As shown in Fig. 1A and B, background precipitation with normal IgG could be ignored, and histone H3 levels were fairly constant.

Active chromatin markers, such as H3K9Ac or H3K4me3, were present at lower levels in the viral genome (Fig. 1C and D, white bars). It is noteworthy that the levels of those active marks were intrinsically higher at the commonly active latent EBNA promoter Qp (Fig. 1C and D, white bars). Elevated levels of histone H3/H4 acetylation and H3K4 methylation at the Qp in latent Raji cells was previously demonstrated by Day et al. as well (12). Relatively high histone H3K4me3 levels in the latent DS, the EBNA1 binding sites in oriP (Fig. 1D, white bars) (12) may reflect EBNA1's function as a transcriptional activator (24).

Lytic induction by TPA, A23187, and sodium butyrate markedly elevated the active histone markers H3K9Ac and H3K4me3 in the viral genome (Fig. 1C and D). The enhancement of H3K4me3 upon the induction of the EBV lytic cycle has never been reported to our knowledge, although such modification is reported for herpes simplex virus (HSV) (21, 45) and Kaposi's sarcoma-associated herpesvirus (KSHV) (18, 52). The enhancement of H3K4me3 at the BZLF1 transcription start site Zp0 (−221 to +12) and Zp +500 upon the induction of the EBV lytic cycle was notably higher (1.5 and 0.85% of input, respectively) than that of the other part of the BZLF1 promoter (less than 0.35%) (Fig. 1D, gray bars). This enhancement of H3K4me3 levels at the proximal part of Zp will be discussed later.

On the other hand, repressive markers, including H3K9me2, H3K9me3, H3K27me3, and H4K20me3, were present overall in latency (Fig. 1E to H). The presence of histones H3K9me3 and H3K27me3 at the Zp and oriLyt in the latent EBV genome of Akata cells was reported quite recently (47). H3K9me2 and H4K20me3 modifications have not been reported for the BZLF1 promoter of EBV to our knowledge.

Unexpectedly, lytic induction did not significantly diminish levels of those repressive markers (Fig. 1E to H), if any (see Fig. 7E). The reason why repressive marks did not noticeably diminish upon lytic induction in Raji cells is not clear, because the lytic induction of KSHV by sodium butyrate (18) or K-Rta (52) caused

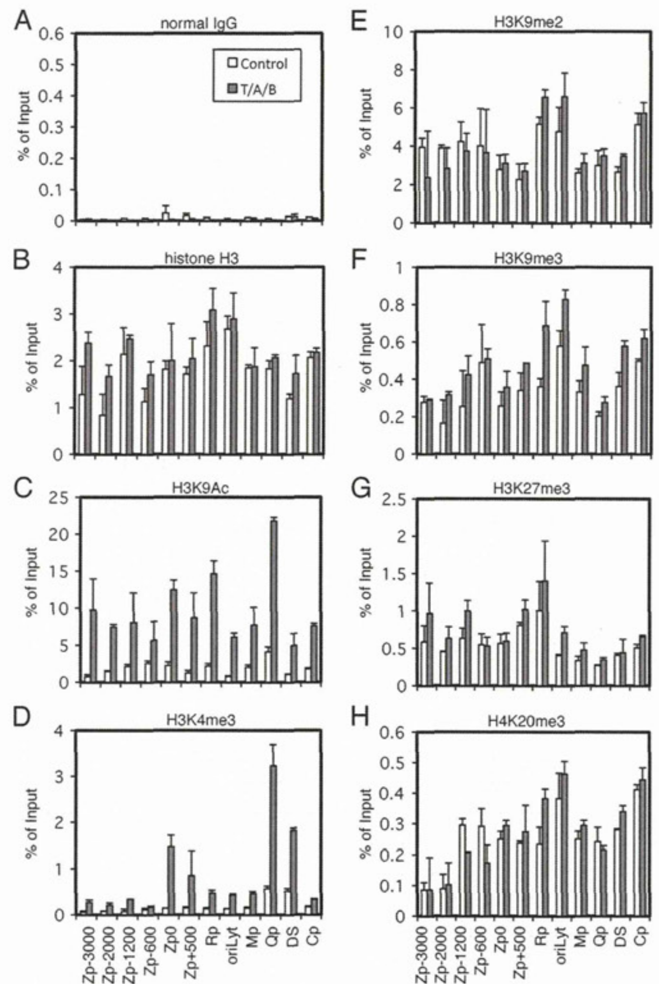

FIG 1 Histone modification pattern of EBV Zp upon lytic reactivation. Raji cells were treated with TPA (20 ng/ml), A23187 (1 μM), and sodium butyrate (5 mM) (T/A/B; gray bar) or the vehicle (Control; white bar) for 20 h. Cells then were cross-linked, and ChIP experiments were performed as described in Materials and Methods using normal IgG (A), anti-histone H3 (B), anti-H3K9Ac (C), anti-H3K4me3 (D), anti-H3K9me2 (E), anti-H3K9me3 (F), anti-H3K27me3 (G), or anti-H4K20me3 (H) antibody, followed by DNA extraction and real-time PCR to detect DNA fragments using the primers as indicated. Zp, BZLF1 promoter; Rp, BRLF1 promoter; oriLyt, origin of lytic DNA replication; Mp, BMRF1 promoter; Qp, one of the EBNA promoters; DS, dyad symmetry, a part of oriP (origin of plasmid replication), containing multiple EBNA1 binding sites; Cp, one of the EBNA promoters. The number of Zp indicates sequence position relative to the transcription start site.

the loss of H3K27me3 at the K-Rta (Orf50) promoter region, at least to some extent. We speculate that this was because EBV in Raji cells cannot replicate at all due to the lack of the BALF2 gene, while the elimination of negative marks might need lytic viral DNA replication. Although Toth and others paid attention to the possible effect of viral genome replication and performed assays within 12 h after K-Rta induction (52), this induction might still touch off undetectable levels of replication. We normalized levels of immunoprecipitated DNA fragments to input levels, which is a very common method of normalization. Because the normalization of the data to a certain internal control, such as the GAPDH promoter (7), might provide better resolution for comparison between the control and lytic induction, we measured the levels of