Title

EPSTEIN–BARR VIRUS (EBV)-ASSOCIATED T/NK LYMPHOPROLIFERATIVE DISEASES IN NON-IMMUNOCOMPROMISED HOSTS: PROSPECTIVE ANALYSIS OF 108 CASES

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Running Title

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Key Words

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Footnotes

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Abstract

Epstein–Barr virus (EBV)-associated T/NK-cell lymphoproliferative disease (T/NK-LPD) is defined as a systemic illness characterized by clonal proliferation of EBV-infected T- or NK-cells. We prospectively enrolled 108 non-immunocompromised patients with this disease (50 men and 58 women; median onset age, 8 years; age range, 1-50 years) evidenced by expansion of EBV+ T/NK cells in peripheral blood. They consisted of T-cell type in 64 cases and NK-cell type in 44, and were clinically categorized into 4 groups: 80 cases of chronic active EBV disease, 15 of EBV-associated hemophagocytic lymphohistiocytosis, 9 of severe mosquito bite allergy, and 4 of hydroa vacciniforme. These clinical profiles were closely linked with the EBV+ cell immunophenotypes. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications. During the follow-up, 13 patients developed overt lymphoma or leukemia characterized by extranodal NK/T cell lymphoma and aggressive NK-cell leukemia. Fifty-nine received hematopoietic stem cell transplantation, 66% of whom were alive. Age at onset of disease (≥ 8 years) and liver dysfunction were risk factors for mortality, while transplanted patients had better prognosis. These data depict clinical characteristics of systemic EBV+ T/NK-LPD and provide insight into the diagnostic and therapeutic approaches for distinct disease.
Introduction

Epstein–Barr virus (EBV)-associated lymphoproliferative diseases (LPDs) have a vast spectrum from reactive to neoplastic processes in the transformation and proliferation of lymphocytes spanning B-, T- and NK-cells, and are clinically complicated by the interaction between the biologic properties of EBV+ lymphocytes and the host immune status. Our understanding of these diseases is now evolving, and has led to the recognition of a variety of EBV+ diseases, including Burkitt lymphoma, age-related EBV+ B-cell LPD, extranodal NK/T cell lymphoma of nasal type (ENKL), aggressive NK-cell leukemia (ANKL), classical Hodgkin lymphoma, and immunodeficiency-associated lymphoproliferative disorders. EBV-associated T- and NK-cell LPD (T/NK-LPD) was first incorporated into the 4th WHO classification of tumours of hematopoietic and lymphoid tissues, in which systemic EBV+ T-cell LPD of childhood and hydroa vacciniforme-like lymphoma are proposed as distinct entities. Historically, based on their broad clinical manifestation, these diseases have been described under various nosological terms covering from indolent (severe mosquito bites allergy and hydroa vacciniforme), to aggressive or fulminant forms (EBV-associated hemophagocytic lymphohistiocytosis [HLH], chronic active EBV disease [CAEBV] of T/NK-cell type, fulminant EBV+ T-cell LPD of childhood, and fatal infectious mononucleosis) based on clinical manifestations.

CAEBV was originally referred to chronic or recurrent infectious mononucleosis-like symptoms. A severe form of CAEBV was found to be prevalent in the East Asian countries and characterized by clonal expansion of the EBV-infected T- or NK-cells, while in the Western countries patients are mostly associated with EBV-infected B cells. EBV-associated HLH was coined to describe hemophagocytosis involving bone marrow or other organs and resulting in pancytopenia in peripheral blood. This disease is also frequently seen in the East Asian countries, and have a clonal expansion of EBV+ T or NK cells which produce inflammatory cytokines inducing the activation of macrophages and hemophagocytosis. Apart from these systemic diseases,
accumulating evidence indicates that two cutaneous diseases, hydroa vacciniforme and severe mosquito bite allergy, are closely associated with EBV+ T- or NK-cells. Hydroa vacciniforme is characterized by recurrent vesiculopapules usually occurring on sun-exposed areas and seen in children and adolescents\textsuperscript{10}. In some of these patients, systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly have been recorded\textsuperscript{24-26}. Severe mosquito bite allergy was determined to be associated with EBV+ NK cells, but rarely with EBV+ T-cells, and to progress into overt lymphoma or leukemia in the long-standing clinical course\textsuperscript{9,27}. It is noted that these EBV+ cutaneous diseases had the same geographical distribution as the other EBV+ T/NK-cell lymphomas and LPDs among the East Asians and Native Americans in Central and South America and Mexico\textsuperscript{8}, and were encountered as a part of the initial and accompanying symptoms of the systemic EBV+ T/NK-LPDs\textsuperscript{28-30}. However, the mutual relationship and clinicopathologic distinctiveness of these EBV+ T/NK-LPDs are unfounded, posing diagnostic and therapeutic problems for pathologists and hematologists, respectively. These patients appear to exist in the grey zone between systemic EBV+ T-cell LPD of childhood and hydroa vacciniforme-like lymphoma according to the 4th WHO classification. The former encompasses CAEBV of T-cell type, EBV+ HLH, and EBV+ T-cell lymphomas with prodromal phase, while the latter may include all cases with EBV+ hydroa vacciniforme despite the presence or absence of the systemic disease in the patient history.

Our aim was clarifying the clinicopathologic characteristics of these EBV+ T/NK-LPDs and the biologic properties of the proliferating cells by analyzing a large number of patients. We previously performed a nation-wide survey for CAEBV of T/NK-cell type and determined its prognostic factors\textsuperscript{29}. Similarly, a nation-wide study for HLH was recently performed in Japan\textsuperscript{31}. However, these studies were retrospective and lacked the precise diagnosis of the current level because of its study design. In 1998, we established an EBV-DNA quantification system by using real-time PCR\textsuperscript{32-33}, which allowed for the determination of the phenotype of EBV-infected cells in peripheral blood with the combination of fractionation to lymphocyte subset\textsuperscript{12,34-35}. More recently,
we developed the simultaneous staining method for surface antigens and nuclear EBV-encoded small RNA (EBER) to more precisely determine EBV-infected cell phenotypes. Using these techniques we enrolled and prospectively followed patients with definitive cases of EBV+ T/NK-LPDs in 1998. In this study, 108 non-immunocompromised patients with EBV+ T/NK-LPDs were analyzed on clinical and virological characteristics for the understanding of their pathogenesis and for refining their classification. Furthermore, prognostic factors and the efficacy of therapeutic interventions including hematopoietic stem cell transplantation (HSCT) were analyzed.

Patients and methods

Eligibility Criteria

Informed consent was obtained from all participants or their guardians in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of Nagoya University Graduate School of Medicine. From 1998 to 2010, patients whose samples were sent to Nagoya University Graduate School of Medicine for determination of the EBV-infected cell phenotype and who fulfilled the following criteria were prospectively enrolled in this study: i) EBV-associated T/NK-LPD was suspected or diagnosed based on clinical and/or histopathological findings, ii) high EBV load was detected in peripheral blood mononuclear cells (PBMCs) by quantitative PCR (≥ 10^2.5 copies/μg EBV-DNA), and iii) EBV-infection in T or NK cells in peripheral blood was confirmed by either immunobead sorting followed by quantitative PCR or flowcytometric in situ hybridization (ISH). Exclusion criteria were: i) pathologically defined ENKL, ANKL, or peripheral T cell lymphoma (PTCL), ii) cases of congenital immunodeficiency, iii) human immunodeficiency virus-positive cases, and iv) other immunodeficient patients who received immunosuppressive therapies or had underlying diseases with potential immunosuppressions. Patients were recruited through an announcement by the Japanese Association for Research on Epstein-Barr Virus and Related Diseases and on the homepage of our institute’s
website. Approximately 240 Hematology Units and 400 Departments of Pediatrics were included in the association.

Upon entry into the study, peripheral blood was collected and sent to Nagoya University Graduate School of Medicine to examine EBV-DNA quantification and EBV-infected cell determination along with detailed clinical data. Clonality analyses were also performed at this time if possible. Primary EBV infection was determined based on serological findings; detection of anti-viral capsid antigen-IgM and seroconversion of either anti-viral capsid antigen-IgG or anti-EBV nuclear antigen. A total of 108 cases from 40 hospitals were enrolled in the study (25 cases from Nagoya University Hospital, 13 cases from Osaka Medical Center and Research Institute for Maternal and Child Health, 9 cases from Fukushima Medical University, and 61 cases from other hospitals). Each patient enrolled in the study was treated according to physician’s decision at each hospital. The physicians completed questionnaires regarding the administered treatment and outcome at every three year (2001, 2004, and 2007); the final questionnaire was sent and collected in December 2010. As compared with data provided by previous national surveys for CAEBV and HLH\textsuperscript{29,31}, we estimated that approximately 15-20\% of systemic EBV\textsuperscript{+} T/NK-LPD cases during the study period were recruited by this registry.

Criteria of patients

Patients were clinically divided into four groups according to the clinical categorization at the 2008 NIH meeting: i) CAEBV of T/NK-cell type, ii) EBV-associated HLH, iii) hydroa vacciniforme, and iv) severe mosquito bite allergy\textsuperscript{39}. The clinical diagnosis was made at entry into the study. Definitions of each clinical category are listed in Table 1. CAEBV was defined according to the previously proposed criteria\textsuperscript{16,29}. HLH was defined based on the criteria proposed by an international treatment study group\textsuperscript{11}. Severe mosquito bite allergy and hydroa vacciniforme were applied for cases with only skin symptoms and lacking systemic symptoms. In this study, ‘severe mosquito bite
allergy’ and ‘hydroa vacciniforme’ were used as clinical categories, while ‘hyper sensitivity to mosquito bites’ and ‘hydroa vacciniforme-like eruptions’ were uses as terms for symptoms. Furthermore, ‘hydroa vacciniforme-like lymphoma’ was used as a term for pathological classification.

Patients were also classified according to the 4th WHO classification for tumours of hematopoietic and lymphoid tissues. The definitions of pathological classification are listed in Table 1. The classification was made both at the diagnosis and at the last follow-up or death. Patients diagnosed with ENKL, ANKL, or PTCL were excluded from the study, but some cases developed to these diseases during the follow-up period. Out of 108 patients, 54 were biopsied (liver, 15 cases; skin, 15 cases; lymph node, 10 cases; intestine, 3 cases; spleen, 2 cases; muscle, 2 cases; others, 7 cases), and 6 were autopsied. For differential diagnosis, bone marrow examination was performed in most cases (79%), even though there were no hematological abnormalities of the peripheral blood. When abnormal findings were detected in bone marrow or peripheral blood, EBER/immunohistochemical staining was performed. Histopathology was reviewed by the Central Pathology Review Board (Shigeo Nakamura, Nagoya University and Koichi Ohshima, Kurume University).

Disease status was defined as follows: stable disease (SD), partial remission (PR), and complete remission (CR). Patients with PR had no symptoms but had significant EBV loads in PBMCs (EBV-DNA $\geq 10^{2.5}$ copies/$\mu$g DNA). CR patients had no symptoms and continuously low or no EBV loads in PBMCs (EBV-DNA $< 10^{2.5}$ copies/$\mu$g DNA). Disease activity was assessed before HSCT and classified as either active or inactive according to the previous study. Active disease was defined by the existence of symptoms and signs such as fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, or progressive skin lesions alongside an elevated EBV load in the peripheral blood. Liver dysfunction was defined as an increase in alanine transaminase levels to 2 times above the upper limit of normal on at least 2 consecutive occasions.
Analyses of EBV and determination of EBV-infected cells

DNA was extracted from $1 \times 10^6$ PBMCs or 200 μL of plasma and real-time quantitative PCR was then performed as described previously\textsuperscript{12,32}. EBV clonality was assessed by Southern blotting with a terminal repeat probe, as described previously\textsuperscript{12,41}. To determine which cell population harbored EBV, either immunobead sorting followed by quantitative PCR or flowcytometric ISH assay were performed. For the former method, PBMCs were fractionated into CD3$^+$, CD4$^+$, CD8$^+$, CD16$^+$, CD19$^+$, CD56$^+$, TCR$\alpha\beta^+$, and TCR$\gamma\delta^+$ cells using an immunobead method (IMag Cell Separation System; BD Biosciences) that resulted in 97%-99% purity\textsuperscript{34-35}. Purified cells were analyzed by real-time quantitative PCR. The infected-cell phenotypes were determined in comparison with unfractionated (whole) PBMCs, as previously described\textsuperscript{34-35}. For example, patients were defined as CD3-positive when CD3-positive cells contained higher amounts of EBV DNA than that of whole PBMCs. The flowcytometric ISH assay was performed as described previously\textsuperscript{36}. Briefly, PBMCs were stained with fluorescence labeled monoclonal antibodies against surface marker, fixed, permeabilized, and hybridized with EBER-specific PNA Probe/FITC (Y5200; Dako). After enhancing fluorescence, stained cells were analyzed using FACSCalibur and Cell Quest software (BD Biosciences). More than 0.1% of EBER-positive cells was considered to be significant and such subsets were designated EBV-positive. This frequency was chosen based on previous data using EBV$^+$ cells lines\textsuperscript{36}.

T cell receptor (TCR) gene rearrangement

TCR gene rearrangement was determined by multiplex PCR using the T cell Gene Rearrangement/Clonality assay (InVivoScribe Technologies), which was developed and standardized in a European BIOMED-2 collaborative study\textsuperscript{42}.
Histopathology

Immunostaining was performed using an avidin-biotin peroxidase complex method with monoclonal antibodies against CD3 (Dako), CD56 (Novocastra Laboratories), perforin (Novocastra Laboratories), T-cell restricted intracellular antigen (TIA)-1 (Immunotech), and granzyme B (Monosan). ISH was performed using the EBER probe (Dako) as previously described. Hybridization was detected using mouse monoclonal anti-fluorescein isothiocyanate antibody (Dako) and a Vectastain ABC kit (Vector).

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 18.0 (SPSS). For univariate analysis, either chi square or Fisher’s exact test on single sided was used to compare categorical variables. To compare quantitative variables, Mann–Whitney U test was used. For multivariate analysis, logistic regression analysis was used. Comparison between quantities of EBV-DNA in PBMCs and plasma were performed by regression analysis. For survival analysis, Kaplan–Meier method and log-lank test were used. In all analyses, \( P < 0.05 \) was taken to indicate statistical significance.

Results

Characteristics of patients with EBV^T/NK-LPD.

A total of 108 patients (50 men and 58 women) were enrolled in this study. Detailed characteristics of each patient are shown in Supplementary Table 1. Age at diagnosis ranged from 1 to 51 years (median, 14 years). At the time of diagnosis the main phenotypes of EBV-infected cells in peripheral blood were T-cell and NK-cell in 64 and 44 cases, respectively. Onset ages ranged from 1 to 50 years (median, 9 years). Most patients (91% of the cases) were children and young adults less than 30 years, but middle aged cases (range, 30 to 50 years old) also existed (Fig. 1A). There was no
difference in their onset age between patients of T-cell type and NK-cell type. The former were further subdivided into CD4\(^+\)T- (18 cases), CD8\(^+\)T- (14 cases), \(\gamma\delta\) T- (7 cases), and other or ill-defined T-cell type (25 cases). In two patients (# 92 and 100, Supplementary Table 1), two lineages of cells were infected with EBV.

Upon entry into the study, patients were clinically categorized into 4 groups based on their clinical symptoms and diagnostic criterion: CAEBV (80 cases), EBV-associated HLH (15 cases), severe mosquito bite allergy (9 cases), and hydroa vacciniforme (4 cases) (Fig.1B). The CAEBV group consisted of 47 T-cell cases (59%) and 33 NK-cell cases (41%), the former of which were further subdivided into CD4\(^+\) T- (21%), CD8\(^+\) T- (8%), and \(\gamma\delta\) T-cell types (5%). On the other hand, 8 of 15 (53%) EBV-associated HLH cases showed the EBV-harboring CD8\(^+\) T cells, which was clearly contrasted with its low or few percentages in the other clinical groups. Additionally, most patients (89%) with severe mosquito bite allergy had EBV-infected NK-cells, while many with hydroa vacciniforme had EBV-infected \(\gamma\delta\) T cells (75%) (Fig. 1B). Thus, clinical profiles were closely linked with the EBV\(^+\) cell immunophenotypes.

Between 1 and 349 months from the onset of disease (median, 46 months), 47 patients had died, while 61 patients were alive for follow-up periods of 13 to 263 months (median, 82 months). The main causes of death were multiple organ failure (10 cases), hepatic failure (6 cases), heart failure (5 cases), pulmonary failure (5 cases), sepsis (5 cases), intracranial hemorrhage (5 cases), intestinal hemorrhage or perforation (3 cases), hemophagocytic syndrome (2 cases), and others (6 cases). Of the 47 patients who died, 20 (42%) died after transplantation. Of 61 surviving patients, 41 were in CR and 4 were in PR without any symptoms, while 16 remained in SD at the last follow-up.

**Clonality analysis.**

At the time of diagnosis, the viral clonality was analyzed by Southern blot analysis using EBV terminal repeat. Out of 76 cases with available DNA, EBV-infected cells were monoclonal in 64
cases (84%) and oligoclonal in 8 cases (11%). Polyclonal EBV-infected cells were detected in only 4 cases (5%). TCR rearrangement was analyzed in 90 cases at the time of diagnosis, 42 of which had monoclonal rearrangements. Six patients with NK-cell infection demonstrated TCR rearrangement. Since this analysis used a PCR-based method, erroneous detection of a seemingly clonal cell population (pseudoclonality) or reduced TCR diversity caused by the prevalence of a few antigen-selected subclones, which are often seen in EBV infection, may occur. Chromosomal aberrations were detected in the peripheral blood or lymph nodes at the diagnosis of 6 patients, while an additional six patients later developed chromosomal aberrations in their clinical course of 1 to 9 years (median, 5 years). Patterns of chromosomal aberrations in each patient are shown in Supplementary Table 2. These results provided additional support to the assertion that patients with EBV+ T/NK-LPDs had clonality at early stages and subsequently developed to overt lymphoma or leukemia with the increase of chromosomal aberrations in their clinical course.

Pathological categories based on the 4th WHO classification.

At the time of diagnosis, based on the 4th WHO classification, 53 and 13 cases were classified into systemic EBV+ T-LPD of childhood and hydroa vacciniforme-like lymphoma, respectively. The proportion of these pathological categories in each clinical group is shown in Fig. 1C. Four cases clinically categorized to hydroa vacciniforme without any cellular atypia or systemic symptoms were classified into hydroa vacciniforme-like lymphoma based on their monoclonality of cells with TCR rearrangements. In systemic EBV+ T-cell LPD, T-cell subsets of EBV-infected cells were variable (Fig. 1D). In hydroa vacciniforme-like lymphoma, 6 out of 13 cases had γδ T-cell infection. On the other hand, 42 cases were not classified to either of these pathological categories because they failed to correspond to criteria in the current WHO classification. Classification of each patient is shown in Supplementary Table 1.

At the last follow-up or death, there were 29 cases that were unclassifiable, most of which
were CAEBV of NK-cell type and severe mosquito bite allergy with NK-cell infection (Fig. 1D). In the clinical course, ENKL developed in 6 cases (# 2, 5, 20, 34, 60, and 81 in Supplementary Table 1) after 9 month to 12 years follow-up from the onset (median, 1.5 years), while ANKL developed in 4 cases (# 8, 43, 66, and 80) after 2 to 17 years follow-up (median, 12 years). Most of them had NK-cell infection. EBV+ PTCL developed in 3 cases after 1 (#83), 5 (#93), and 20 (#53) years follow-up. The EBV+ PTCL in this study were characterized by their expression of cytotoxic molecules, nodal manifestation, lack of CD56 expression, and TCR gene rearrangement. These features suggest a pathological distinction between these EBV+ PTCL and extranasal ENKL.

Representative results of histological examinations are shown in Fig. 2. Histological findings and number of EBER-positive cells varied among patients. EBER-positive lymphocytes were detected at varying frequencies. Infiltrating cells (presumably EBV-infected) expressed cytotoxic molecules such as TIA-1, perforin, and granzyme B. Bone marrow aspirations showed various findings, but most patients showed normocellular marrow without any abnormal findings. Patients with EBV-associated HLH showed normoplastic or hyperplastic marrow with mild or moderate hemophagocytosis. In all patients, however, bone marrow findings showed an absence of hematologic malignant disorders at the time of diagnosis.

**Differences between T-cell infection and NK-cell infection cases.**

We compared clinical and virological differences between T- and NK-cell infections (Table 2). T-cell infection was characterized with higher rates of primary EBV infection and TCR rearrangement. On the other hand, a significant number (43%) of patients with NK-cell infection had hypersensitivity to mosquito bites (Table 2). Interestingly, 5 patients had both hypersensitivity to mosquito bites and hydroa vacciniforme-like eruption. These patients had NK-cell infection (Table 2). On the other hand, 8 out of 10 patients with hydroa vacciniforme-like eruption but without hypersensitivity to mosquito bites had T-cell infection (Table 2).
A comparison of viral load in peripheral blood between patients with T- and NK-cell infections detected similar levels of EBV-DNA in both PBMCs and plasma (Table 2). Correlation of viral loads between PBMCs and plasma was estimated (Fig. 3A). Quantity of EBV-DNA in PBMCs was significantly correlated with that in plasma in both T-cell and NK-cell infections, although EBV-DNA was not detected from plasma in 15 patients. We also compared viral load among clinical groups (Fig. 3B-C). Interestingly, quantity of EBV-DNA in PBMCs was significantly higher in patients with severe mosquito bite allergy and hydroa vacciniforme, although they did not have any systemic symptoms.

Efficacy of therapeutic interventions.

Each patient received a variety of therapies. HSCT was administered to 59 patients. HSCT induced sustained CR in 63% of patients with CAEBV, 60% of HLH patients, and 57% of severe mosquito bite allergy patients (Fig. 4A). Seventy cases received chemotherapy, such as etoposide/cyclosporine A/dexamethasone, cyclophosphamide/doxorubicin/vincristine/prednisolone (CHOP), CHOP plus etoposide, and high dose of cytosine arabinoside therapy. Chemotherapies were effective in some patients, but its effect was usually transient and failed to induce sustained CR in most cases. Chemotherapy induced sustained CR in only 5 patients, 4 of which were HLH cases (Fig. 4A). Immunomodulating therapies, such as prednisolone, cyclosporine A, high-dose intravenous immunoglobulin, and methyl prednisolone pulse therapy were administered to 58 patients. The immunomodulating therapies induced sustained CR in 2 patients with HLH (Fig. 4A). In patients with HLH, both chemotherapies and immunomodulating therapies induced sustained CR more frequently compared to those with CAEBV ($P = 0.002$ and $P = 0.02$, respectively). Anti-viral therapies, such as acyclovir, adenine arabinoside, and ganciclovir were administered to 32 patients. In 2 patients (#11 and 45 in Supplementary Table 1), sustained CR was achieved during oral acyclovir therapy and weekly intravenous administration of adenine arabinoside (Fig. 4A). However, it was not
clear whether CR was induced by these anti-viral therapies or spontaneously achieved since anti-viral therapies had been administered for a long time. Effects of each therapy among cell types are shown in Fig. 4B. There was no statistical difference in the CR rate of each therapy among cell types.

Factors associated with mortality.

The factors associated with mortality were analyzed (Table 3). By univariate analysis, sex (female), onset age (≥ 8 years), liver dysfunction, splenomegaly, anemia, and thrombocytopenia were significantly associated with mortality. On the other hand, HSCT was inversely correlated with mortality rate (odds ratio, 0.67), and this was statistically significant only in patients with T-cell infection. Multivariate analysis using factors whose \( P \) values were less than 0.10 revealed that onset age and liver dysfunction were independently significant factors that increased mortality (Table 3). Again, HSCT was an independent factor that decreased mortality rate.

We compared overall survival rates between each subgroup to confirm association of the above factors with mortality (Fig. 5). Overall survival rate in patients whose onset was more than 8 years was significantly low (\( P < 0.001 \)). Patients with liver dysfunction at the time of diagnosis had lower survival rate (\( P = 0.031 \)). When patients were divided into five groups based on EBV-infected cells, patients with CD4+ T-cell infection had significantly lower survival rate compared to those with NK-cell infection (\( P = 0.002 \)). However, there was no statistical difference in survival rate among clinical groups, although the numbers in some groups were small. Patients who received HSCT survived longer (\( P = 0.001 \)), and again, this was statistically significant only in patients with T-cell infection (\( P = 0.003 \)).

Characteristics of patients with HSCT

Out of 59 patients who underwent HSCT, 39 patients (66%) survived 1 to 144 months after
transplantation (median, 35.5 months). On the other hand, 20 patients (34%) died 1 day to 48 months after transplantation (median, 1.8 months). Detailed characteristics of each patient are shown in Supplementary Table 3. Main causes of death were multiple organ failure (5 cases), intracranial hemorrhage (5 cases), sepsis (2 cases), and others (8 cases). Of the 20 deaths, 15 were considered to be treatment related deaths. We compared various factors between alive and dead cases with HSCT (Table 4). Univariate analysis showed that age at HSCT was higher and patients with active disease status at HSCT were more in the dead cases (Table 4). Time from disease onset to HSCT and intensity of the conditioning regimen (either myeloablative or reduced) were marginally associated with death ($P = 0.059$ and $P = 0.086$, respectively). To determine independent risk factors, we performed multivariate analysis using factors whose $P$ value was less than 0.10, and found that none of them were independent risk factors for death (data not shown).

We compared overall survival rates (Fig. 6A) and event free survival rates (Fig. 6B) of transplanted patients between each subgroup. Although disease status at HSCT was not an independent risk factor by multivariate analysis, overall survival rate was significantly higher in inactive cases at HSCT ($P = 0.014$); however, its significance diminished in the event free survival rate. Patients who received HSCT at an age less than 15 years old were significantly higher in both overall ($P = 0.013$) and event free survival rates ($P = 0.015$). Moreover, patients whose time from onset to HSCT was less than 30 months had significantly higher survival rates in both overall ($P = 0.036$) and event free survival ($P = 0.033$). Interestingly, these were statistically significant only in patients with T-cell infection.

**Discussion**

Determining the phenotype of EBV-infected cells is mandatory for our further understanding of the pathogenesis of EBV$^+$ T/NK-LPDs and related biologic behaviors. In the present study, we used unfixed peripheral blood to determine the phenotypes of EBV-infected cells. One caveat of this study
is that we may have missed EBV-associated T/NK-LPDs in cases where EBV-infected cells failed to migrate to the peripheral blood\textsuperscript{33}. Furthermore, EBV-infected cells in peripheral blood might be different from those existing in tissues, although there was no discordant result between tissue biopsy and peripheral blood.

In the present study, EBV-infected cells in EBV\textsuperscript{+} T/NK-LPDs were immunophenotypically divided to CD4\textsuperscript{+} T, CD8\textsuperscript{+} T, γδ T, and NK cells, the variable proportion of which were observed in each of the clinical categories. Kasahara et al reported that CAEBV and EBV-associated HLH were largely caused by CD4\textsuperscript{+} T or NK cells and CD8\textsuperscript{+} T cells, respectively\textsuperscript{22}. We demonstrated that CAEBV was caused by not only CD4\textsuperscript{+} T and NK cells but also CD8\textsuperscript{+} T and γδ T cells. We also demonstrated that EBV-infected cells in nearly half of hydroa vacciniforme or hydroa vacciniforme-like lymphoma were γδ T cells in accordance with our previous observations\textsuperscript{36}. Interestingly, all these cells express molecules characteristic of cytotoxic cells. In fact, EBER-positive lymphocytes in EBV\textsuperscript{+} T/NK-LPDs usually express cytotoxic molecules including perforin, granzyme B, and TIA-1, as shown in this study and previous reports\textsuperscript{7,44}. The mechanism underlying EBV-infection of T- and NK-cells, which do not express CD21, remains unresolved. It has been shown that NK cells activated by EBV-infected B cells acquire CD21 by synaptic transfer, and these ectopic receptors allow EBV binding to NK cell hosts\textsuperscript{45}. It is plausible that killer cells that closely contact with EBV-infected B cells may acquire EBV infection directly and then proliferate with clonality.

In this study, we evaluated prognostic factors among patients with EBV\textsuperscript{+} T/NK-LPDs. Multivariate analysis showed that age at onset of disease (≥ 8 years) and liver dysfunction were independent risk factors for mortality, and that transplanted patients had better prognosis. We previously identified that older onset age (≥ 8 years) was associated with mortality in patients with CAEBV\textsuperscript{29}. Furthermore, a recent report demonstrated that adult patients with CAEBV had progressive and more aggressive courses than those of childhood onset cases\textsuperscript{46}. Interestingly, patients
with CD4⁺ T-cell infection had shorter survival than those with NK infection, while clinical categories were irrespective of 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 the 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 of EBV-associated HLH patients well responded to chemotherapy and immunomodulating therapies⁴⁷, but patients with CAEBV were generally refractory to chemotherapy. Similar findings were reported in patients with CAEBV in the United States²⁰. Furthermore, Kaplan–Meier estimates indicated that shorter time from onset to HSCT (< 30 months) and inactive disease at HSCT resulted in long survival, suggesting that earlier HSCT in good condition is preferred. Patients with CAEBV have a higher risk of transplantation-related complications⁴¹,⁴⁸. Recently, Kawa et al reported excellent outcome of HSCT with reduced-intensity conditioning⁴⁰. Although the superiority of reduced-intensity conditioning over myeloablative one did not reach the statistical significance in this study, it seems that reduced-intensity regimen is suitable to avoid transplantation-related death⁴⁰,⁴⁹.

The concept of EBV⁺ T/NK-LPD was initially proposed by Kawa et al and followed by other researchers²⁷,⁴⁴. This umbrella term encompasses specific clinical diseases of 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, i.e., ANKL and extranasal ENKL, from EBV⁺ NK-LPDs, because EBV⁺ proliferating cells are indistinguishable in morphology and phenotype. Recently, this
issue was first highlighted by Takahashi et al\textsuperscript{50}. Interestingly, 4 patients of the present series
developed ANKL in their clinical course, 2 of who 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 \textit{de novo} ENKL was its early onset (median age, 8.5 years). Three
patients had hypersensitivity to mosquito bites. There were no differences in pathological features
between these cases and \textit{de novo} ENKL cases\textsuperscript{50}. Furthermore, new development of chromosomal
aberrations was seen in 6 patients during follow-up. In this study, most of the patients with EBV\textsuperscript{+}
T/NK-LPDs had clonality of EBV-infected cells. These results indicate that clonally expanding
EBV-infected T or NK cells in EBV\textsuperscript{+} 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,
Bethesda, to better define the pathogenesis, classification, and treatment of EBV-associated LPDs in
nonimmunocompromised hosts\textsuperscript{39}. At the 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\textsuperscript{+} T-LPD of childhood
were listed as EBV\textsuperscript{+} T/NK-LPDs under an umbrella term of CAEBV of T/NK-cell type\textsuperscript{39}. In the
present study, EBV\textsuperscript{+} T/NK-LPD is characterized by the systemic distribution of EBV\textsuperscript{+} 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 despite the fact that
they comprise about 40\% of the cases with EBV\textsuperscript{+} T/NK-LPD. 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.
Acknowledgements

This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (21591384) and a Health and Labour Science Research Grant on intractable diseases from the Ministry of Health, Labour and Welfare of Japan (H22-Nanchi-080) to H.K.

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Hospital, Tsukuba University Hospital, The Institute of Medical Sciences, The University of Tokyo, The University of Tokyo Hospital, Yamagata University, Yamaguchi University Hospital, University of Miyazaki Hospital, Yokohama City University Hospital, Yokohama Minami Kyousai Hospital, and Wakayama Medical University Hospital.

Authorship Contributions and Disclosure of Conflicts of Interest

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

Conflict-of-interest disclosure: The authors declare no competing financial interest.
References


<table>
<thead>
<tr>
<th>Disease</th>
<th>Eligibility criteria</th>
<th>Exclusion criteria</th>
<th>Lineages/clonality</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical categories</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic active EBV disease (CAEBV) of T/NK-cell type</td>
<td>1) Illness $\geq 3$ months duration (EBV-related illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, $^1$hydroa vacciniforme-like eruptions, and $^1$hypersensitivity to mosquito bites).&lt;br&gt;2) Increased amounts of EBV was detected by Southern blot hybridization or EBER-positive cells in affected tissues or peripheral blood; $\geq 10^{2.5}$ copies/$\mu$g EBV DNA in peripheral blood mononuclear cells.</td>
<td>1) No evidence of previous immunological abnormalities or other recent infection that might explain the observed condition.&lt;br&gt;2) Cases of congenital immunodeficiency including X-linked lymphoproliferative disorders.</td>
<td>T/NK-cell&lt;br&gt;Polyclonal&lt;br&gt;Monoclonal</td>
<td>12&lt;br&gt;16&lt;br&gt;29</td>
</tr>
</tbody>
</table>

<p>| Hemophagocytic lymphohistiocytosis (HLH) | 1) Clinical criteria (fever and splenomegaly)&lt;br&gt;2) Laboratory criteria (cytopenia affecting two of three lineages in the peripheral blood, hypertriglyceridemia, and/or hypofibrinogenemia).&lt;br&gt;3) Histological criteria (hemophagocytosis in the bone marrow, spleen, or lymph nodes). | 1) Cases with hemophagocytic syndrome in accelerated phase of CAEBV of T/NK-cell type.&lt;br&gt;2) Cases of congenital immunodeficiency including familial HLH. | T/NK-cell&lt;br&gt;Polyclonal&lt;br&gt;Monoclonal | 11 |</p>
<table>
<thead>
<tr>
<th>Severe mosquito bite allergy (SMBA)</th>
<th>1) Hypersensitivity to mosquito bites characterized by high fever after bites, ulcers, necrosis, and scarring.</th>
<th>Patients with any systemic symptoms in addition to the cutaneous lesions were categorized to CAEBV of T/NK-cell type.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroa vacciniforme (HV)</td>
<td>1) Recurrent vesiculopapules with central umbilication and crust formation, mimicking herpetic vesicles usually occurring on the sun-exposed areas.</td>
<td>Patients with any systemic symptoms in addition to the cutaneous lesions were categorized to CAEBV of T/NK-cell type.</td>
</tr>
</tbody>
</table>

**Pathological classification**

<table>
<thead>
<tr>
<th>Systemic EBV-positive T-cell LPD (SETLPD)</th>
<th>1) Illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, hemophagocytosis, interstitial pneumonia. 2) It can occur shortly after primary EBV infection or in the setting of CAEBV. 3) Monoclonal expansion of EBV-infected T cells with an activated cytotoxic phenotype in tissues or peripheral blood.</th>
<th>Other overt leukemia and lymphoma such as extranodal NK/T cell lymphoma, aggressive NK cell leukemia, and peripheral T cell lymphoma.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T-cell 7</td>
</tr>
</tbody>
</table>

Monoclonal
Hydroa vacciniforme-like lymphoma (HVL)

1) Recurrent vesiculopapules with central umbilication and crust formation usually occurring on the sun-exposed areas with or without systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly.
2) Monoclonality of EBV-infected cells.

Other overt leukemia and lymphoma such as extranodal NK/T cell lymphoma, aggressive NK cell leukemia, and peripheral T cell lymphoma

Monoclonal T/NK-cell

NOTE 1’severe mosquito bite allergy’ and ‘hydroa vacciniforme’ were used as clinical categories, while ‘hyper sensitivity to mosquito bites’ and ‘hydroa vacciniforme-like eruptions’ were used to designate symptoms.
<table>
<thead>
<tr>
<th>Factors</th>
<th>Total (n=108)</th>
<th>T-cell (n=64)</th>
<th>NK-cell (n=44)</th>
<th>P value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>50/58</td>
<td>27/37</td>
<td>23/21</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age at disease onset, years (mean±SD)</td>
<td>12.1±10.6</td>
<td>12.7±10.3</td>
<td>11.3±11.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Clinical category at diagnosis, cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic active EBV disease</td>
<td>80</td>
<td>47</td>
<td>33</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemophagocytic lymphohistiocytosis</td>
<td>15</td>
<td>12</td>
<td>3</td>
<td>0.066</td>
</tr>
<tr>
<td>Severe mosquito bite allergy</td>
<td>9</td>
<td>1</td>
<td>8</td>
<td>0.003</td>
</tr>
<tr>
<td>Hydroa vacciniforme</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Past history of infectious mononucleosis, cases (%)</td>
<td>37 (34)</td>
<td>24 (22)</td>
<td>13 (12)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Primary infection at diagnosis, cases (%)</td>
<td>19 (18)</td>
<td>16 (15)</td>
<td>3 (3)</td>
<td>0.012</td>
</tr>
<tr>
<td>EBV DNA quantity in peripheral blood at diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mononuclear cells (log copies/µg DNA, mean±SD)</td>
<td>4.3±0.9</td>
<td>4.2±0.9</td>
<td>4.5±0.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>plasma (log copies/mL, mean±SD)</td>
<td>3.3±1.7</td>
<td>3.5±1.6</td>
<td>3.1±2.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>EBV clonality (monoclonal/oligoclonal/polyclonal)</td>
<td>64/8/4</td>
<td>36/4/3</td>
<td>28/4/1</td>
<td>n.s.</td>
</tr>
<tr>
<td>TCR rearrangement (any rearrangement/ none)</td>
<td>42/48</td>
<td>36/20</td>
<td>6/28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chromosomal aberration (abnormal/normal cases)</td>
<td>6/84</td>
<td>4/50</td>
<td>2/34</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
## Symptoms and signs at diagnosis, cases (%)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>98 (91)</td>
<td>59 (92)</td>
<td>39 (89)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Liver dysfunction</td>
<td>83 (77)</td>
<td>49 (77)</td>
<td>34 (77)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>64 (59)</td>
<td>39 (61)</td>
<td>25 (57)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>47 (44)</td>
<td>26 (41)</td>
<td>21 (48)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Anemia</td>
<td>46 (43)</td>
<td>29 (45)</td>
<td>17 (39)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>41 (38)</td>
<td>27 (42)</td>
<td>14 (32)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemophagocytic syndrome</td>
<td>38 (36)</td>
<td>23 (36)</td>
<td>15 (34)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hypersensitivity to mosquito bites (HMB)</td>
<td>32 (30)</td>
<td>3 (5)</td>
<td>29 (43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hydroa vacciniforme-like eruption (HV-LE)</td>
<td>15 (14)</td>
<td>8 (13)</td>
<td>7 (16)</td>
<td>n.s.</td>
</tr>
<tr>
<td>HMB+ HV-LE+</td>
<td>5 (5)</td>
<td>0 (0)</td>
<td>5 (11)</td>
<td>0.001</td>
</tr>
<tr>
<td>HMB− HV-LE+</td>
<td>10 (9)</td>
<td>8 (13)</td>
<td>2 (5)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Chemotherapy, cases (%)</td>
<td>70 (65)</td>
<td>45 (70)</td>
<td>25 (57)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hematopoietic stem cell transplantation, cases (%)</td>
<td>59 (55)</td>
<td>32 (50)</td>
<td>27 (61)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

## Outcome, cases (%)

<table>
<thead>
<tr>
<th>Status</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead</td>
<td>47 (44)</td>
<td>27 (42)</td>
<td>20 (45)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Alive</td>
<td>61 (57)</td>
<td>37 (58)</td>
<td>27 (61)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Stable disease</td>
<td>11 (10)</td>
<td>8 (13)</td>
<td>3 (7)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Patient Status</td>
<td>Numbers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>---------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete remission</td>
<td>46 (43) 26 (41) 20 (20) n.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial remission</td>
<td>4 (4) 3 (5) 1 (2) n.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** $^1P$ values less than 0.10 are shown and those less than 0.05 (shown as bold and italic) are statistically significant. n.s.: not significant, SD: standard deviation.
Table 3. Univariate and multivariate analyses of factors associated with mortality in 108 patients with EBV+ T/NK lymphoproliferative disease

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (95% CI)</td>
<td>P value(^1)</td>
</tr>
<tr>
<td>Sex (female)</td>
<td>1.40 (0.98-1.97)</td>
<td>0.048</td>
</tr>
<tr>
<td>Age at disease onset ( (\geq 8) years)</td>
<td>1.63 (1.17-2.28)</td>
<td>0.003</td>
</tr>
<tr>
<td>Past history of infectious mononucleosis</td>
<td>0.62 (0.35-1.11)</td>
<td>0.093</td>
</tr>
<tr>
<td>Primary infection at diagnosis</td>
<td>0.47 (0.18-1.20)</td>
<td>0.079</td>
</tr>
<tr>
<td>Clinical entity at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic active EBV disease</td>
<td>1.12 (0.90-1.39)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemophagocytic lymphohistiocytosis</td>
<td>0.65 (0.24-1.77)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Severe mosquito bite allergy</td>
<td>1.04 (0.30-3.65)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hydroa vacciniforme</td>
<td>0.43 (0.05-4.03)</td>
<td>n.s.</td>
</tr>
<tr>
<td>T-cell infection</td>
<td>1.13 (0.69-1.71)</td>
<td>n.s.</td>
</tr>
<tr>
<td>NK-cell infection</td>
<td>0.95 (0.69-1.30)</td>
<td>n.s.</td>
</tr>
<tr>
<td>EBV DNA in mononuclear cells ((\geq 10^{4.5}) copies/(\mu)g DNA)</td>
<td>1.16 (0.79-1.71)</td>
<td>n.s.</td>
</tr>
<tr>
<td>EBV DNA in plasma ((\geq 10^{3.5}) copies/mL)</td>
<td>1.23 (0.84-1.72)</td>
<td>n.s.</td>
</tr>
<tr>
<td>EBV monoclonality</td>
<td>1.08 (0.89-1.31)</td>
<td>n.s.</td>
</tr>
<tr>
<td>TCR rearrangement</td>
<td>1.13 (0.73-1.76)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
### Chromosomal aberration

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>p-value</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.92(0.34-10.9)</td>
<td>n.s.</td>
<td>-</td>
</tr>
</tbody>
</table>

### Symptoms and signs at diagnosis (cases)

<table>
<thead>
<tr>
<th>Symptoms and signs at diagnosis</th>
<th>Value</th>
<th>p-value</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>1.10 (0.98-1.24)</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Liver dysfunction</td>
<td>1.33 (1.09-1.63)</td>
<td>0.006</td>
<td>4.25 (1.23-14.7)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>1.38 (1.01-1.88)</td>
<td>0.033</td>
<td>-</td>
</tr>
<tr>
<td>Anemia</td>
<td>1.84 (1.18-2.88)</td>
<td>0.005</td>
<td>1.36 (0.31-6.01)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>1.75 (1.13-2.71)</td>
<td>0.009</td>
<td>1.80 (0.44-7.33)</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>1.24 (0.77-2.00)</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Hemophagocytic syndrome</td>
<td>1.30 (0.72-2.32)</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Hypersensitivity to mosquito bites</td>
<td>0.89 (0.69-1.15)</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Hydroa vacciniforme-like eruption</td>
<td>0.86 (0.34-1.97)</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.84 (0.53-1.34)</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Hematopoietic stem cell transplantation</td>
<td>0.67 (0.045-0.98)</td>
<td>0.022</td>
<td>0.34 (0.12-0.96)</td>
</tr>
<tr>
<td>in T-cell infection group</td>
<td>0.54 (0.30-0.97)</td>
<td>0.021</td>
<td>-</td>
</tr>
<tr>
<td>in NK-cell infection group</td>
<td>0.83 (0.51-1.34)</td>
<td>n.s.</td>
<td>-</td>
</tr>
</tbody>
</table>

**NOTE.**  
1. P values less than 0.10 are shown and those less than 0.05 (shown as bold and italic) are statistically significant.  
2. For multivariate analysis, factors with P value < 0.10 were included.  
3. P values less than 0.05 (shown as bold and italic) are statistically significant.  
4. Stratified onset ages were analyzed in advance, and ≥ 8 years was chosen as the age factor.  
5. Splenomegaly was excluded from multivariate analysis, because this factor was closely associated with anemia, thrombocytopenia, and liver dysfunction. CI: confidence interval, n.s.: not significant.
Table 4. Comparison of characteristics based on outcome in 59 patients with transplantation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Total (n=59)</th>
<th>Alive (n=39)</th>
<th>Dead (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>29/30</td>
<td>22/17</td>
<td>7/13</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age at disease onset, years (mean±SD)</td>
<td>11.8±9.2</td>
<td>11.0±9.0</td>
<td>13.6±9.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Clinical category at diagnosis, cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic active EBV disease</td>
<td>46</td>
<td>32</td>
<td>14</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemophagocytic lymphohistiocytosis</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Severe mosquito bite allergy</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hydroa vacciniforme</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>n.s.</td>
</tr>
<tr>
<td>EBV DNA quantity in peripheral blood at diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in mononuclear cells (log copies/μg DNA, mean±SD)</td>
<td>4.5±0.8</td>
<td>4.4±0.9</td>
<td>4.5±0.89</td>
<td>n.s.</td>
</tr>
<tr>
<td>in plasma (log copies/mL, mean±SD)</td>
<td>3.3±1.6</td>
<td>3.3±1.3</td>
<td>3.3±2.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>T-cell infection, cases</td>
<td>32</td>
<td>23</td>
<td>9</td>
<td>n.s.</td>
</tr>
<tr>
<td>NK-cell infection, cases</td>
<td>27</td>
<td>16</td>
<td>11</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age at HSCT, years (mean±SD)</td>
<td>17.5±9.23</td>
<td><strong>15.6±9.1</strong></td>
<td><strong>21.2±8.3</strong></td>
<td>0.034</td>
</tr>
<tr>
<td>Time from onset to HSCT, months (mean±SD)</td>
<td>65.0±68.2</td>
<td>52.2±54.7</td>
<td>90.0±84.8</td>
<td>0.059</td>
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<tr>
<td>Disease status at transplantation (active/inactive)</td>
<td>25/34</td>
<td><strong>13/26</strong></td>
<td><strong>12/8</strong></td>
<td><strong>0.046</strong></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Category</th>
<th>% Preceded chemotherapy</th>
<th>% Stem cell source (bone marrow/peripheral blood/cord blood)</th>
<th>% Donor (MRD/MUD/MMRD/MMUD)</th>
<th>% Number of mismatched HLA (mean±SD)</th>
<th>% Preconditioning regimen (myeloablative/reduced)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>42 (71)</td>
<td>35/11/13</td>
<td>18/11/4/26</td>
<td>0.76±0.9</td>
<td>21/38</td>
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<tr>
<td></td>
<td>27 (69)</td>
<td>22/8/9</td>
<td>10/9/3/17</td>
<td>0.76±0.9</td>
<td>11/28</td>
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<tr>
<td></td>
<td>15 (75)</td>
<td>13/3/4</td>
<td>8/2/1/9</td>
<td>0.75±0.9</td>
<td>10/10</td>
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**NOTE.** *P* values less than 0.10 are shown and those less than 0.05 (shown as bold and italic) are statistically significant.
Figure legends

Figure 1. EBV-infected cell phenotypes of EBV+ T/NK lymphoproliferative diseases. A: Age distribution of patients with T-cell and NK-cell types. B: EBV-infected cells among categories of clinical groups. Infected T cells were further divided into CD4+ T, CD8+ T, γδ T, and “other T cells”. The 25 cases of “other T cells” were defined as either phenotypically different T cell subsets (2 cases were CD4-CD8-, 1 case was CD4+CD8+, and 1 case had two lineages consisting of CD4+CD8- and CD4-CD8+ cells) or ill-defined T cells (21 cases). In the majority of the ill-defined T-cell cases, antibodies against CD4 or CD8 could not be used to define their CD4/CD8 phenotype because the number of recovered PBMCs was not sufficient. CAEBV, chronic active EBV disease; HLH, hemophagocytic lymphohistiocytosis; SMBA, severe mosquito bite allergy; and HV, hydroa vacciniforme. C: The 4th WHO pathological classification of each clinical group at the time of diagnosis. SETLPD, systemic EBV-positive T-cell lymphoproliferative disease of childhood and HVL, hydroa vacciniforme-like lymphoma. D: EBV-infected cells among categories of the pathological classification at the diagnosis (At diagnosis) and at the last follow-up or death (At last). Patients in complete remission were classified according to the data and status before remission. ENKL, extranodal NK/T lymphoma, nasal type; ANKL, aggressive NK cell leukemia; and PTCL, peripheral T cell lymphoma.

Figure 2. Histopathological findings of representative cases. A: Cervical lymph node from a 6-year-old boy with chronic active EBV disease with T-cell infection (Patient #3). Follicles and paracortical hyperplasia including a mild increase in transformed lymphocytes were seen. Focal epithelioid reactions were detected (arrows). Medium sized transformed lymphocytes in paracortex were positive for EBV-encoded small RNA (EBER). TIA-1 and perforin were positive, but granzyme B was negative. B: Spleen from a 13-year-old boy with chronic active EBV disease with NK-cell infection (Patient #6). White pulp was atrophic and red pulp showed congestion. Small
lymphocytes infiltrating in the red pulp were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. C: Bone marrow from a 25-year-old female with chronic active EBV disease with T-cell infection (Patient #17). In the mild hyperplastic marrow, small sized lymphocytes were positive for EBER. TIA-1, perforin, and granzyme B were positive. D: Liver from a 42-year-old female with chronic active EBV disease with NK-cell infection (Patient #60). Small sized lymphocytes infiltrating in vessels and sinusoid were positive for EBER. TIA-1, perforin, and granzyme B were positive. HE: hematoxylin-eosin staining.

**Figure 3. Viral load in peripheral blood at time of diagnosis.** EBV-DNA was quantified by real-time PCR. A: Correlation of viral load between peripheral blood mononuclear cells (PBMCs) and plasma. The correlation was separately estimated in patients with T-cell infection and those with NK-cell infection. B: Quantity of EBV-DNA in PBMCs among categories of clinical groups. *P < 0.05. C: Quantity of EBV-DNA in plasma among categories of clinical groups. CAEBV, chronic active EBV disease; HLH, hemophagocytic lymphohistiocytosis; SMBA, severe mosquito bite allergy; and HV, hydroa vacciniforme.

**Figure 4. Efficacy of therapeutic interventions.** A: Number of cases treated with each therapy and cases that maintained complete remission (CR) are shown among categories of clinical groups. CAEBV, chronic active EBV disease; HLH, hemophagocytic lymphohistiocytosis; SMBA, severe mosquito bite allergy; and HV, hydroa vacciniforme. HSCT (hematopoietic stem cell transplantation) *P = 0.002. **P = 0.02. B: Numbers of cases who received each therapy and those who maintained sustained CR are shown among categories of EBV-infected cells.

**Figure 5. Probability of survival rates from time of disease onset.** Overall survival rates from onset (n = 108) were calculated from Kaplan–Meier estimates between each subgroup (onset age ≥
8 years or < 8 years, with or without liver dysfunction, EBV-infected cell types, clinical categories, and with or without hematopoietic stem cell transplantation [HSCT]). HSCT patients were divided into groups based on T-cell infection (n = 64) and NK-cell infection (n = 44) and independently analyzed. CAEBV, chronic active EBV disease; HLH, hemophagocytic lymphohistiocytosis; SMBA, severe mosquito bite allergy; and HV, hydroa vacciniforme.

Figure 6. Probability of survival rates after hematopoietic stem cell transplantation (HSCT).
Survival rates after HSCT were calculated from Kaplan–Meier estimates between each subgroup (inactive or active cases at HSCT, reduced or myeloablative conditioning, transplant age ≥ 15 years or < 15 years, and time from onset to HSCT ≥ 30 months or < 30 months). Stratified transplanted ages were analyzed in advance, and ≥15 years was chosen as the age factor. Similarly stratified times from onset to HSCT were analyzed in advance, and ≥ 30 months was chosen as the time factor. 
**A:** Overall survival rate after HSCT (n = 59). **B:** Event free survival rate after HSCT (n = 59). For time from onset to HSCT, patients were divided into T-cell infection (n = 32) and NK-cell infection (n = 27) groups and independently analyzed.
Figure 2

A

Lymph node/HE

TIA-1 Perforin Granzyme B

B

Spleen/HE

TIA-1 Perforin Granzyme B

C

Bone marrow/HE

TIA-1 Perforin Granzyme B

D

Liver/HE

TIA-1 Perforin Granzyme B

Scale bars: large, 200nm; small, 20nm
Figure 3

A

![Graph A](image)

**Plasma (log copy/mL)** vs. **PBMCs (log copy/µg DNA)**

- NK: $y = 1.28x - 2.78$, $R^2 = 0.31$
- T: $y = 0.59x + 0.95$, $R^2 = 0.11$

B

![Bar Chart B](image)

B: CAEBV, HLH, SMBA, HV

C

![Bar Chart C](image)

C: CAEBV, HLH, SMBA, HV
Figure 5

Overall survival rate

Onset age
- <8 years
  - 15 yr 59.7%
- ≥8 years
  - 15 yr 27.0%

Liver function
- Normal
  - 15 yr 73.7%
- Abnormal
  - 15 yr 37.2%

EBV-infected cell type
- P = 0.002 between CD4+T and NK
- γδ T
- CD8+T
- NK
- CD4+T
- Other T

Clinical categories
- HLH
- HV
- SMBA
- CAEBV

HSCT
- P = 0.001
- Transplant +
  - 15 yr 60.6%
- Transplant -
  - 15 yr 25.7%

HSCT/T-cell infection
- P = 0.003
- Transplant +
  - 15 yr 56.9%
- Transplant -
  - 15 yr 36.1%

HSCT/NK-cell infection
- P = 0.12
- Transplant +
  - 15 yr 63.5%
- Transplant -
  - 15 yr 17.3%
Figure 6

A. Overall survival rate after HSCT

1. Status at HSCT
   - P = 0.014
   - Inactive cases: 60 mo 71.2%
   - Active cases: 60 mo 48.9%

2. Conditioning regimen
   - P = 0.14
   - Reduced: 60 mo 70.3%
   - Myeloablative: 60 mo 49.5%

3. Age at HSCT
   - P = 0.013
   - <15 years: 60 mo 81.1%
   - ≥15 years: 60 mo 45.8%

4. Time from onset to HSCT
   - P = 0.036
   - <30 months: 60 mo 80.2%
   - ≥30 months: 60 mo 48.0%

5. Time from onset to HSCT
   - T-cell infection
     - P = 0.009
     - <30 months: 60 mo 88.9%
     - ≥30 months: 60 mo 41.7%

6. Time from onset to HSCT
   - NK-cell infection
     - P = 0.95
     - <30 months: 60 mo 62.5%
     - ≥30 months: 60 mo 54.9%

B. Event free survival rate after HSCT

1. Status at HSCT
   - P = 0.077
   - Inactive cases: 60 mo 60.8%
   - Active cases: 60 mo 49.8%

2. Conditioning regimen
   - P = 0.32
   - Reduced: 60 mo 55.4%
   - Myeloablative: 60 mo 48.2%

3. Age at HSCT
   - P = 0.015
   - <15 years: 60 mo 72.6%
   - ≥15 years: 60 mo 44.3%

4. Time from onset to HSCT
   - P = 0.033
   - <30 months: 60 mo 76.0%
   - ≥30 months: 60 mo 35.4%

5. Time from onset to HSCT
   - T-cell infection
     - P = 0.015
     - <30 months: 60 mo 83.0%
     - ≥30 months: 60 mo 34.7%

6. Time from onset to HSCT
   - NK-cell infection
     - P = 0.79
     - <30 months: 60 mo 62.5%
     - ≥30 months: 60 mo 27.4%
Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative diseases in non-immunocompromised hosts: prospective analysis of 108 cases

Hiroshi Kimura, Yoshinori Ito, Shinji Kawabe, Kensei Gotoh, Yoshiyuki Takahashi, Seiji Kojima, Tomoki Naoe, Shinichi Esaki, Atsushi Kikura, Akihisa Sawada, Keisei Kawa, Koichi Ohshima and Shigeo Nakamura