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BJD original article

Survival Rates and Prognostic Factors of Epstein-Barr Virus-Associated Hydroa Vacciniforme and Hypersensitivity to Mosquito Bites

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Running head: Prognostic factors in hydroa vacciniforme

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Conflicts of interest: None declared.

Abbreviations: EBV : Epstein-Barr virus, HV: hydroa vacciniforme, HMB: hypersensitivity to mosquito bites, LPDs :lymphoproliferative disorders, PBMCs : peripheral blood mononuclear cells, EBER :EBV-encoded small nuclear RNA, CTLs : cytotoxic T lymphocytes, cHV : classical HV, sHV :systemic HV, CAEBV :chronic active EBV infection, HLH :haemophagocytic lymphohistiocytosis, PCR : polymerase chain reaction, BMRF1:BamHI M region, qRT: quantitative reverse transcriptase, HSCT: hematopoietic stem cell transplantation, WBC :white blood cell count, Hb : hemoglobin, AST :aspartate aminotransferase, ALT : alanine aminotransferase, LD : lactate dehydrogenase, VCA : viral capsid antigen, EBNA : EBV nuclear antigen, EA : early antigen

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What's already known about this topic?

Epstein-Barr virus-associated T/NK lymphoproliferative disorders are a group of diseases that include classical and systemic hydroa vacciniforme (HV), and hypersensitivity to mosquito bites (HMB).

Patients with systemic HV and HMB usually present with fever, liver damage and hematological abnormalities, and often have a fatal outcome.

What does this study add?

Our patients with classical HV showed a favourable prognosis, while approximately one-third of the patients with systemic HV or HMB died over in the 10-year follow-up.

Late onset over 9 years of age and an EBV reactivation signal BZLF-1 mRNA expression were both related to more severe phenotypes of the disease, and a poor prognosis.

Abstract

Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative disorders are a group of diseases that include classical and systemic hydroa vacciniforme (HV), and hypersensitivity to mosquito bites (HMB). Patients with systemic HV (sHV) and HMB often have a poor prognosis, although little is known about the prognostic factors.

In order to elucidate the prognostic factors of HV and HMB, we studied clinicopathologic manifestations, routine laboratory findings, anti-EBV titres, EBV DNA load, and EBV-encoded gene expression, including expression of BZLF1, in 50 patients with classical HV (cHV), sHV, HMB only, and HMB with HV (HMB+HV), and further analysed 30 patients who were available for follow-up. The median age of disease onset was 5 years (age range: 1-74 years). A follow-up study indicated that fatal outcomes were observed in 3 of 8 sHV patients, 2 of 6 HMB only patients, and 2 of 5 patients with HMB+HV; main causes of death were complications from hematopoietic stem cell transplantation, and multi-organ failure. There were no fatalities among the 11 cHV patients. Univariate analysis revealed 2 poor prognostic indicators: onset age of over 9 years, and the expression of an EBV-encoded immediate-early gene transcript, *BZLF1* mRNA, in the skin lesions ($p<0.001$ and $p=0.003$, respectively). No prognostic correlation was observed in EBV-infected lymphocyte subsets, anti-EBV antibody titres, or EBV DNA load. Late onset and EBV reactivation are both related to more severe phenotypes of the disease, and thus may predict a poor prognosis.

Introduction

Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative disorders (LPDs) are a group of diseases that include hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB). EBV-associated HV lesions contain a number of EBV-encoded small nuclear RNA $(EBER)^+$ T cells, together with larger numbers of $EBER^-$ cytotoxic T lymphocytes (CTLs); meanwhile, NK cells are absent or occur at background levels in such lesions[1,2]. Although there are no systemic symptoms or abnormalities in the routine laboratory tests of classical HV (cHV) patients, EBV DNA load and EBV^+ γδT cells are increased in the peripheral blood mononuclear cells (PBMCs)[3]. In contrast, patients with HV-like ulcerative cutaneous eruptions often present with systemic symptoms such as fever, hepatic damage and lymphadenopathy (systemic HV: sHV), and show dense inflammatory cell infiltrates that reach the subcutaneous tissue. As reported previously, patients with cHV may progress to sHV in the clinical course[2].

HMB is an EBV-associated T/NK LPD characterized by intense local skin reactions and systemic symptoms, including high fever, lymphadenopathy, hepatosplenomegaly, and hemophagocytic syndrome[4]. These clinical symptoms can be induced by mosquito bites, other insect bites or vaccination. Patients with HMB usually have EBV^+ NK cell lymphocytosis[3,5], and HV-like eruptions may occur over the course of the disease. We previously examined differences in cellular events between HMB and HV-like eruptions[3]. Our results indicated that many $CD56^+$ NK cells and T-cells are present in the subcutaneous infiltrates in HMB, but no $CD56^+$ NK cells occur in HV dermal infiltrates.

Unlike cHV, both sHV and HMB have been reported in Asian and Latin American countries[6–8]. The nomenclature of EBV-associated T/NK LPDs, however, has been controversial. Patients with sHV in the present study may be synonymous with HV-like lymphoma in WHO classification[9], and be overlapped with chronic active EBV infection (CAEBV) and EBV-associated haemophagocytic

lymphohistiocytosis (HLH)[10]. Because of the diagnostic value of HV-like cutaneous signs, we have used the terms, HV and HMB in our classification, excluding the diagnoses of CAEBV and HLH.

Although no prognostic markers have been elucidated, previous reports of CAEBV indicate that patients with the EBV^+ T-cell-predominant type have a poor prognosis compared to those with EBV^+ NK cell-predominant type, and that late onset may be a risk factor[10]. In the present study, we attempted to clarify cellular and molecular markers related to the prognosis of cutaneous EBV-associated T/NK LPDs in a series of patients with HV and HMB, and to verify the validity of diagnostic criteria to distinguish benign from malignant types for the purposes of prognosis.

Materials and Methods

Patients

Fifty patients were categorized into 4 groups: cHV (23 cases), sHV (12 cases), HMB only (9 cases), and HMB+HV (6 cases), according to the criteria in Table 1. Briefly, patients with cHV presented with lesions defined as vesiculopapules on sun-exposed areas without any systemic symptoms or abnormalities in routine laboratory test results. Patients with sHV presented with HV-like eruptions associated with systemic symptoms such as fever and lymphadenopathy and/or abnormalities in routine blood examinations at diagnosis. In our series, 1 of 12 patients with sHV initially had skin symptoms without systemic symptoms, although the patient had an increased percentage of NK cells (>30% of lymphocytes) in the blood test. HMB is defined as an intense skin response to mosquito bites, insect bites or vaccination associated with systemic symptoms and/or abnormalities in routine blood tests.

Skin biopsy materials, crusts and blood samples were obtained for diagnosis, and used for *in situ* hybridization with the EBV-encoded small RNA (EBER) and quantitative reverse transcriptase-polymerase reaction (qRT-PCR) to detect EBV infection in skin lesions. This study was approved by the ethical board committee (the institutional review board of Okayama University Hospital (No. 419, 2011) in accordance with the 1975 Declaration of Helsinki.

Assay for EBV DNA load in PBMCs

DNA was extracted from 1×10^6 PBMCs using a QIAamp™ Blood Kit (Qiagen; Netherlands), and the polymerase chain reaction (PCR) amplification was performed using QuantiTect™ Probe PCR (Qiagen; Netherlands) by Roche Light cycler (Roche; Switzerland). The PCR primers for this assay were selected in the BamHI M region (BMRF1). The upstream and downstream primer sequences were 5'-GTGCCAATCTTGAGGTTTTAC-3' and 5'-CACCCGGGGACTTTTATC-3', respectively.

The fluorogenic probes used were probe A, 5'-GACCTGCCGTTGGATCTTAGTG-3', and probe B, 5'-TATTTTATTTAACCACGCCTCCGAAGA-3 phosphol. Amplification was carried out at 95 ℃ for 15 minutes, followed by 50 cycles consisting of 95 ℃ for 15 seconds, 56 ℃ for 20 seconds, and 72 ℃ for 15 seconds. The semiquantitative amounts of EBV DNA copies in patients' samples were determined from the standard curve obtained by PCR amplification of serial 10-fold dilutions of the template plasmid DNA solution.

Primer sets for reverse transcriptase (RT)-PCR

RNA was extracted from the samples with TRIZOL™ reagent (GIBCOBRL, Gaithersburg, MD), and the cDNA was amplified by PCR using EBER1-specific and BARTs-specific primers, as described previously[11,12]. The integrity of the RNA was checked by the parallel amplification of the beta-2-microglobulin (β2-MG). To detect EBV reactivation, BZLF1 was amplified by RT-PCR, using BZLF1-specific outer primers: sense, 5'-CATGTTTCAACCGCTCCGACTGG-3'; and antisense, 5'-GCGCAGCCTGTCATTTTCAGATG-3'. Amplification consisted of 40 cycles of 94 ℃ for 45 seconds, 64 ℃ for 30 seconds, and 72 ℃ for 1 minute. BZLF1-specific inner primers: sense, 5'-TCCCAGTCTCCGACATAACCCA-3'; and antisense, 5'-AGCAGCGACCTCACGGTAGT-3'; amplification involved 28 cycles consisting of 94 ℃ for 45 seconds, 58 ℃ for 30 seconds, and 72 ℃ for 1 minute. Ampilcation gave 167 bp for EBER1 cDNA, 142 bp for BARTs cDNA, 295bp for β2-MG cDNA, and 332 bp for BZLF1 cDNA (639 bp for BZLF1 DNA).

Labeling for EBER *in situ* hybridization

Lymphoid cells containing EBER1 were detected by *in situ* hybridization on paraffin-embedded sections, as described previously[13]. The density of EBER-positive cells was classified into 4

subgroups according to the percentage of positive cells: 1+ (1% to less than 5% positivity in the infiltrate); 2+ (5% to less than 25%); 3+ (25% to less than 50%); and 4+ (50% or more).

Immunophenotyping of infiltrating cells

Deparafinized biopsy specimens were incubated with monoclonal antibodies to CD3ε, CD4, CD8, CD20, CD30 (DAKO, Tokyo) and CD56 (Novocastra Laboratories, Ltd; Newcastle Upon Tyne), as described previously[14].

Flow cytometric analysis for lymphocyte subsets

Blood samples from the patients were reacted with fluorescence-conjugated antibodies to CD3, CD56, TCRαβ, and TCRγδ (BECKMAN COULTER; California), and analyzed using a FACS Calibur flow cytometer and CellQuest software, version 5.2.1 (Becton Dickinson; New Jersey).

Statistical analysis

Analyses were performed using SPSS for Windows version 20.0 (SPSS). For univariate analyses, a one-sided Fisher's exact test was used to compare the categorical variable. To compare the quantitative variable, the Mann-Whitney U test was used. For survival analysis, the Kaplan-Meier method and log-rank test were used. In all analyses, the $p<0.05$ was considered significant.

Results

Clinical observations of the patients

Fifty patients (26 males and 24 females) were enrolled in the current study. They were classified into 4 groups: cHV, sHV, HMB only, or HMB+HV, according to our tentative diagnostic criteria as described elsewhere[3] (Table 1). Age of onset ranged from 1-74 years (median: 5 years). The median

onset ages and gender of the 4 groups were as follows: cHV, 5 years (13 males and 10 females); sHV, 8 years (5 males and 7 females); HMB only, 8 years (6 males and 3 females); and HMB+HV, 3.5 years (2 males and 4 females). Of 23 patients with cHV, 21 (91.3%) had cutaneous lesions that presented within their first decade. The cutaneous signs of cHV occurred at younger ages than those of sHV and HMB+HV (p=0.022 and p=0.026, respectively).

Mucocutaneous symptoms such as conjunctivitis and oral aphthous stomatitis/gingivitis were observed in 6 (26.1%) of 23 cHV patients and 5 (41.7%) of 12 sHV patients, but were not observed in any (0%) of the 9 HMB patients or any (0%) of the 6 HMB+HV. Of 27 patients in the sHV, HMB only, and HMB+HV patients, 22 patients presented with systemic symptoms, including fever (22 patients; 81.5%), diarrhea (2 patients; 7.4%), intestinal perforation (1 patient; 3.7%), hepatosplenomegaly (7 patients; 25.9%), myocarditis (2 patients; 7.4%), and hemophagocytic syndrome (2 patients; 7.4%) (Table 2).

Follow-up study

Of 50 patients enrolled in the present study, a follow-up study was possible in a total of 30 patients; time to follow-up ranged from 1 to 26 years (median: 6.5 years). Median follow-up times for the 4 groups were: 8 years for cHV, 7 years for sHV, 3 years for HMB only, and 12 years for HMB+HV. All 11 patients with cHV were alive at follow-up, with or without disease, and 9 of the 11 patients had been treated only with sunscreen. One of the 2 remaining patients, a 4-year-old girl with cHV, progressed to sHV in the follow-up period due to an episode of fever associated with rash, and underwent hematopoietic stem cell transplantation (HSCT) [15]. The patient had no BZLF1 mRNA expression in the skin lesions, and 83.6% of the γδT cell fraction were positive for EBV infection.

Of 19 patients with sHV, HMB, and HMB+HV, 7 (36.8%) died during the follow-up period, including 3 of 8 patients (37.5%) with sHV, 2 of 6 patients (33.3%) with HMB only, and 2 of 5

patients (40%) with HMB+HV. The main cause of death was HSCT-related in at least 2 patients and was multi-organ failure in another 3 patients.

Fatalities were observed only in groups with systemic symptoms such as sHV, HMB only and HMB+HV, and not in the cHV group (Fig. 1). The log-rank test demonstrated a poor prognosis in patients with sHV and HMB only compared to those with cHV (p=0.016 and p=0.015, respectively). Patients with cHV were distinct from the other 3 groups in terms of prognosis (p=0.026), but no significant difference in prognosis was observed between the HV group and HMB group (p=0.286).

The number of fatal cases increased gradually over 10 years and did not plateau, except in patients with cHV, who showed no fatalities. The cumulative survival rates reached below 50% in 4 years in patients with HMB only, 9 years in sHV, and 14 years in HMB+HV. There was a significant difference in cumulative survival between the groups with HMB only and sHV (p=0.031).

Blood test results related to each group

Routine laboratory test results upon diagnosis showed no significant differences between the groups in white blood cell count (WBC), hemoglobin (Hb) or aspartate aminotransferase (AST) (Table 2). In the HMB only group, however, 3 of 6 patients (50%) had platelet counts below 15×10^4/ μL and higher serum levels of lactate dehydrogenase (LD) than the cHV or sHV subgroups (p<0.05, respectively). Patients with sHV showed elevated levels of AST, ALT and LD to varying degrees, and hematologic abnormalities such as leukopenia and thrombocytopenia, suggestive of hemophagocytic syndrome.

Lymphocyte subsets in PBMCs, taken together with the additional data described in our previous research[3], revealed that 10 of 11 patients (90.9%) with HV-like cutaneous lesions, whether in cHV or sHV, showed elevated γδT cell percentages of over 5% (range: 2.18% to 25%), while 12 of 13

patients (92.3%) with HMB only or HMB+HV showed NK cell lymphocytosis of over 30% of PBMCs (range: 2% to 85%).

Antibody titres against EBV-related antigens as determined by immunofluorescence study demonstrated no differences in EBV nuclear antigen (EBNA); however, IgG titres against anti-early antigen (EA) and IgG-class anti-viral capsid antigen (VCA) were slightly higher in the HMB+HV subgroup than those in the cHV and sHV ($p<0.05$) , and those in the cHV and HMB only groups ($p<0.05$), respectively

The EBV DNA load in PBMCs, as determined by qRT-PCR, was less than 100 copies/μg DNA in healthy individuals, but higher than the reference values in all 26 samples from our patients. The range was from 770 to 720,000 copies /μg DNA, with a mean value of 67,420 ± 140,224 copies /μg DNA. There were no statistically significant differences among the patient's groups (Table2).

Histopathologic examinations

The percentages of $EBER^+$ cells varied even though in the same disease group, ranging from 1% to 25% of the infiltrating mononuclear cells in all but 2 of 21 patients. In the 2 exceptional cases, more than 25% $EBER^+$ cells were observed in the infiltrates in one sHV and one cHV patients. No difference was observed in the number of $EBER^+$ cells among the cHV, sHV, and HMB only groups. The number of samples in the HMB+HV group was insufficient for statistical analysis.

In agreement with our previous report[3], $CD3\varepsilon^+$ and $CD56^-$ T-cells were predominantly infiltrating in HV lesions, without correlation to the severity of the cutaneous lesions. In HMB lesions, however, in addition to $CD3\varepsilon^+$ and $CD56^-$ T-cells, many CD56+ cells suggestive of NK cells were present in 6 of the 7 cases examined. The numbers of reactive T or NK cells negative for EBER were usually larger than the numbers of $EBER^+$ cells.

Onset age as a prognostic factor

We also evaluated prognostic factors by univariate analysis (Table 3). We analysed the 30 cases that were available for follow-up, which consisted of 11 cHV cases, 8 sHV cases, 6 HMB only cases and 5 HMB+HV cases. Onset age over 9 years was significantly correlated with mortality by univariate analysis (p<0.001), and this association was stronger than that between mortality and onset age over 8 years (p=0.026). No significant correlation was observed regarding gender, clinical symptoms such as fever, splenomegaly, or lymphadenopathy, routine blood test results or HSCT treatment.

The mortality rate for patients under 9 years was 8.3%, much lower than for those over 9 years, in whom it jumped to 83.3% (p=0.001) (Fig. 2). Among patients with cHV and sHV, a poor prognosis was observed in those over 9 years of age (p=0.0041). Because a good prognosis is expected with cHV, we recalculated the mortality rates excluding cHV patients. However, the significance of the age-related difference (those under versus over 9 years) remained (p=0.0095). This suggests that an onset age over 9 years is a risk factor.

BZLF-1 as a molecular indicator of poor prognosis

The expression of EBV-encoded BZLF-1 mRNA, an immediate-early gene product, was detected in the skin lesions of patients in the sHV, HMB only, and HMB+HV groups, while no BZLF1 mRNA expression was observed in any of the 13 cHV patients (Fig. 3). The positivity rate of BZLF-1 mRNA expression was significantly high in the 3 groups with systemic symptoms (33%, p=0.047). No difference was observed in BZLF-1 mRNA expression among the 3 groups.

Among EBV-related molecules, the expression of EBV-encoded BZLF-1 in the skin lesions was statistically correlated to a poor prognosis (p=0.003): 4 of 5 (80%) BZLF1 mRNA$^+$ patients, and 2 of 23 (8.7%) BZLF-1 mRNA$^-$ patients died during follow-up, respectively (Fig. 3). Among the 25 patients who could be followed-up, Kaplan-Meier analysis confirmed that BZLF1 mRNA$^+$ patients

showed a worse prognosis compared to those without this expression (p=0.012). The mortality rate excluding cHV patients was higher in the BZLF-1 mRNA$^+$ group (4 of 5 patients: 80%) than in the BZLF-1 mRNA$^-$ group (1 of 9 patients; 11.1%), but there was no statistical significance (p=0.367). The survival rate of BZLF-1 mRNA$^+$ patients was 80% in the first 5 years, but decreased to 26.7% in 10 years. No correlation was found in other EBV-related markers, including anti-EBV antibody titres, EBV DNA load in PBMCs, the number of EBER$^+$ cells, or the subsets of infiltrating cells in the cutaneous lesions. Furthermore, neither an increase in the number of γδT cells nor an increase in NK cells among PBMCs was correlated with mortality (p=0.75, and p =0.86).

Discussion

Our research demonstrated that patients with cHV showed a favourable prognosis with 100% survival, and were distinct in this way from the other 3 groups, sHV, HMB only, and HMB+HV, in which approximately one-third of patients had died by end of the follow-up period. Therefore, the cHV criteria used for the present study, i.e., '*typical cutaneous lesions of HV without systemic symptoms or abnormalities in routine laboratory tests results*,' are valid criteria for distinguishing benign disease from those with fatal potential. However, it is important to remember that patients with typical HV lesions may progress to systemic forms with fatal outcomes, as previously reported[2]. In terms of disease progression, HMB only may result in fatal outcome within the first 5 years, sHV may progress later, and cases with episodes of both HV and HMB may also require a longer period for progression.

Our univariate analysis of patients demonstrated 2 risk factors: 1) onset age of over 9 years, and 2) the expression of BZLF-1 mRNA in the skin lesions (p<0.001 and p=0.003, respectively). In contrast, no prognostic correlation was observed for EBV-infected lymphocyte subsets, anti-EBV antibody titres, or EBV DNA load, although these parameters would be useful for a diagnostic test or

for monitoring of EBV^+ cell numbers.

There was a clear difference in mortality rate between age groups: the rate for patients under 9 years was 8.3%, while that for patients over 9 years was 83.3% ($p<0.001$). Because cHV with a favourable prognosis occurred in the first decade, we recalculated the mortality rates excluding cHV patients. The results still showed a significant difference between the under/over 9-years categories ($p=0.0041$). We, therefore, conclude that onset age may be a risk factor. This supports the findings of a previous report on CAEBV in which onset age over 8 years was one of the prognostic factors[10]. Our analyses indicate that the possibility of disease progression should be considered for patients over 9 years at onset.

In addition to late onset, BZLF-1 mRNA expression was closely related to more severe disease conditions and poorer prognosis. BZLF-1 is an immediate early gene product that induces EBV reactivation, with subsequent generation of the lytic cycle infection-associated viral antigens, which evoke CTL responses[16]. Although a previous report has described that BZLF-1 expression was associated with the proliferation of transformed lymphocytes[17], our observations indicate that fatal outcomes may be related to HPS and multi-organ failure mediated by host immune reactions, but not to the tumour burden evaluated by EBV DNA load or serum LD levels. Therefore, BZLF-1 may be important as both a pathogenic molecule that accounts for systemic symptoms and a prognostic marker.

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REFERENCES

1. Morizane S, Suzuki D, Tsuji K, *et al.* The role of CD4 and CD8 cytotoxic T lymphocytes in the formation of viral vesicles. *Br J Dermatol* 2005: **153**:981-6.
2. Iwatsuki K, Satoh M, Yamamoto T, *et al.* Pathogenic link between hydroa vacciniforme and Epstein-Barr virus-associated hematologic disorders. *Arch Dermatol* 2006: **142**:587-95.
3. Hirai Y, Yamamoto T, Kimura H, *et al.* Hydroa Vacciniforme is associated with increased numbers of Epstein-Barr Virus-infected γδT cells. *J Invest Dermatol* 2012: **132**:1401-1408.
4. Asada H, Saito-Katsuragi M, Niizeki H, *et al.* Mosquito salivary gland extracts induce EBV-infected NK cell oncogenesis via CD4 T cells in patients with hypersensitivity to mosquito bites. *J Invest Dermatol* 2005: **125**:956-61.
5. Tokura Y, Ishihara S, Tagawa S, *et al.* Hypersensitivity to mosquito bites as the primary clinical manifestation of a juvenile type of Epstein-Barr virus-associated natural killer cell leukemia/lymphoma. *J Am Acad Dermatol* 2001: **45**:569-78.
6. Magana M, Sangueza P, Gil-Beristain J, *et al.* Angiocentric cutaneous T-cell lymphoma of childhood (hydroa-like lymphoma): a distinctive type of cutaneous T-cell lymphoma. *J Am Acad Dermatol* 1998: **38**:574-9.
7. Cho KH, Lee SH, Kim CW, *et al.* Epstein-Barr virus-associated lymphoproliferative lesions presenting as a hydroa vacciniforme-like eruption: an analysis of six cases. *Br J Dermatol* 2004: **151**:372-80.
8. Rodriguez-Pinilla SM, Barrionuevo C, Garcia J, *et al.* EBV-associated cutaneous NK/T-cell lymphoma: review of a series of 14 cases from peru in children and young adults. *Am J Surg Pathol* 2010: **34**:1773-82.

9. Swerdlow, S.H., Campo, E., Harris, N.L., *et al.* WHO Classification of tumours of Haematopoietic and Lymphoid Tissues. *In: EBV-positive T-cell lymphoproliferative disorders of childhood* (L.Quintanilla-Martinez , H.Kimura, E.S. Jaffe, eds) Press; 2008 IARC, 278-80.

10. Kimura H, Ito Y, Kawabe S, *et al*. EBV-associated T/NK-cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases. *Blood* 2012.**119**: 673-86

11. Yamamoto T, Tsuji K, Suzuki D, *et al*. A novel, noninvasive diagnostic probe for hydroa vacciniforme and related disorders: detection of latency-associated Epstein-Barr virus transcripts in the crusts. *J Microbiol Methods* 2007: **68**: 403-7.

12. Yamamoto T, Iwatsuki K. Diversity of Epstein-Barr virus BamHI-A rightward transcripts and their expression patterns in lytic and latent infections. *J Med Microbiol.* 2012: **61**:1445-53

13. Weiss LM, Chen YY, Liu XF, Shibata D. (1991). Epstein-Barr virus and Hodgkin's disease. A correlative in situ hybridization and polymerase chain reaction study. *Am J Pathol* **139**:1259-65.

14. Iwatsuki K, Yamamoto T, Tsuji K, *et al*. A spectrum of clinical manifestations caused by host immune responses against Epstein-Barr Virus infections. *Acta Med Okayama*. 2004;**58**: 169-180.

15. Tanaka C, Hasegawa M, Fujimoto M, et al. Phenotypic analysis in a case of hydroa vacciniforme-like eruptions associated with chronic active Epstein-Barr virus disease of γδ T cells. *Br J Dermatol* 2012: **166**: 216-8.

16. E K Flemington, A E Goldfeld and S H Speck. Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein

synthesis. J Virol. 1991: **65**: 7073-7077

17. Fu Z, Cannon MJ. Functional analysis of the CD4(+) T-cell response to Epstein-Barr virus: T-cell-mediated activation of resting B cells and induction of viral BZLF1 expression. J Virol. 2000 : **74**: 6675-9

Figure legends

Fig. 1. Survival time of the 4 groups by the Kaplan-Meier method.

All patients with cHV survived during the observation period. Patients with cHV showed significantly better prognosis than patients with sHV or HMB only by log-rank test (p=0.016 and p=0.015), and rather good prognosis as compared with the other 3 groups (p=0.026) (a, b). There was no significant difference of the survival time between patients with only HV or HV-like eruptions and patients with HMB (p=0.286) (c).

Fig. 2. Onset age and mortality rates.

There was a clear difference in mortality rates: patients under 9 years had mortality of 8.3%, while those over 9 years had a mortality of 83.3% (p<0.001) (a). In the group of patients with cHV or sHV, patients over 9 years also showed a poor prognosis (p=0.0041) (b). Even when cHV patients with favorable outcome were excluded, the significant difference in mortality rates between patients under and over 9 years remained (p=0.0095) (c).

Fig. 3. BZLF-1 mRNA expression in disease types and prognosis.

No BZLF-1 mRNA expression was observed in the skin lesions of the 13 patients with cHV, while BZLF-1 mRNA was detected in 5 of the 15 (33.3%) of patients with sHV, HMB only, and HMB+HV (p=0.047) (a). The log rank test demonstrated that BZLF-1 mRNA$^+$ cases showed significantly worse prognosis than BZLF-1 mRNA$^-$ cases (p=0.012). Among the 25 patients who could be followed-up, the 10-year survival rate was significantly worse in the BZLF-1 mRNA$^+$ cases by log-rank test (26.7% vs 95%, p=0.003) (b).

Table 1. Criteria for classical and systemic HV, and HMB

	Classical HV	Systemic HV	HMB
Cutaneous lesion	vesiculopapular lesion	ulceronecrotic lesion	Swollen erythema or skin ulcer following mosquito bites, insect bites or vaccination
EBER$^+$cells	+	+	+
systemic symptoms * or Abnormality in routine laboratory findings**	−	+	+

* Systemic symptoms include high grade fever, lymphoadenopathy, and hepatosplenomegaly. **Laboratory abnormalities include hepatic damage, hematological findings suggestive of hemophagocytic syndrome and NK lymphocytosis (>30%).

Table 2. Clinical background and laboratory data of patients with HV and/or HMB

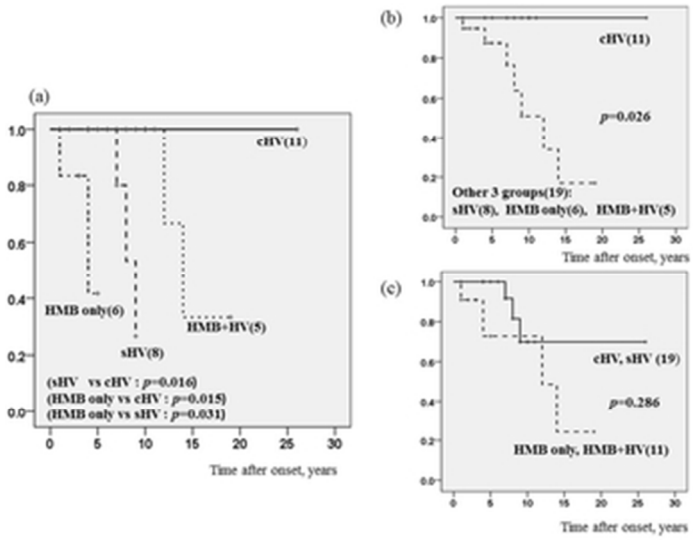
	cHV	sHV	HMB only	HMB+HV
cases (n=50)	n=23	n=12	n=9	n=6
sex (m:f)	13:10	5:7	6:3	2:4
onset age (median)	5	8	8	3.5
clinical symptoms (No.)				
fever	0/23	8/12	9/9	5/6
lymphoadenopathy	0/23	2/12	7/9	1/6
hepatosplenomegaly	0/23	4/12	3/9	4/6
aphthous stomatitis/gingivitis	6/23	5/12	0/9	0/6
conjunctivitis/iritis	4/23	1/12	0/9	0/6
NK lymphocytes (>30%) (No.)	0/9	2/7	6/7	6/6
γδT cell (≧5%) (No.)	7/8	3/3	0/1	N.D
followed-up cases (n=30)	n=11	n=8	n=6	n=5
follow up time (median)	1-26y(8y)	4-9y(7y)	1-5y(3y)	1-19(12y)
laboratory data(mean±SD)				
WBC (/μl)	7141.8±2685.8	5273.8±2343.9	5873.3±2169.8	5718.0±1759.0
Hb (g/dl)	12.7±0.7	11.8±1.8	12.4±1.5	13.3±1.3
Plt (10*4/μl)	28.0±6.3	20.5±8.2	13.0±5.6 *	27.0±6.9
LDH (U/l)	258.6±79.7	289.0±201.8	455.5±176.8 *	265.3±91.2
AST (U/l)	26.6±10.1	32.5±12.9	74.8±78.0	53.0±51.9
ALT (U/l)	19.4±12.7	31.9±21.2	117.0±145.0	53.5±65.8
NK lymphocytes (>30%) (No.)	0/8	2/6	6/6	5/5
γδT cell (≧5%) (No.)	4/5	3/3	0/1	N.D
EBV antibody titer (FA method) (median±SD)				
VCA IgG (titres)	120±97.4	80±432.0	40±1031.6	640±1210.1*
EA IgG (titres)	0±5.3	10±70.3	5±5.5	20± 27.9*
EBNA (titres)	15±101.9	15±25.6	15±71.1	10±8.4
EBV DNA load (PBMC) copies/μg DNA	97,200±222,332	56,140 ± 37,587	36,400 ± 58,812	51,500 ± 54,126
mortarity rates (%)	0/11(0)	3/8(37.5)	2/6(33.3)	2/5(40)
complications (No.)	-	HPS(1) myocarditis(2) gastrointestinal bleeding(2)	unkown	HPS(1)
Cases examined by immunostainig (n=19)	n=6	n=5	n=4	n=4
Dominant lymphocyte subset in skin lesion	CD3ε+CD56-(6/6)	CD3ε+CD56-(5/5)	CD3ε+CD56+(4/4)	HV(1) HMB(3) CD3ε+CD56- (1) (1) CD3ε+CD56+ (0) (2)

* means a significant difference from some other subgroups ($p<0.05$)

Table 3. Prognostic factors

Univariate analysis of factors related to the mortality of cHV, sHV, HMB only and HMB+HV			
Factor	alive	dead	*P* value
Onset (years, mean±SD)	5.6±3.1	12.6±8.6	0.077
Onset (≥9 y/<9 y, No.) total	1/22	5/2	<0.001
Onset (≥9 y/<9 y, No.) cHV+sHV	1/15	3/0	0.004
Onset (≥9 y/<9 y, No.) cases excluding cHV	1/11	5/2	0.0095
Onset (≥8 y/<8 y, No.) total	5/18	5/2	0.026
Man/Woman (No.)	11/12	2/4	0.663
Fever +/- (No.)	9/14	5/2	0.204
Splenomegaly +/- (No.)	2/20	3/4	0.075
Lymphoadenoma +/- (No.)	3/20	3/4	0.12
WBC (/μl, mean±SD)	5570.8±1968.7	6201.4±2016.9	0.85
Hgb (g/dl, mean±SD)	12.3±1.2	13.2±1.8	0.377
Plt (10*4/μl, mean±SD)	24.0±9.1	17.8±5.1	0.071
LDH (U/l, mean±SD)	299.4±128.8	344±254.2	0.694
AST (U/l, mean±SD)	43.2±45.4	36.5±33.2	0.232
ALT (U/l, mean±SD)	47.7±83.1	47.7±46.7	0.414
DNA load (copies/μg DNA mean±SD)	73435±155300	41780±36127	0.753
VCA IgG (titres, median±SD)	80±282.3	160±1331.8	0.226
VCA IgM (titres, median±SD)	10±5.1	5±5.5	0.932
EA IgG (titres, median±SD)	10±33.5	10±59.7	0.381
EA IgM (titres, median±SD)	0±2.2	0±4.1	0.7
EBNA (titres, median±SD)	10±69.9	20±63.4	0.678
BZLF1 mRNA +/- in skin lesion (No.)	1/21	4/2	0.003
BARTs mRNA +/- in PBMC (No.)	10/1	3/1	0.476
HSCT +/- (No.)	3/19	3/4	0.13
EBER *in situ* score (mean)	1.44	2	0.20
CD56+ infiltrating cells +/- (No.)	5/11	1/3	1.00
γδT cell in PBMC (%, mean±SD)	13.2±7.8	17.3	0.75
NK cell in PBMC (%, mean±SD)	30.2±25.2	27.2±22.4	0.86

Abbreviations: VCA, viral capsid antigen; EA, early antigen; EBNA, Epstein-Barr nuclear antigen; HSCT, hematopoietic stem cell transplantation; **P* value were obtained by use of either Fisher exact test or Mann–Whitney U test. Laboratory data were determined at the time of diagnosis.



All patients with cHV survived during the observation period. Patients with cHV showed significantly better prognosis than patients with sHV or HMB only by log-rank test (p=0.016 and p=0.015), and rather good prognosis as compared with the other 3 groups (p=0.026) (a, b). There was no significant difference of the survival time between patients with only HV or HV-like eruptions and patients with HMB (p=0.286) (c).
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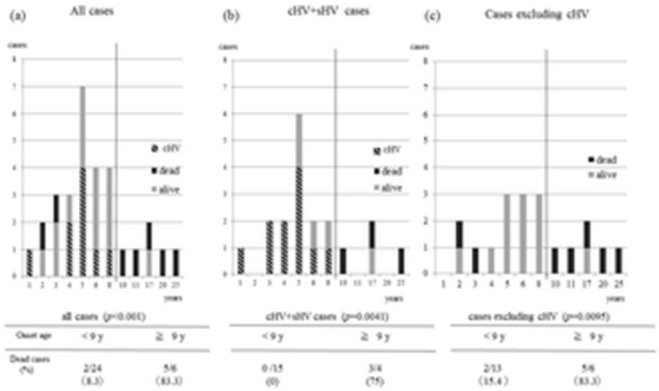
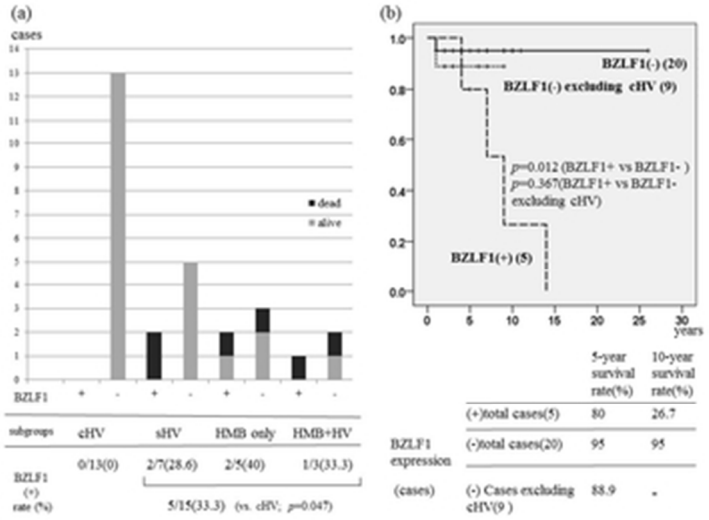


There was a clear difference in mortality rates: patients under 9 years had mortality of 8.3%, while those over 9 years had a mortality of 83.3% ($p<0.001$) (a). In the group of patients with cHV or sHV, patients over 9 years also showed a poor prognosis ($p=0.0041$) (b). Even when cHV patients with favorable outcome were excluded, the significant difference in mortality rates between patients under and over 9 years remained ($p=0.0095$) (c).
30x17mm (300 x 300 DPI)



No BZLF-1 mRNA expression was observed in the skin lesions of the 13 patients with cHV, while BZLF-1 mRNA was detected in 5 of the 15 (33.3%) of patients with sHV, HMB only, and HMB+HV (p=0.047) (a). The log rank test demonstrated that BZLF-1 mRNA+ cases showed significantly worse prognosis than BZLF-1 mRNA- cases (p=0.012). Among the 25 patients who could be followed-up, the 10-year survival rate was significantly worse in the BZLF-1 mRNA+ cases by log-rank test (26.7% vs 95%, p=0.003) (b).
30x22mm (300 x 300 DPI)

	Classical HV	Systemic HV	HMB
Cutaneous lesion	vesiculopapular lesion	ulceronecrotic lesion	Swollen erythema or skin ulcer following mosquito bites, insect bites or vaccination
EBER$^+$cells	+	+	+
systemic symptoms * and/or Abnormality in routine laboratory findings**	−	+	+

* Systemic symptoms include high grade fever, lymphoadenopathy, and hepatosplenomegaly. **Laboratory abnormalities include hepatic damage, hematological findings suggestive of hemophagocytic syndrome and NK lymphocytosis (>30%).

	cHV
cases (n=50)	n=23
sex (m:f)	13:10
onset age (median)	5
clinical symptoms (No.)	
fever	0/23
lymphoadenopathy	0/23
hepatosplenomegaly	0/23
aphthous stomatitis/gingivitis	6/23
conjunctivitis/iritis	4/23
NK lymphocytes (>30%) (No.)	0/9
γδT cell (≧5%) (No.)	7/8
followed-up cases (n=30)	n=11
follow up time (median)	1-26y(8y)
laboratory data(mean±SD)	
WBC (/μl)	7141.8±2685.8
Hb (g/dl)	12.7±0.7
Plt (10*4/μl)	28.0±6.3
LDH (U/l)	258.6±79.7
AST (U/l)	26.6±10.1
ALT (U/l)	19.4±12.7
NK lymphocytes (>30%) (No.)	0/8
γδT cell (≧5%) (No.)	4/5
EBV antibody titer (FA method) (median±SD)	
VCA IgG (titres)	120±97.4
EA IgG (titres)	0±5.3
EBNA (titres)	15±101.9
EBV DNA load (PBMC) copies/μg DNA	97,200±222,332
mortarity rates (%)	0/11(0)
complications (No.)	-
Cases examined by immunostainig (n=19)	n=6
Dominant lymphocyte subset in skin lesion	CD3ε+CD56-(6/6)

* means a significant difference from some other subgroups (p <0.05)

sHV	HMB only	HMB+HV
n=12	n=9	n=6
5:7	6:3	2:4
8	8	3.5
8/12	9/9	5/6
2/12	7/9	1/6
4/12	3/9	4/6
5/12	0/9	0/6
1/12	0/9	0/6
2/7	6/7	6/6
3/3	0/1	N.D
n=8	n=6	n=5
4-9y(7y)	1-5y(3y)	1-19(12y)
5273.8±2343.9	5873.3±2169.8	5718.0±1759.0
11.8±1.8	12.4±1.5	13.3±1.3
20.5±8.2	13.0±5.6 *	27.0±6.9
289.0±201.8	455.5±176.8 *	265.3±91.2
32.5±12.9	74.8±78.0	53.0±51.9
31.9±21.2	117.0±145.0	53.5±65.8
2/6	6/6	5/5
3/3	0/1	N.D
80±432.0	40±1031.6	640±1210.1*
10±70.3	5±5.5	20± 27.9*
15±25.6	15±71.1	10±8.4
56,140 ± 37,587	36,400 ± 58,812	51,500 ± 54,126
3/8(37.5)	2/6(33.3)	2/5(40)
HPS(1) myocarditis(2) gastrointestinal bleeding(2)	unkown	HPS(1)
n=5	n=4	n=4
CD3ε+CD56-(5/5)	CD3ε+CD56+(4/4)	HV(1) HMB(3) CD3ε+CD56- (1) (1) CD3ε+CD56+ (0) (2)

Univariate analysis of factors related to the mortality of cHV, sHV, HMB only and H

Factor	alive	dead
Onset (years, mean±SD)	5.6±3.1	12.6±8.6
Onset (≥9 y/<9 y, No.) total	1/22	5/2
Onset (≥9 y/<9 y, No.) cHV+sHV	1/15	3/0
Onset (≥9 y/<9 y, No.) cases excluding cHV	1/11	5/2
Onset (≥8 y/<8 y, No.) total	5/18	5/2
Man/Woman (No.)	11/12	2/4
Fever +/- (No.)	9/14	5/2
Splenomegaly +/- (No.)	2/20	3/4
Lymphoadenoma +/- (No.)	3/20	3/4
WBC (/μl, mean±SD)	5570.8±1968.7	6201.4±2016.9
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BZLF1 mRNA +/- in skin lesion (No.)	1/21	4/2
BARTs mRNA +/- in PBMC (No.)	10/1	3/1
HSCT +/- (No.)	3/19	3/4
EBER *in situ* score (mean)	1.44	2
CD56+ infiltrating cells +/- (No.)	5/11	1/3
γδT cell in PBMC (%, mean±SD)	13.2±7.8	17.3
NK cell in PBMC (%, mean±SD)	30.2±25.2	27.2±22.4

Abbreviations: VCA, viral capsid antigen; EA, early antigen; EBNA, Epstein-Barr nuclear antigen; HSC transplantation; *P value were obtained by use of either Fisher exact test or Mann−Whitney U test. Laborat time of diagnosis.

MB+HV
P value
0.077
<0.001
0.004
0.0095
0.026
0.663
0.204
0.075
0.12
0.85
0.377
0.071
0.694
0.232
0.414
0.753
0.226
0.932
0.381
0.7
0.678
0.003
0.476
0.13
0.20
1.00
0.75
0.86

CT, hematopoietic stem cell
ory data were determined at the