



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

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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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1 Conflicts of interest: None declared.

2 Abbreviations: EBV : Epstein-Barr virus, HV: hydroa vacciniforme, HMB:

3 hypersensitivity to mosquito bites, LPDs :lymphoproliferative disorders, PBMCs :

4 peripheral blood mononuclear cells, EBER :EBV-encoded small nuclear RNA, CTLs :

5 cytotoxic T lymphocytes, cHV : classical HV, sHV :systemic HV, CAEBV :chronic

6 active EBV infection, HLH :haemophagocytic lymphohistiocytosis, PCR : polymerase

7 chain reaction, BMRF1:BamHI M region, qRT: quantitative reverse transcriptase,

8 HSCT: hematopoietic stem cell transplantation, WBC :white blood cell count, Hb :

9 hemoglobin, AST :aspartate aminotransferase, ALT : alanine aminotransferase, LD :

10 lactate dehydrogenase, VCA : viral capsid antigen, EBNA : EBV nuclear antigen, EA :

11 early antigen

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1 What’s already known about this topic?

2 Epstein-Barr virus-associated T/NK lymphoproliferative disorders are a group of

3 diseases that include classical and systemic hydroa vacciniforme (HV), and

4 hypersensitivity to mosquito bites (HMB).

5 Patients with systemic HV and HMB usually present with fever, liver damage and

6 hematological abnormalities, and often have a fatal outcome.

7

8 What does this study add?

9 Our patients with classical HV showed a favourable prognosis, while approximately

10 one-third of the patients with systemic HV or HMB died over in the 10-year follow-up.

11 Late onset over 9 years of age and an EBV reactivation signal BZLF-1 mRNA

12 expression were both related to more severe phenotypes of the disease, and a poor

13 prognosis.

Abstract

Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative disorders are a group of diseases that include classical and systemic hydra vacciniiforme (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.

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1 **Introduction**

2 Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative disorders (LPDs) are a group of

3 diseases that include hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB).

4 EBV-associated HV lesions contain a number of EBV-encoded small nuclear RNA (EBER)⁺ T cells,

5 together with larger numbers of EBER⁺ cytotoxic T lymphocytes (CTLs); meanwhile, NK cells are

6 absent or occur at background levels in such lesions^{1,2}. Although there are no systemic symptoms or

7 abnormalities in the routine laboratory tests of classical HV (cHV) patients, EBV DNA load and

8 EBV⁺ $\gamma\delta$ T cells are increased in the peripheral blood mononuclear cells (PBMCs)³. In contrast,

9 patients with HV-like ulcerative cutaneous eruptions often present with systemic symptoms such as

10 fever, hepatic damage and lymphadenopathy (systemic HV: sHV), and show dense inflammatory cell

11 infiltrates that reach the subcutaneous tissue. As reported previously, patients with cHV may

12 progress to sHV in the clinical course².

13 HMB is an EBV-associated T/NK LPD characterized by intense local skin reactions and

14 systemic symptoms, including high fever, lymphadenopathy, hepatosplenomegaly, and

15 hemophagocytic syndrome⁴. These clinical symptoms can be induced by mosquito bites, other insect

16 bites or vaccination. Patients with HMB usually have EBV⁺ NK cell lymphocytosis^{3,5}, and HV-like

17 eruptions may occur over the course of the disease. We previously examined differences in cellular

18 events between HMB and HV-like eruptions³. Our results indicated that many CD56⁺ NK cells and

19 T-cells are present in the subcutaneous infiltrates in HMB, but no CD56⁺ NK cells occur in HV

20 dermal infiltrates.

21 Unlike cHV, both sHV and HMB have been reported in Asian and Latin American countries⁶⁻⁸.

22 The nomenclature of EBV-associated T/NK LPDs, however, has been controversial. Patients with

23 sHV in the present study may be synonymous with HV-like lymphoma in WHO classification⁹, and

24 be overlapped with chronic active EBV infection (CAEBV) and EBV-associated haemophagocytic

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

3 Although no prognostic markers have been elucidated, previous reports of CAEBV indicate that
4 patients with the EBV⁺ T-cell-predominant type have a poor prognosis compared to those with EBV⁺
5 NK cell-predominant type, and that late onset may be a risk factor¹⁰. In the present study, we
6 attempted to clarify cellular and molecular markers related to the prognosis of cutaneous
7 EBV-associated T/NK LPDs in a series of patients with HV and HMB, and to verify the validity of
8 diagnostic criteria to distinguish benign from malignant types for the purposes of prognosis.

1 **Materials and Methods**

2 **Patients**

3 Fifty patients were categorized into 4 groups: cHV (23 cases), sHV (12 cases), HMB only (9 cases),
4 and HMB+HV (6 cases), according to the criteria in Table 1. Briefly, patients with cHV presented
5 with lesions defined as vesiculopapules on sun-exposed areas without any systemic symptoms or
6 abnormalities in routine laboratory test results. Patients with sHV presented with HV-like eruptions
7 associated with systemic symptoms such as fever and lymphadenopathy and/or abnormalities in
8 routine blood examinations at diagnosis. In our series, 1 of 12 patients with sHV initially had skin
9 symptoms without systemic symptoms, although the patient had an increased percentage of NK cells
10 (>30% of lymphocytes) in the blood test. HMB is defined as an intense skin response to mosquito
11 bites, insect bites or vaccination associated with systemic symptoms and/or abnormalities in routine
12 blood tests.

13 Skin biopsy materials, crusts and blood samples were obtained for diagnosis, and used for *in situ*
14 hybridization with the EBV-encoded small RNA (EBER) and quantitative reverse transcriptase-
15 polymerase reaction (qRT-PCR) to detect EBV infection in skin lesions. This study was approved by
16 the ethical board committee (the institutional review board of Okayama University Hospital (No.
17 419, 2011) in accordance with the 1975 Declaration of Helsinki.

19 **Assay for EBV DNA load in PBMCs**

20 DNA was extracted from 1×10^6 PBMCs using a QIAamp™ Blood Kit (Qiagen; Netherlands), and
21 the polymerase chain reaction (PCR) amplification was performed using QuantiTect™ Probe PCR
22 (Qiagen; Netherlands) by Roche Light cycler (Roche; Switzerland). The PCR primers for this assay
23 were selected in the BamHI M region (BMRF1). The upstream and downstream primer sequences
24 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 °C for 15 minutes, followed by 50 cycles consisting of 95 °C for 15 seconds, 56 °C for 20 seconds, and 72 °C 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 °C for 45 seconds, 64 °C for 30 seconds, and 72 °C for 1 minute. BZLF1-specific inner primers: sense, 5'-TCCCAGTCTCCGACATAACCCA-3'; and antisense, 5'-AGCAGCGACCTCACGGTAGT-3'; amplification involved 28 cycles consisting of 94 °C for 45 seconds, 58 °C for 30 seconds, and 72 °C for 1 minute. Amplification 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 previously¹³. 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

Deparaffinized 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 previously¹⁴.

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 elsewhere³ (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 $\gamma\delta$ T 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

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

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

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

13 **Blood test results related to each group**

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

21 Lymphocyte subsets in PBMCs, taken together with the additional data described in our previous
22 research³, revealed that 10 of 11 patients (90.9%) with HV-like cutaneous lesions, whether in cHV or
23 sHV, showed elevated $\gamma\delta\text{T}$ cell percentages of over 5% (range: 2.18% to 25%), while 12 of 13

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

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

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

12 **Histopathologic examinations**

13 The percentages of EBER⁺ cells varied even though in the same disease group, ranging from 1% to
14 25% of the infiltrating mononuclear cells in all but 2 of 21 patients. In the 2 exceptional cases, more
15 than 25% EBER⁺ cells were observed in the infiltrates in one sHV and one cHV patients. No
16 difference was observed in the number of EBER⁺ cells among the cHV, sHV, and HMB only groups.
17 The number of samples in the HMB+HV group was insufficient for statistical analysis.

18 In agreement with our previous report³, CD3 ϵ ⁺ and CD56⁺ T-cells were predominantly infiltrating
19 in HV lesions, without correlation to the severity of the cutaneous lesions. In HMB lesions, however,
20 in addition to CD3 ϵ ⁺ and CD56⁺ T-cells, many CD56⁺ cells suggestive of NK cells were present in 6
21 of the 7 cases examined. The numbers of reactive T or NK cells negative for EBER were usually
22 larger than the numbers of EBER⁺ cells.

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1 **Onset age as a prognostic factor**

2 We also evaluated prognostic factors by univariate analysis (Table 3). We analysed the 30 cases that
3 were available for follow-up, which consisted of 11 cHV cases, 8 sHV cases, 6 HMB only cases and
4 5 HMB+HV cases. Onset age over 9 years was significantly correlated with mortality by univariate
5 analysis ($p<0.001$), and this association was stronger than that between mortality and onset age over
6 8 years ($p=0.026$). No significant correlation was observed regarding gender, clinical symptoms such
7 as fever, splenomegaly, or lymphadenopathy, routine blood test results or HSCT treatment.

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

14 **BZLF-1 as a molecular indicator of poor prognosis**

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

20 Among EBV-related molecules, the expression of EBV-encoded BZLF-1 in the skin lesions was
21 statistically correlated to a poor prognosis ($p=0.003$): 4 of 5 (80%) BZLF1 mRNA⁺ patients, and 2 of
22 23 (8.7%) BZLF-1 mRNA⁻ patients died during follow-up, respectively (Fig. 3). Among the 25
23 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 $\gamma\delta$ T 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 reported². 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

1 for monitoring of EBV⁺ cell numbers.

2 There was a clear difference in mortality rate between age groups: the rate for patients under 9
3 years was 8.3%, while that for patients over 9 years was 83.3% ($p<0.001$). Because cHV with a
4 favourable prognosis occurred in the first decade, we recalculated the mortality rates excluding cHV
5 patients. The results still showed a significant difference between the under/over 9-years categories
6 ($p=0.0041$). We, therefore, conclude that onset age may be a risk factor. This supports the findings of
7 a previous report on CAEBV in which onset age over 8 years was one of the prognostic factors¹⁰.
8 Our analyses indicate that the possibility of disease progression should be considered for patients
9 over 9 years at onset.

10 In addition to late onset, BZLF-1 mRNA expression was closely related to more severe disease
11 conditions and poorer prognosis. BZLF-1 is an immediate early gene product that induces EBV
12 reactivation, with subsequent generation of the lytic cycle infection-associated viral antigens, which
13 evoke CTL responses¹⁶. Although a previous report has described that BZLF-1 expression was
14 associated with the proliferation of transformed lymphocytes¹⁷, our observations indicate that fatal
15 outcomes may be related to HPS and multi-organ failure mediated by host immune reactions, but not
16 to the tumour burden evaluated by EBV DNA load or serum LD levels. Therefore, BZLF-1 may be
17 important as both a pathogenic molecule that accounts for systemic symptoms and a prognostic
18 marker.

19
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For Peer Review

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, lymphadenopathy, 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

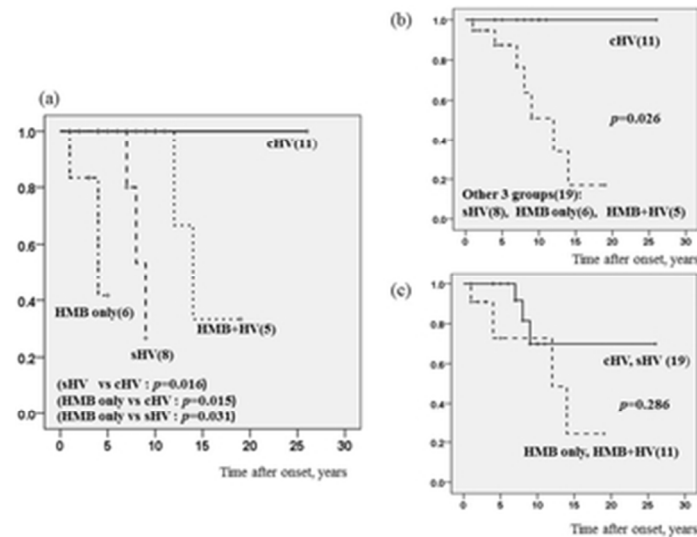
	cHV	sHV	HMB only	HMB+HV
cases (n=50)	n=23	n=12	n=9	n=6
sex (mf)	13:10	5:7	6:3	2:4
onset age (median)	5	8	8	3.5
clinical symptoms (No.)				
fever	0/23	8/12	9/9	5/6
lymphadenopathy	0/23	2/12	7/9	1/6
hepatosplenomegaly	0/23	4/12	3/9	4/6
aphthous stomatitis/gingivitis	6/23	5/12	0/9	0/6
conjunctivitis/iritis	4/23	1/12	0/9	0/6
NK lymphocytes (>30%) (No.)	0/9	2/7	6/7	6/6
$\gamma\delta$ T cell ($\geq 5\%$) (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 $\pm$ SD)				
WBC (/ μ l)	7141.8 $\pm$ 2685.8	5273.8 $\pm$ 2343.9	5873.3 $\pm$ 2169.8	5718.0 $\pm$ 1759.0
Hb (g/dl)	12.7 $\pm$ 0.7	11.8 $\pm$ 1.8	12.4 $\pm$ 1.5	13.3 $\pm$ 1.3
Plt (10 ⁴ / μ l)	28.0 $\pm$ 6.3	20.5 $\pm$ 8.2	13.0 $\pm$ 5.6 *	27.0 $\pm$ 6.9
LDH (U/l)	258.6 $\pm$ 79.7	289.0 $\pm$ 201.8	455.5 $\pm$ 176.8 *	265.3 $\pm$ 91.2
AST (U/l)	26.6 $\pm$ 10.1	32.5 $\pm$ 12.9	74.8 $\pm$ 78.0	53.0 $\pm$ 51.9
ALT (U/l)	19.4 $\pm$ 12.7	31.9 $\pm$ 21.2	117.0 $\pm$ 145.0	53.5 $\pm$ 65.8
NK lymphocytes (>30%) (No.)	0/8	2/6	6/6	5/5
$\gamma\delta$ T cell ($\geq 5\%$) (No.)	4/5	3/3	0/1	N.D
EBV antibody titer (FA method) (median $\pm$ SD)				
VCA IgG (titres)	120 $\pm$ 97.4	80 $\pm$ 432.0	40 $\pm$ 1031.6	640 $\pm$ 1210.1*
EA IgG (titres)	0 $\pm$ 5.3	10 $\pm$ 70.3	5 $\pm$ 5.5	20 $\pm$ 27.9*
EBNA (titres)	15 $\pm$ 101.9	15 $\pm$ 25.6	15 $\pm$ 71.1	10 $\pm$ 8.4
EBV DNA load (PBMC) copies/ μ g DNA	97,200 $\pm$ 222,332	56,140 $\pm$ 37,587	36,400 $\pm$ 58,812	51,500 $\pm$ 54,126
mortality rates (%)	0/11(0)	3/8(37.5)	2/6(33.3)	2/5(40)
complications (No.)	-	HPS(1) myocarditis(2) gastrointestinal bleeding(2)	unknown	HPS(1)
Cases examined by immunostaining (n=19)	n=6	n=5	n=4	n=4
Dominant lymphocyte subset in skin lesion	CD3e+CD56-(6/6)	CD3e+CD56-(5/5)	CD3e+CD56-(4/4)	HV(1) HMB(3) CD3e+CD56- (1) (1) CD3e+CD56+ (0) (2)

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

1 **Table 3. Prognostic factors**

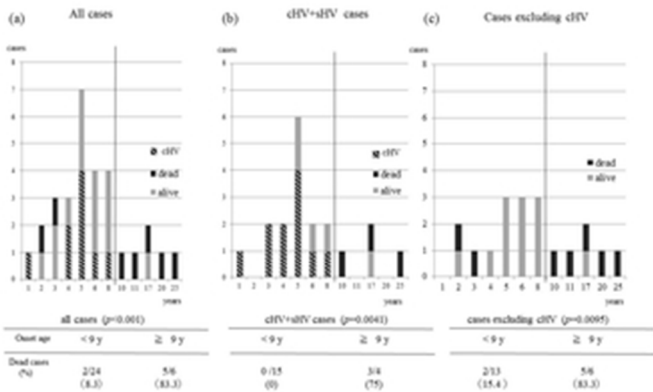
Univariate analysis of factors related to the mortality of cHV, sHV, HMB only and HMB+HV			
Factor	alive	dead	<i>P</i> value
Onset (years, mean±SD)	5.6±3.1	12.6±8.6	0.077
Onset (≥9 y/<9 y, No.) total	1/22	5/2	<0.001
Onset (≥9 y/<9 y, No.) cHV+sHV	1/15	3/0	0.004
Onset (≥9 y/<9 y, No.) cases excluding 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
Lymphadenoma +/- (No.)	3/20	3/4	0.12
WBC (/ μ l, 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 / μ l, 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/ μ g 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 <i>in situ</i> score (mean)	1.44	2	0.20
CD56+ infiltrating cells +/- (No.)	5/11	1/3	1.00
γ δT 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; * <i>P</i> value were obtained by use of either Fisher exact test or Mann-Whitney U test. Laboratory data were determined at the time of diagnosis.			

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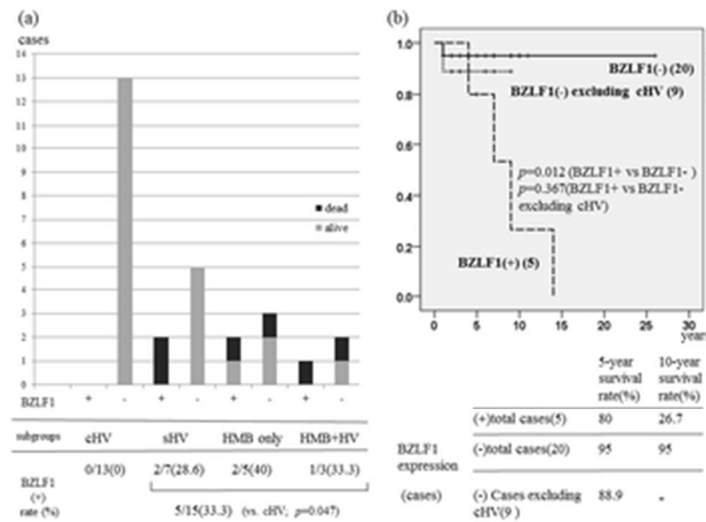
All patients with cHV survived during the observation period. Patients with cHV showed significantly better prognosis than patients with sHV or HMB only by log-rank test ($p=0.016$ and $p=0.015$), and rather good prognosis as compared with the other 3 groups ($p=0.026$) (a, b). There was no significant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

30x22mm (300 x 300 DPI)



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, lymphadenopathy, 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
lymphadenopathy	0/23
hepatosplenomegaly	0/23
aphthous stomatitis/gingivitis	6/23
conjunctivitis/iritis	4/23
NK lymphocytes (>30%) (No.)	0/9
$\gamma\delta$ T cell ($\geq 5\%$) (No.)	7/8
followed-up cases (n=30)	n=11
follow up time (median)	1-26y(8y)
laboratory data(mean $\pm$ SD)	
WBC (/ μ l)	7141.8 $\pm$ 2685.8
Hb (g/dl)	12.7 $\pm$ 0.7
Plt (10^4 / μ l)	28.0 $\pm$ 6.3
LDH (U/l)	258.6 $\pm$ 79.7
AST (U/l)	26.6 $\pm$ 10.1
ALT (U/l)	19.4 $\pm$ 12.7
NK lymphocytes (>30%) (No.)	0/8
$\gamma\delta$ T cell ($\geq 5\%$) (No.)	4/5
EBV antibody titer (FA method) (median $\pm$ SD)	
VCA IgG (titres)	120 $\pm$ 97.4
EA IgG (titres)	0 $\pm$ 5.3
EBNA (titres)	15 $\pm$ 101.9
EBV DNA load (PBMC) copies/ μ g DNA	97,200 $\pm$ 222,332
mortality rates (%)	0/11(0)
complications (No.)	-
Cases examined by immunostainig (n=19)	n=6
Dominant lymphocyte subset in skin lesion	CD3 ϵ +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)	unkown	HPS(1)
gastrointestinal bleeding(2)		
n=5	n=4	n=4
		HV(1) HMB(3)
CD3ε+CD56-(5/5)	CD3ε+CD56+(4/4)	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
Lymphadenoma +/- (No.)	3/20	3/4
WBC (μl, mean±SD)	5570.8±1968.7	6201.4±2016.9
Hgb (g/dl, mean±SD)	12.3±1.2	13.2±1.8
Plt (10 ⁴ /μl, mean±SD)	24.0±9.1	17.8±5.1
LDH (U/l, mean±SD)	299.4±128.8	344±254.2
AST (U/l, mean±SD)	43.2±45.4	36.5±33.2
ALT (U/l, mean±SD)	47.7±83.1	47.7±46.7
DNA load (copies/μg DNA mean±SD)	73435±155300	41780±36127
VCA IgG (titres, median±SD)	80±282.3	160±1331.8
VCA IgM (titres, median±SD)	10±5.1	5±5.5
EA IgG (titres, median±SD)	10±33.5	10±59.7
EA IgM (titres, median±SD)	0±2.2	0±4.1
EBNA (titres, median±SD)	10±69.9	20±63.4
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 <i>in situ</i> 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. Laboratory 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