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Reappraisal of EBV in diffuse large B-cell lymphoma (DLBCL): comparative analysis between EBV-positive and -negative DLBCL with EBV-positive bystander cells

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Running title: DLBCL with EBV+ bystander cells

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Conflict of interest

None of the authors have a conflict of interest to report.

Abstract

Aims: Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL), not otherwise specified (NOS) is defined as monoclonal EBV^+ B-cell proliferation affecting patients without any known immunosuppression. Non-neoplastic EBV^+ cells proliferating in or adjacent to EBV^- DLBCL were reported recently, but their clinical significance is unclear. Thus, we investigated the prognostic impact of EBV^+ cells in DLBCL.

Methods and Results: We compared the clinico-pathological characteristics of 30 EBV^+ DLBCL patients and 29 and 604 EBV^- DLBCL patients with and without EBV^+ bystander cells (median age of onset 71, 67, and 62 years, respectively). Both of EBV^+ DLBCL patients and EBV^- DLBCL patients with EBV^+ bystander cells tended to have high and high-intermediate International Prognostic Index scores (60% and 59%, respectively), compared with only 46% of EBV^- DLBCL patients without EBV^+ bystander cells. EBV^- DLBCL patients with EBV^+ bystander cells exhibited a significantly higher incidence of lung involvement than those without EBV^+ bystander cells (10% vs. 2%, $P<0.05$). Furthermore, EBV^+ DLBCL patients and EBV^- DLBCL patients with EBV^+ bystander cells had poorer prognosis than patients without any detectable EBV^+ cells (median overall survival [OS] 100 and 40 months vs. not

reached, P<0.01). Notably, cases of EBV^+ DLBCL and EBV^- DLBCL with EBV^+ bystander cells treated with rituximab demonstrated overlapping survival curves (OS, P=0.77, progression-free survival, P=1.0).

Conclusions: EBV^- DLBCL with bystander EBV^+ cells has similar clinical characteristics as EBV^+ DLBCL. DLBCL with EBV^+ bystander cells may be related to both age and microenvironment-related immunological deterioration.

Keywords: Epstein-Barr virus, diffuse large B-cell lymphoma, senescence, bystander cells

Introduction

Epstein-Barr virus (EBV), a member of the human herpes virus family, infects >90% of humans in a chronic asymptomatic manner.[1,2] This infection mostly persists for life, but is well known to be associated with a number of malignancies, including various types of malignant lymphomas and carcinomas.[3,4] EBV-driven B-cell lymphoproliferative disorders (LPDs), such as post-transplantation and methotrexate

(MTX)-associated LPDs, occur in immunosuppressed patients with primary immunodeficiency, HIV infection or iatrogenic immunosuppression.[4,5]

EBV$^+$ diffuse large B-cell lymphoma (DLBCL) of the elderly is a provisional entity in the 2008 WHO classification[4] defined as monoclonal EBV$^+$ B-cell proliferation affecting patients over 50 years of age without any known immunodeficiency.[6-10] EBV$^+$ DLBCL was postulated to be associated with immune senescence occurring in parallel with aging but is now recognized among younger patients.[11,12] This new information has led to a substitution of the modifier "elderly" with "not otherwise specified" in the revised 2016 WHO classification.[13]

EBV$^+$ DLBCL has been reported to be more aggressive than EBV$^-$ DLBCL in regards to its biological behavior, especially in the elderly,[7-9,11] but remains controversial in the English literature. Some reports have indicated that the EBV association with DLBCL has little prognostic significance.[12,14,15] These contradicting results may be due to a difference in objective cohorts, including variable cut-off values and the geographic heterogeneity of EBV infection. Therefore, we questioned whether the harbouring of EBV in tumour or bystander cells would impact prognosis. Klapper's and Quintanilla-Martinez's groups reported an unusual EBV expression

pattern in which a small number of non-neoplastic EBV$^+$ B-cells proliferate in or adjacent to EBV$^-$ DLBCL tumour cells.[16-18] The former asserted that this is the result of local immune escape by non-neoplastic B-cells in the lymphoma microenvironment,[16] but little is known about the clinical implications. Here, we assembled pathological and clinical data pooled from two cohorts, the Adult Lymphoma Treatment Study Group (ATLSG) and Fujita Health University Hospital (FHUH), yielding 30 cases of EBV$^+$ DLBCL and 29 cases of EBV$^-$ DLBCL with bystander EBV$^+$ B-cell proliferation, and a control group of DLBCL patients without any EBV$^+$ cells (n=604) to perform a clinico-pathological comparison.

Materials and methods

Patient samples

The institutional review board of Nagoya University approved the study protocol. This study included 526 DLBCL patients sequentially diagnosed in the ALTSG case files in Japan from January 1998 to August 2004 and 141 DLBCL patients diagnosed at Fujita Health University Hospital before February 2012.

Histological and immunohistochemical staining

Tissue samples were fixed in 10% formalin and embedded in paraffin, followed by staining of 5-μm-thick sections with haematoxylin and eosin. All cases were reviewed by two pathologists (A.O. and S.N.) according to the diagnostic criteria of the WHO classification.[5,13]

Formalin-fixed paraffin sections were subjected to immunoperoxidase studies using an avidin-biotin peroxidase complex method. Staining was performed on an automated immunostainer (Ventana Medical Systems and/or Leica Biosystems). The following monoclonal antibodies were used after antigen retrieval following microwave oven heating: anti-CD3, CD10, L26/CD20, Ber-H2/CD30, CD79a, BCL-2, BCL-6, MUM1.

In situ hybridization

The presence of EBV small ribonucleic acids was examined by in situ hybridization using EBER oligonucleotides on formalin-fixed, paraffin-embedded sections as described previously.[18] Cases were considered EBER-positive if nuclear expression

of EBER was observed in 80% or more of the malignant cells. Immunohistochemical double-staining for EBER-ISH and CD3 or CD79a was performed in selected cases. We also evaluated the presence of bystander EBER+ cells in the background of tumour cells.

Statistical analysis

Correlations between groups were determined using the Kruskal-Wallis test, χ^2 test and the Fisher exact test. Survival curves were estimated by Kaplan-Meier method and compared using the log-rank test. Overall survival (OS) was defined as the time from initial diagnosis to the date of death from any cause or last contact. Progression-free survival (PFS) was calculated from the date of diagnosis to the first day of disease progression, relapse, death as a result of any cause, or last date of follow-up. $P < 0.05$ was considered significant. All statistical analyses were performed using the STATA software package v.11 (Stata Corporation, College Station, Texas).

Results

Patient characteristics

We examined EBV harbouring in 526 ATLSG cases and 141 FHUH cases, finding EBV$^+$ tumour cells in 34 cases (5.1%).Three of these cases were excluded from the analysis due to pyothorax-associated lymphoma in two cases and MTX-associated LPD with rheumatoid arthritis in one case. We enrolled 30 of the remaining 31 cases (31/664, 4.7%), for which clinical information was available, as EBV$^+$ DLBCL according to the 2016 WHO classification. An additional 29 cases (29/664, 4.7%) had a small number of EBV$^+$ non-neoplastic B lymphocytes. These EBV$^+$ cells had a scattered distribution, were generally small or medium-sized, and easily judged as non-neoplastic bystander B-cells. Figure 1 summarizes the age distribution of the patients with DLBCL according to subtypes determined by the EBV$^+$ pattern. The percentage of patients with EBV$^+$ neoplastic cells increased in parallel with patient age, reaching 19% in patients over 81years of age. The ratio of DLBCL patients with EBV$^+$ bystander B-cells also increased with age, with a peak incidence (9.0%) in those aged 71-80 years.

The characteristics of 30 EBV$^+$ DLBCL patients and 29 and 604 EBV$^-$ DLBCL patients with and without EBV$^+$ bystander cells, respectively, are provided in Table 1. Compared to EBV$^-$ DLBCL patients without EBV$^+$ bystander cells, patients with EBV$^+$ DLBCL had an older distribution (median 71 years vs. 62 years ; P<0.01). Patients with EBV$^-$ DLBCL with EBV$^+$ bystander cells also had a tendency to be elderly, but no significant difference from those without EBV$^+$ bystander cells was found (median 67 years vs. 62 years; P=0.11). There were no significant differences in clinical variables, although both EBV$^+$ groups were more frequently categorized into high and high-intermediate International Prognostic Index (IPI) score groups (60% and 59% vs.46%; P=0.13). The total incidences of extranodal involvement was similar among the groups (23%,14%, and 22%) without any significant difference between EBV$^-$ DLBCL patients with and without EBV$^+$ bystander cells except for the lungs (10% vs.2%; P<0.05). Cases with lung involvement (n=3) were also found in EBV+ DLBCL group, especially posing their differential diagnostic problem from grade 3 lymphomatoid granulomatosis. However, all of them had a nodal disease with the other extranodal involvement, such as the GI tract, liver and bone marrow. Based on their systemic disease, our preferred diagnosis was EBV$^+$ DLBCL for them.

Histopathological characteristics

EBV$^+$ DLBCL presented with a large cell morphology composed of centroblasts and immunoblasts (Fig.2A). These large transformed cells were positive for B-cell markers (i.e.,CD20). Twelve cases (40%) had a polymorphic appearance with scattered HRS-like giant cells positive for CD30. All 11 cases fully examined by immunohistochemistry were positive for MUM1, and only one (9%) was categorized as germinal centre B-cell (GCB) type based on weak expression of CD10 according to the Hans Algorithm, meaning the remaining 10 were non-GCB type. A majority (>80%) of the tumour cells exhibited strong harbouring of EBV in their nuclei (Fig. 2B).

EBV$^-$ DLBCL cases with EBV$^+$ bystander cells were indistinguishable from ordinal EBV$^-$ DLBCL in regards to morphology , which comprised large CD20$^+$ tumour cells (Fig. 3A, B). Peri- and intra-tumoural lymphocytes were positive for EBV, generally small or medium-sized, and easily distinguished from neoplastic large cells, with consistent concordance between the two reviewers (A.O. and S.N.) (Fig. 3C). The number of EBV$^+$ cells ranged from a few to less than 5%. These EBV$^+$ background cells were positive for CD20 (Fig. 3B). The B-cell nature of these cells

was confirmed by double-staining using EBER-ISH and immunohistochemistry for CD79a in five cases. All eight cases fully examined by immunohistochemistry were positive for MUM1, 2 (25%) of which were categorized as GCB type based on CD10 expression.

Therapy and survival

Out of 29 evaluable cases of EBV$^+$ DLBCL, 26 patients were treated with combination chemotherapy including anthracycline, eight of whom also received rituximab. Additional radiotherapy was given in three patients. One patient did not receive any therapy and another received only a steroid drug because of poor performance status; both died of disease within a month of the diagnosis. Complete remission (CR) was achieved in 20 patients (77%), and 6 (23%) were refractory to chemotherapy (Table 2.). Out of the 22 evaluable cases of EBV$^-$ DLBCL with EBV$^+$ bystander cells, 19 patients were treated with combination chemotherapy including anthracycline, and six patients were additionally treated with rituximab. A total of 13 patients (68%) achieved CR with initial therapy, and the remaining 6 (32%) were refractory to chemotherapy (Table 2). One patient from each group was lost to follow-up. As a control group, 122 EBV$^-$ DLBCL patients without EBV$^+$ bystander cells from the

FHUH cohort, for which information on the treatment response was available, were also analyzed, containing 51 GCB and 65 non-GCB type tumours according to the Hans Algorithm.

Figure 4 shows the unadjusted OS and PFS curves for each of the three groups. Patients with EBV^+ DLBCL and EBV^- DLBCL with EBV^+ bystander cells had inferior survival compared to patients without any detectable EBV^+ cells (median OS 100 and 40 months, vs. not reached, $P<0.01$). In contrast to expectations, patients with EBV^- DLBCL with EBV^+ bystander cells had a poorer prognosis than EBV^+ DLBCL patients, but the difference was not significant (OS, $P = 0.14$; PFS, $P = 0.24$). Compared with the control group of EBV-undetectable DLBCL of non-GCB type (n=65), significant differences were also preserved with EBV+ neoplastic and bystander groups (OS and PFS, $P<0.01$) (Figure 5). Figure 6 shows the survival curves for cases treated with immunochemotherapy containing rituximab. The OS and PFS curves of EBV^+ DLBCL and EBV^- DLBCL with EBV^+ bystander cells overlapped ($P=0.77$ and 1.0, respectively), and were significantly inferior to those of EBV^- DLBCL without EBV^+ bystander cells ($P<0.01$).

Discussion

We performed a clinico-pathological analysis of DLBCL with different EBV$^+$ patterns, i.e., tumour or bystander cells harbouring EBV. The proportion of EBV$^+$ DLBCL patients in our series (4.7%) was lower than in previous studies from Asian countries (8.7%[7] and 9.0%[8]), but the tendency to affect elderly immunocompetent patients was present as originally asserted by our group.

In this study, we first addressed the clinicopathological features of EBV$^-$ DLBCL cases with EBV$^+$ bystander cells compared to EBV$^+$ DLBCL and EBV$^-$ DLBCL cases without EBV$^+$ bystander cells among Japanese patients. The prevalence of DLBCL with EBV$^+$ bystander cells (29/664, 4.4%) was equal to that of EBV$^+$ DLBCL (31/664, 4.7%) in our series. In addition, patients with EBV$^-$ DLBCL with EBV$^+$ bystander cells had a poor prognosis, with 33% (7/21) of patients having a lethal clinical course within 2 years after diagnosis even with multi-agent chemotherapy. Rituximab has been reported to improve the outcome of DLBCL patients, but has not demonstrated sufficient efficacy in EBV$^+$ DLBCL.[20-22] In our study, immunochemotherapy appeared to improve the prognosis of EBV$^-$ DLBCL patients with EBV$^+$ bystander cells more so than EBV$^+$ DLBCL, but the difference was not significant. In addition, the present EBV+ DLBCL group achieved CR in 77% of the

patients, which was similar to 82% reported by Nicolae A et al. among young cases with this disease.[11] These percentages appeared to be higher than that previously described by us without rituximab usage in 2007.[7] This issue should be clarified, though it was difficult to draw any definitive conclusions because of the paucity of cases enrolled in the present study.

EBV$^+$ bystander cells have been reported in some lymphomas, such as Hodgkin lymphoma and T cell lymphoma.[25-28] These bystander cells may represent an outgrowth of EBV$^+$ B-cells due to lymphoma-induced immunosuppression in the tumour microenvironment, but the clinical significance remains to be elucidated. In this study, 29 cases of EBV$^-$ DLBCL had a small number of bystander EBV$^+$ B-cells, which is the largest number documented in the English literature to date. Quintanilla-Martinez et al. reported an incidentally higher level of EBV$^+$ bystander cells in DLBCL patients in Mexico (7/136, 5.1%) than Germany (1/169, 0.6%). In their study, EBV$^+$ bystander cells were present in less than 20% of cells and consisted of small lymphocytes and large transformed cells.[18] In our study, the prevalence of DLBCL with EBV$^+$ bystander cells (4.4%) was in line with that of the Mexican cohort (5.1%). This may reflect the higher susceptibility of Asian and Mexican populations to EBV infection, as the prevalence of EBV$^+$ DLBCL is also epidemiologically higher in these populations than in Western countries.[7,8,14,18] EBV$^+$ DLBCL is postulated to be

associated with immune senescence as a part of systemic immunological deterioration in the aging process. On the other hand, EBV^+ bystander cells are representative of local immune dysregulation in the microenvironment of DLBCL lesions. However, the overlap in clinico-pathological findings and biological behaviour suggests that the phenomenon of lymphoma-associated immunodeficiency is highly influenced by systemic immune senescence in the host.

Recently, Klapper et al. reported four cases of EBV^- DLBCL in German patients with a small proportion (<10%) of EBV^+ B-cells with blastoid morphology[16]; three of them achieved CR and PR with immunochemotherapy. Their follow-up period ranged from 4 to 11 months. Because of this limited follow-up period, it was difficult to exactly predict the clinical outcome of survivors. Whether intra- and peri-tumoural EBV^+ bystander cells indicate a poor outcome was questioned. The 76-month follow-up of the longest surviving EBV^- DLBCL patient with EBV^+ bystander cells in our study clearly showed the poor prognosis of this disease. We speculate that microenvironment-related lymphoma-induced immune dysregulation and host immune senescence may cooperatively contribute to the existence of bystander EBV^+ cells and the unfavourable prognosis of patients, but further investigation is needed to clarify the precise mechanism.

EBER-ISH is mandatory for the diagnosis of EBV+ DLBCL, but the criteria for defining EBV+ DLBCL has varied in previous studies. Nicolae et al.[11] suggested using a cut-off value of >90% tumour cells. Hofscheiner et al.[18] also suggested using a cut-off of >80%, as whether EBV drives the tumour in cases with a low percentage of tumour cells is questionable. Some studies have applied a 10% threshold for the diagnosis of EBV+ DLBCL,[14,15,21,29,30] but this raises the question of whether these EBV+ cells are bystander non-neoplastic B-cells. Unfortunately, this issue has not been well addressed in past reports. The four cases of DLBCL with transformed EBV+ B-cells reported by Klapper et al. were revealed to be bystander cells, clonally different from lymphoma cells based on an immunoglobulin rearrangement test using microdissected specimens of the formalin-fixed paraffin-embedded tissue.[16] Thus, the controversial findings of EBV+ cells among DLBCL patients may be biased by the interpretation of EBV+ bystander cells, i.e., its inclusion in either EBV+ or EBV− groups. Our data suggest that EBV− DLBCL with EBV+ bystander cells is distinct from DLBCL without any EBV+ cells.

In summary, we highlighted the clinico-pathological characteristics of EBV− DLBCL with EBV+ bystander cells in a comparison with EBV+ DLBCL. DLBCL cases with a small number of EBV+ cells may be erroneously interpreted as EBV+ DLBCL. The recognition of this disorder will lead to more accurate classification and

appropriate treatments for patients. Further investigations with a large number of patients are expected in the future to clarify the clinico-pathological and biological significance of EBV^+ bystander cells.

Author contributions

A. Ohashi: collected data, reviewed the histopathology of all tumours, interpreted the results of immunostaining, analysed the data and wrote the paper.

S.Kato: proposed the study, analysed the data, reviewed and revised the manuscript.

A. Satou and T. Tsuzuki: joined the discussion for the consensus diagnosis with multi-headed microscope, and reviewed the manuscript.

A.Okamoto, Y. Inaguma, N. Emi and M. Okamoto : collected the data and interpreted the results of immunostaining and reviewed the data.

S. Nakamura: proposed the study, reviewed the histopathology of all tumours, interpreted the results of immunostaining, reviewed and revised the manuscript.

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Table 1. Patient characteristics at diagnosis

	EBV$^+$ DLBCL (n=30)	DLBCL with EBV$^+$ bystander cells (n=29)	DLBCL without EBV$^+$ bystander cells (n=604)	$P^\dagger$
Median age, years (range)	71 (40-91) **	67 (34-87)	62 (17-93)	<0.05
Sex, male/female	19 /11	19 /10	347 /257	0.58
Ann Arbor Stage, III/IV (%)	22/30 (73)	17/29(59)	321/603 (53)	0.087
B symptoms, presence (%)	10/30 (33)	12/29 (41)	174/600 (29)	0.34
LDH level, high (%)	23/30 (77)	18/29 (62)	348/601 (58)	0.11
Performance status, 2-4 (%)	12/30 (40)	10/29 (34)	187/603 (31)	0.55
Extranodal involvement (>1 site) (%)	7/30 (23)	4/29 (14)	135/603 (22)	0.54
Extranodal site (%)				

Skin	3/30(10)	1/29 (3)	36/603 (6)	
GI tract	6/30 (20)	5/29(17)	139/603 (23)	
Lung	3/30 (10)	3/29 (10)*	15/603 (2)	<0.05
Pleural effusion	5/30 (17)	1/29 (3)	33/603 (2)	
Bone marrow	4/30 (13)	3/29 (10)	92/603 (15)	
Thyroid gland	2/30 (7)	2/29 (7)	18/603 (3)	
Salivary gland	1/30 (3)	2/29 (7)	7/603 (1)	
IPI risk group				
High-intermediate/high (%)	18/30 (60)	17/29 (59)	274/601 (46)	0.13

GI, gastrointestinal; LDH, lactate dehydrogenase; IPI, International Prognostic Index.

†Comparing EBV+ DLBCL vs EBV$^-$ DLBCL with EBV$^+$ bystander cells vs EBV$^-$ DLBCL without EBV$^+$ bystander cells.

**P<0.01 for DLBCL without EBV$^+$ bystander cells

*P<0.05 for DLBCL without EBV$^+$ bystander cells

Table 2. Treatment strategies and responses

	EBV$^+$ DLBCL (n=29)	DLBCL with EBV$^+$ bystander cells (n=22)	DLBCL without EBV$^+$ bystander cells (n=122)
Treatment (%)			
CT with rituximab	8/29 (28%)	6/22 (27%)	116/122 (95%)
CT without rituximab	18/29 (62%)	13/22 (59%)	2/122 (1%)
CT with RT	3/29 (10%)	0/22 (0%)	0/122 (0%)
RT alone	0/29 (0%)	2/22 (9%)	0/122 (0%)
No therapy	2/29 (7%)	0/22 (0%)	3/122 (2%)
Response (%)			
CR	20/26 (77%)	13/19 (68%)	96/118 (81%)
PR	4/26 (15%)	3/19 (16%)	15/118 (13%)
SD/PD	2/26 (8%)	3/19 (16%)	7/118 (6%)

CT, chemotherapy; RT, radiotherapy; CR complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.

Figure legends

Figure 1. Age distribution of patients with EBV$^+$ cells.

(A) The number of DLBCL cases with or without EBV$^+$ cells. **(B)** The percentage of all DLBCL cases examined with EBV$^+$ cells. Closed bars indicate cases with EBV$^+$ lymphoma cells. Striped bars indicate cases with background cells positive for EBV. Blank bars indicate cases negative for EBV.

Figure 2. Histopathological features of EBV$^+$ DLBCL.

(A) Diffuse proliferation of large lymphoma cells with blastoid morphology (haematoxlyin and eosin). **(B)** EBER-ISH positive nuclei (in situ hybridization). Original magnification x400.

Figure 3. Histopathological features of EBV$^-$ DLBCL with bystander EBV$^+$ cells.

(A) Diffuse proliferation of large lymphoma cells admixed with scattered reactive cells in the background (haematoxlyin and eosin). **(B)** Lymphoma cells are strongly positive for CD20. Note the CD20-positivity of small or medium-sized B lymphocytes

in the background of the tumour cells (immunoperoxidase). **(C)** Although lymphoma cells are negative for EBV, some background cells harbour EBV based on EBER-ISH positivity (in situ hybridization).

Original magnification x400.

Figure 4. **Survival curves.**

(A) Overall survival (OS) and **(B)** progression-free survival (PFS) for EBV^+ DLBCL (n=28, dashed line), EBV^- DLBCL with EBV^+ bystander cells (n=21, solid line), and EBV^- DLBCL without EBV^+ bystander cells (n=122, dotted line). EBV^+ DLBCL and EBV^- DLBCL with EBV^+ bystander cells had poorer prognoses than EBV^- DLBCL without EBV^+ bystander cells (OS, PFS; $P<0.01$). Patients with EBV^- DLBCL with EBV^+ bystander cells had a poorer prognosis than those with EBV^+ DLBCL, but the differences in survival were not significant (OS, $P=0.14$; PFS,$P=0.24$).

Figure 5. **Survival curves.**

(A) Overall survival (OS) and **(B)** progression-free survival (PFS) for EBV^+ DLBCL (n=28, dashed line), EBV^- DLBCL with EBV^+ bystander cells (n=21, solid line), and EBV-undetectable DLBCL of non-GCB type (n=65, dotted line). EBV^+ DLBCL and EBV^- DLBCL with EBV^+ bystander cells had poorer prognoses than EBV-undetectable DLBCL of non-GCB type (OS, PFS; $P<0.01$).

Figure 6. Survival curves.

(A) Overall survival (OS) and **(B)** progression-free survival (PFS) for EBV^+ DLBCL (n=8, dashed line), EBV^- DLBCL with EBV^+ bystander cells (n=6, solid line), and EBV^- DLBCL without EBV^+ bystander cells (n=116, dotted line) treated with immunochemotherapy (rituximab and multi-agent chemotherapy). EBV^+ DLBCL and EBV^- DLBCL with EBV^+ bystander cells had poorer prognosis than EBV^- DLBCL without EBV^+ bystander cells (OS, PFS; P<0.01), and overlapped mutually (OS, P=0.77; PS, P=1.00).