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Prognostic significance of Epstein–Barr virus DNA detection in pretreatment serum in diffuse large B-cell lymphoma

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It is still a matter of debate whether detection of Epstein–Barr virus (EBV) DNA in pretreatment serum has clinical implications for diffuse large B-cell lymphoma. For this study, we measured EBV DNA load in pretreatment serum from 127 diffuse large B-cell lymphoma patients without any underlying immunodeficiency to evaluate its effects on clinical manifestations and prognosis. Anthracycline-based chemotherapy in combination with rituximab was given as initial therapy for 119 patients (94%). Epstein–Barr virus DNA was detected in 15 patients (12%), who were older ($P = 0.005$) and tended to be at a more advanced disease stage ($P = 0.053$). They showed significantly worse progression-free survival (PFS) and overall survival (OS) than other patients ($P < 0.001$ each). This effect remained significant ($P = 0.004$ and $P = 0.027$, respectively) after adjustment for age, lactate dehydrogenase, performance status, stage, and extranodal sites. The status of EBV-encoded small RNA *in situ* hybridization was known for 123 patients; 6 of 8 positive patients (75%) and 9 of 115 negative patients (8%) had detectable EBV DNA in pretreatment serum. While patients positive for EBV-encoded small RNA had significantly worse PFS and OS than negative patients ($P = 0.001$ and $P = 0.029$, respectively), EBV DNA detection in pretreatment serum was associated with poorer PFS and OS even for the 115 patients negative for EBV-encoded small RNA ($P < 0.001$ each). These findings suggest that EBV DNA detection in pretreatment serum may have an adverse prognostic impact for patients with diffuse large B-cell lymphoma.

Diffuse large B-cell lymphoma (DLBCL), the most common subtype of non-Hodgkin's lymphoma in adults, is a potentially curable disease. The past few decades have witnessed significant improvements in outcomes for DLBCL patients owing to the widespread use of rituximab in combination with chemotherapy,[1–7] and this treatment has been established as the current standard of care. However, outcomes remain unsatisfactory for a subset of patients, and this points to the importance of identifying those who are deemed likely to show a poor prognosis, because such patients may need a therapy different from what is considered the current standard. Although several prognostic factors have been reported on the basis of analyses of DLBCL patients treated after the introduction of rituximab,[7–10] the prognostication is not sufficiently thorough, thus warranting investigation of additional prognostic markers.

Epstein–Barr virus (EBV) is a human herpes virus that is associated with lymphoid malignancies such as Hodgkin's lymphoma, natural killer (NK)/T-cell lymphoma, and DLBCL. Detection of EBV DNA in pretreatment plasma or serum has been shown to help in predicting outcomes for Hodgkin's lymphoma,[11] and NK/T-cell lymphoma,[12] although data are sparse regarding its prognostic impact on DLBCL. With this background, we measured EBV DNA load in pretreament serum from DLBCL patients diagnosed at our hospital, and investigated its effects on clinical manifestations and prognosis in comparison with results of EBV-encoded small RNA *in situ* hybridization (EBER-ISH).

Patients and Methods

Patients. Eligibility criteria for enrolment in this study were: newly diagnosed with DLBCL at the Fujita Health University Hospital (Toyoake, Japan) between October 2007 and March 2012; age 18 years or older at DLBCL diagnosis; and no history of any type of lymphoma, HIV infection, or rheumatoid arthritis treated with methotrexate. Pretreatment serum was available for 127 of the 140 patients, and became the subject of the subsequent analyses. The diagnosis of DLBCL was based on the WHO classification,[13] and pathologic evaluations were carried out by two of the authors (Y.M. and S.N.). The immunophenotypes were classified into the germinal center B-cell-like (GCB) or non-GCB groups according to the published criteria.[14] The institutional review board of the

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Fujita Health University School of Medicine approved this study.

Quantification of EBV DNA load. DNA was extracted from cryopreserved pretreatment serum using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The EBV DNA load was quantified by means of a StepOne sequence detector (Applied Biosystems, Foster City, CA, USA). In brief, DNA samples were mixed with the primers and the TaqMan probe and amplified using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Primer sequences were based on the *BALF5* gene encoding the DNA polymerase of EBV (5′-CGGAAGCCC TCTGGACTTC-3′, 5′-CCCTGTTTATCCGATGGAATG-3′), and the probe sequence corresponded to a region between the primers (5′-TGTACACGCACGAGAAATGCGCC-3′). The EBV DNA load for each sample was calculated automatically by StepOne software version 2.2.2 (Applied Biosystems). A positive control of DNA from the Namalwa cell line and a negative control of water blanks were included in each analysis.

Epstein–Barr virus-encoded small RNA *in situ* hybridization. Biopsy specimens were fixed in formalin, dehydrated, embedded in paraffin, and sectioned with routine methods. The INFORM EBER probe (Ventana Medical Systems, Tucson, AZ, USA) was used for EBER-ISH. Slides were stained on an automated stainer (Benchmark XT; Ventana Medical Systems), and for visualization the ISH iView Blue Detection Kit (Ventana Medical Systems) with alkaline phosphatase and NBT/BCIP substrate was used, with Nuclear Fast Red (Ventana Medical Systems) for contrast. Specimens in which nuclear expression of EBER was observed in 20% or more of the malignant cells were considered EBER-positive.[15]

Statistical analysis. Distributions of characteristics of patients with or without EBV DNA detected in their pretreatment serum were compared by using the χ^2- test or Fisher's exact test for categorical variables, and the Wilcoxon rank-sum test for continuous variables. Overall survival (OS) was calculated from the date of diagnosis to death, and progression-free survival (PFS) from the date of diagnosis to progression or death, with patients without any event censored at the last follow-up. The probabilities of OS and PFS were estimated with the aid of the Kaplan–Meier estimator, and differences between groups were compared by using the log–rank test. The Cox proportional hazards regression model was constructed for univariate and multivariate analyses, and hazard ratios (HRs) were calculated together with the corresponding 95% confidence intervals (CIs). All statistical analyses were carried out using Easy R (EZR),[16] a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Table 1. Baseline characteristics of diffuse large B-cell lymphoma patients with or without Epstein–Barr virus (EBV) DNA detected in pretreatment serum

	EBV DNA-negative (n = 112)	EBV DNA-positive (n = 15)	*P*-value
Age, years, median (range)	67 (33–89)	77 (51–91)	0.005
Sex, male/female	65/47	8/7	0.785
LDH, ≤ULN/>ULN	46/66	4/11	0.401
Performance status, 0–1/2–4	75/37	8/7	0.387
Stage, I–II/III–IV	54/58	3/12	0.053
Extranodal sites, 0–1/≥2	80/32	10/5	0.765
B symptom, present/absent	83/29	11/4	1.000
IPI score, 0–2/3–5	60/52	3/12	0.025
Immunophenotype, GCB/non-GCB	51/61	4/11	0.267
Initial treatment, rituximab with anthracycline-based chemotherapy/others	106/6	13/2	0.240

GCB, germinal center B-cell-like; IPI, international prognostic index; LDH, lactate dehydrogenase; ULN, upper limit of normal.

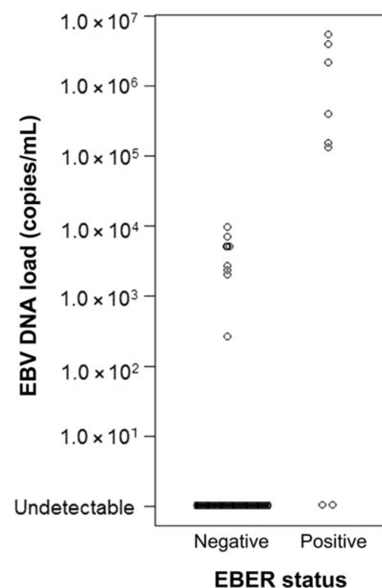

Fig. 1. Epstein–Barr virus (EBV) DNA load in pretreatment serum for patients with diffuse large B-cell lymphoma who were positive or negative for EBV-encoded small RNA (EBER). The vertical axis is shown on a log scale.

Results

Patients. Among the 127 patients eligible for analysis, EBV DNA was detected in pretreatment serum from 15 patients (12%), with a median EBV DNA load of 7.5×10^3 copies /mL (range, 2.5×10^2 to 5.6×10^6 copies/mL). Table 1 shows baseline characteristics of patients who were positive or negative for serum EBV DNA. No significant intergroup imbalances were observed in terms of performance status, serum level of lactate dehydrogenase, and number of extranodal sites. However, patients who were positive for serum EBV DNA were older (P = 0.005) and tended to be at a more advanced stage (P = 0.053), which resulted in higher scores for this group for the international prognostic index (IPI) (P = 0.025). Anthracycline-based chemotherapy in combination with rituximab was used as initial treatment for 119 patients (94%): 106 of 112 EBV DNA-negative patients and 13 of 15 EBV DNA-positive patients (P = 0.240). The remaining patients were treated with anthracycline-based chemotherapy without rituximab (n = 2), non-anthracycline-based chemotherapy with rituximab (n = 1), rituximab monotherapy (n = 1), radiation therapy (n = 2), and supportive care alone (n = 2).

Serum EBV DNA by EBER status. Results of EBER-ISH of diagnostic specimens were available for 123 patients. Figure 1 shows distributions of the EBV DNA load in pretreatment serum by EBER status. Epstein–Barr virus DNA was detectable in the serum of 6 of 8 (75%) EBER-positive patients and

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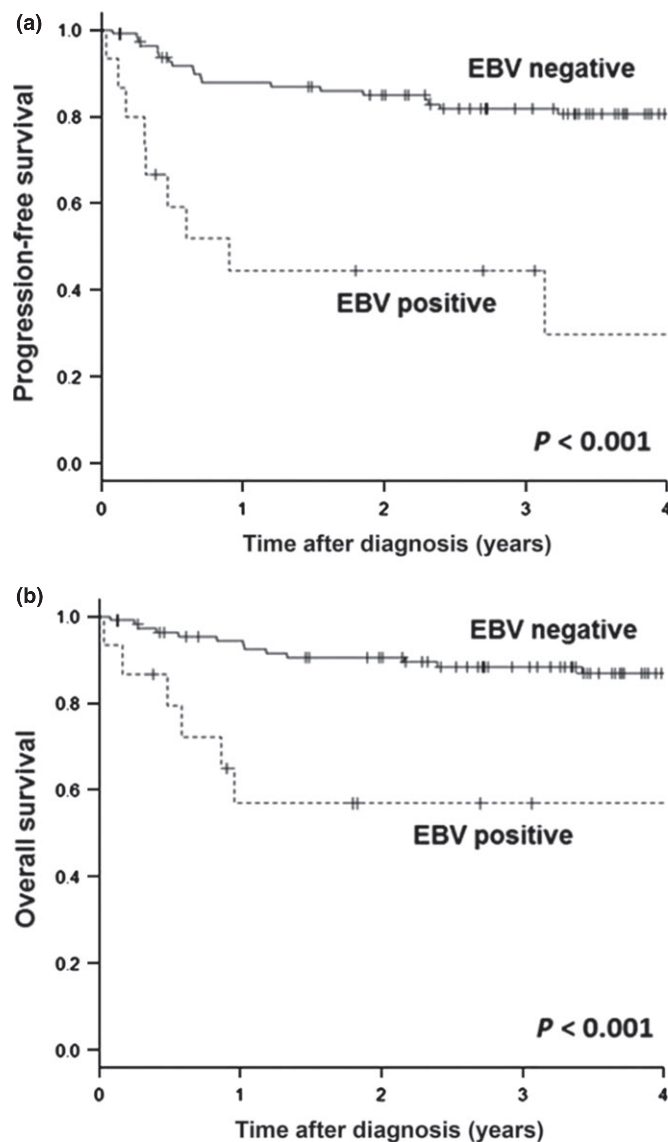

Fig. 2. Kaplan–Meier estimates of progression-free survival (a) and overall survival (b) for diffuse large B-cell lymphoma patients with (n = 15) or without (n = 112) Epstein–Barr virus (EBV) DNA detected in pretreatment serum.

in 9 of 115 (8%) EBER-negative patients. The serum EBV DNA load was more than 1.0×10^5 copies/mL for all six EBER-positive patients (median, 1.2×10^6 copies/mL; range, 1.2×10^5 to 5.6×10^6 copies/mL), and 1.0×10^4 copies/mL or less for all nine EBER-negative patients (median, 4.9×10^3 copies/mL; range, 2.5×10^2 to 1.0×10^4 copies/mL).

Outcomes. During a median follow-up period of 44.4 months (range, 1.5–78.0 months) for surviving patients, deaths were documented in 15 and 6 patients whose pretreatment serum was negative and positive for EBV DNA, respectively. The causes of death were lymphoma (n = 9), infectious complications (n = 2), interstitial pneumonitis (n = 1), alcoholic cirrhosis (n = 1), asphyxia (n = 1), and other malignancies (n = 1) for EBV DNA-negative patients, whereas all deaths were attributed to lymphoma (n = 6) for EBV DNA-positive patients. No patient in this study died as a result of EBV-related complications. Figure 2 shows survival curves for patients with or without EBV DNA detected in pretreatment serum. Patients who had detectable EBV DNA had significantly worse PFS and OS than those who did not (PFS at 4 years, 30% *vs*. 81%, P < 0.001; OS at 4 years, 57% *vs*. 87%, P < 0.001), and this finding remained the same after adjustment for age, serum level of lactate dehydrogenase, performance status, stage, and extranodal sites. Multivariate analysis revealed that EBV DNA detection in pretreatment serum was significantly associated with inferior PFS (HR, 3.53; 95% CI, 1.49–8.32; P = 0.004) and OS (HR, 3.06; 95% CI, 1.13–8.27; P = 0.027; Table 2).

Table 2. Univariate and multivariate analysis for overall survival in patients with diffuse large B-cell lymphoma

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age, years				
<60	1.00	0.100	1.00	0.180
≥60	2.50 (0.84–7.47)		2.24 (0.69–7.29)	
LDH				
<ULN	1.00	0.016	1.00	0.188
≥ULN	3.83 (1.29–11.38)		2.12 (0.69–6.53)	
Performance status				
0–1	1.00	0.008	1.00	0.152
≥2	3.13 (1.35–7.28)		1.89 (0.79–4.52)	
Stage				
I–II	1.00	0.002	1.00	0.005
III–IV	10.15 (2.37–43.49)		8.86 (1.91–40.96)	
Extranodal site				
0–1	1.00	0.280	1.00	0.331
≥2	1.62 (0.68–3.86)		0.63 (0.24–1.61)	
Serum EBV DNA				
Negative	1.00	<0.001	1.00	0.027
Positive	5.29 (2.13–13.12)		3.06 (1.13–8.27)	

CI, confidence interval; EBV, Epstein–Barr virus; HR, hazard ratio; LDH, lactate dehydrogenase; ULN, upper limit of normal.

When outcomes were compared as a function of EBER status, both PFS and OS were significantly worse for EBER-positive patients than for EBER-negative patients (PFS at 4 years, 38% *vs*. 78%, P = 0.001; OS at 4 years, 63% *vs*. 86%, P = 0.029). Given this finding and the positive interaction between EBER status and serum EBV DNA detection, we next assessed whether outcomes for EBER-negative patients could be differentiated by means of serum EBV DNA detection. Figure 3 shows survival curves for EBER-negative patients who did or did not have detectable EBV DNA in their pretreatment serum. It was found that, even among EBER-negative patients, serum EBV DNA detection was significantly associated with worse PFS and OS (PFS at 4 years, 27% *vs*. 82%, P < 0.001; OS at 4 years, 51% *vs*. 88%, P < 0.001).

Discussion

Outcomes for patients with DLBCL have been significantly improved by the advent of rituximab;[1–7] however, a certain percentage of patients still show insufficient response to this therapy and die of the disease. To ameliorate prognositic prediction primarily based on the IPI,[17] recent studies have attempted to identify biologically distinct subgroups associated with poor prognosis, such as non-GCB subtype[14,18–21] and double-hit lymphoma.[22–27] Enhancement of prognostification

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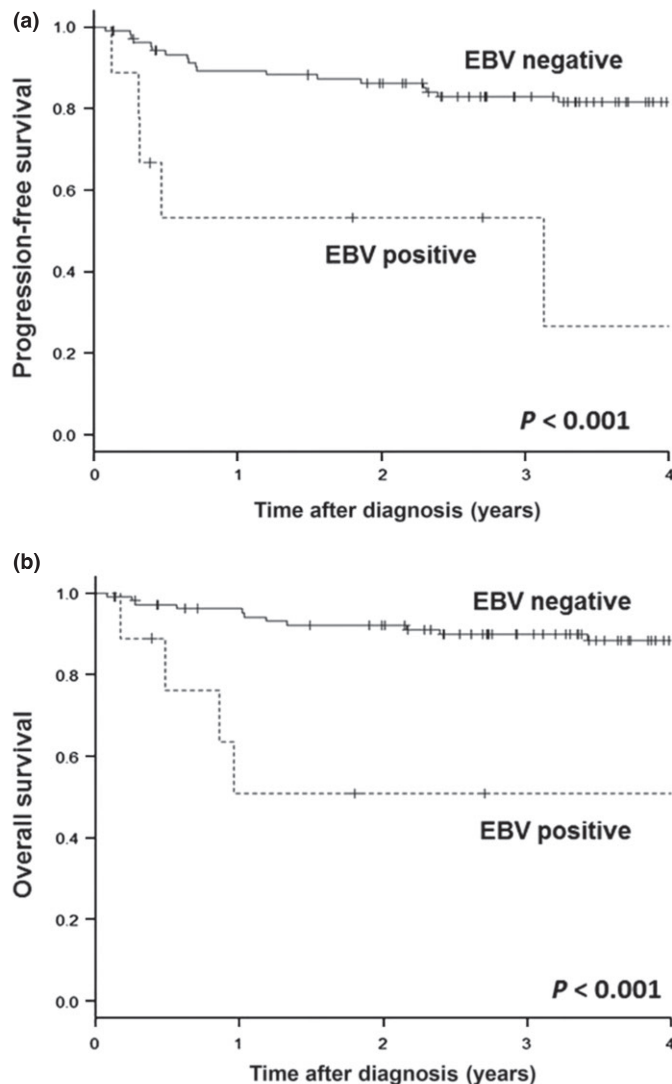

Fig. 3. Kaplan–Meier estimates of progression-free survival (a) and overall survival (b) for Epstein–Barr virus (EBV)-encoded small RNA (EBER)-negative patients with diffuse large B-cell lymphoma with (n = 9) or without (n = 106) EBV DNA detected in pretreatment serum.

based on information available at diagnosis is clinically important because it facilitates therapeutic optimization. A good example is primary mediastinal large B-cell lymphoma, the outcome of which has been shown to improve by using dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab.[28]

Epstein–Barr virus-positive DLBCL of the elderly is a subtype of DLBCL that has been newly incorporated into the 2008 WHO classification as a provisional entity,[29] and its prognosis has been shown to be inferior to that of other types of DLBCL.[15,30–34] Although EBER-ISH is the currently accepted method to evaluate EBV involvement, quantification of EBV DNA by means of the PCR assay may become an effective alternative to EBER-ISH because of its high sensitivity and objectivity. Several studies of patients with Hodgkin's lymphoma and NK/T-cell lymphoma have investigated the prognostic significance of serum or plasma EBV DNA status, and have reported that outcomes are worse for patients with higher EBV DNA load.[11,12] However, the effect of serum or plasma EBV DNA status on prognosis for DLBCL has not been studied thoroughly. This situation prompted us to measure EBV DNA load in cryopreserved pretreatment serum of our DLBCL patients to evaluate its prognostic significance.

Epstein–Barr virus DNA was detectable in pretreatment serum from 15 (12%) of the 127 DLBCL patients without underlying immunodeficiency who were included in the current study. Of these 15 patients, 6 were EBER-positive and 9 were EBER-negative. As shown in Figure 1, all of the EBER-negative patients showed an EBV DNA load of 1.0×10^4 copies /mL or less, which was in marked contrast with the much higher levels observed in the majority of EBER-positive patients. This could raise a concern that the high sensitivity of the PCR assay may have resulted in the inclusion of false-positive results for our EBER-negative patients due to the detection of EBV DNA in non-malignant cells. With this concern in mind, we excluded patients with underlying immunodeficiency from analysis, although this does not guarantee that the possibility was removed. Regarding the origin of EBV DNA detected in blood, previous studies have shown that the EBV genome detected in the plasma from patients with lymphoma and other EBV-related malignancies was not encapsidated but naked DNA,[35,36] indicating that it was released from malignant cells. Furthermore, a recent study to evaluate the kinetics of EBV DNA in blood in DLBCL patients showed that the EBV DNA load declined over time to undetectable levels in those who responded well to chemotherapy, but not in those who did not respond.[34] These findings may support the notion that EBV DNA detected in the pretreatment serum derived from lymphoma cells; however, it remains inconclusive because of a lack of direct evidence for the origin of EBV DNA detected in pretreatment serum from our EBER-negative patients. Another possibility has been suggested in a recent study, in which the proliferation of EBV-positive B cells was observed within or adjacent to lymphoma cells in a fraction of patients with EBV-negative DLBCL.[37] Also of interest, that study shows that the EBV-positive B cells were not clonally identical to the lymphoma cells.

The primary objective of this study was to determine whether EBV DNA status in pretreatment serum is predictive for outcomes for DLBCL patients. In fact, our data showed that EBV DNA detection in pretreatment serum was significantly associated with worse prognosis, and that this effect remained the same after adjustments for well-known risk factors that are included in the IPI. Although the prognostic significance of EBER status in DLBCL has been thoroughly investigated, little information is available with respect to how EBV DNA status in pretreatment serum affects outcomes. Very recently, Liang *et al.*[34] reported results of their retrospective study that examined the relationship between EBV DNA load in pretreatment whole blood and prognosis. Although they used whole blood instead of serum to measure the EBV DNA load, their findings were consistent with ours in that patients who were positive for EBV DNA showed worse prognosis than EBV DNA-negative patients. Furthermore, in our study, the adverse prognostic impact of EBV DNA detection in pretreatment serum was observed even on EBER-negative patients, suggesting that assessment of EBV DNA in pretreatment serum with the PCR assay can provide significant prognostic information that may be missed by EBER-ISH.

When interpreting our data, it should be kept in mind that this study has several limitations. Even though 94% of our patients were initially treated with anthracycline-based chemotherapy in combination with rituximab, the retrospective

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study design and heterogeneity of treatments may have affected our analysis. Additionally, as this was a single center study, the sample size is necessarily limited, so that the results presented here need to be validated for a larger number of patients. However, our study has certain advantages in that all the enrolled patients were diagnosed and treated after the advent of rituximab and that the prognostic significance of EBV DNA detection in serum was evaluated in contrast with that of EBER-ISH. Moreover, because pretreatment serum was available from most of our patients, we could include almost consecutive patients with DLBCL diagnosed at our hospital within the study period.

In summary, the findings of this study show that EBV DNA detection in pretreatment serum has an adverse prognostic impact on DLBCL patients. Although this conclusion needs to be validated in larger studies, patients with EBV DNA detectable in pretreatment serum may constitute potential candidates for whom development of alternative therapies is warranted.

Disclosure Statement

The authors have no conflict of interest.

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