



Clinical impact of cell-free serum Epstein–Barr virus status in patients with newly diagnosed malignant lymphoma

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Background

We analyzed cell-free serum Epstein–Barr virus (EBV) DNA to identify its prognostic role in patients with newly diagnosed lymphoma.

Methods

We retrospectively reviewed patients diagnosed with lymphoma between January 2014 and July 2020. Patients were enrolled according to the following criteria: i) pathologically confirmed lymphomas according to the World Health Organization criteria, ii) age over 18 years, iii) serum EBV DNA measurement using polymerase chain reaction prior to first-line therapy, and iv) receipt of curative standard chemotherapy. In total, 263 patients met these criteria and were included in this study.

Results

Serum EBV DNA was detected in 79 patients (30.0%). Patients with positive serum EBV tended to be older ($P=0.090$), and the proportion of T-cell lineage lymphomas was higher than that of B-cell lymphomas ($P=0.003$). EBV positivity was significantly associated with more advanced disease based on the Ann Arbor staging system ($P=0.008$) and the International Prognostic Index ($P=0.009$). EBV positivity was also associated with higher disease relapse ($P=0.038$) and death rates ($P=0.005$). EBV-positive lymphomas further showed inferior long-term survival outcomes in terms of progression-free survival (PFS) ($P=0.053$) and overall survival (OS) ($P=0.014$). In the subgroup analyses, serum EBV positivity was a significant prognostic factor for patients with B-cell lineage lymphomas in terms of PFS ($P=0.003$) and OS ($P=0.033$).

Conclusion

We demonstrated that cell-free serum EBV DNA status at the time of diagnosis has potential as a prognostic biomarker for patients with newly diagnosed malignant lymphomas.

Key Words Epstein–Barr virus, Lymphoma, Prognosis, Biomarker

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus that is widely disseminated in humans, with approximately 90% of adults testing EBV seropositive. EBV persists asymptotically for a lifetime as a latent EBV infection and is known to be linked to a variety of malignancies, including lymphomas [1, 2]. The World Health Organization (WHO) classified EBV as an oncovirus in 1997 [3].

EBV-associated lymphomas are a heterogeneous hematologic malignancy group that shares features of latent EBV infection within cancer cells. In general, EBV preferentially infects B-lymphocytes, and thus primarily occurs as B-cell lymphomas, such as Burkitt lymphoma, diffuse large B-cell lymphoma (DLBCL), and Hodgkin lymphoma (HL), although any type of EBV-associated lymphoma can develop [1].

EBV has been shown to be a potential biomarker for predicting the prognosis and therapeutic targets in gastric cancer [4]. Recent studies have been conducted to identify the con-

tribution of latent EBV infection to disease development and prognosis in lymphomas. In a study of 437 patients with HL, EBV positivity was a significant prognostic factor affecting long-term survival [5]. Park *et al.* [6] demonstrated that patients with EBV-positive DLBCL had poorer clinical outcomes. However, most studies analyzed lymphoid tissues and considered EBV positivity based on EBV-encoded RNA (EBER) using the *in situ* hybridization (ISH) technique. Although some studies have shown the potential role of serum EBV viral load as a biomarker to determine disease burden, the clinical usefulness of cell-free serum EBV DNA in lymphomas has not been fully established [7, 8].

The detection and quantification of serum EBV DNA has recently become possible using real-time quantitative polymerase chain reaction (RQ-PCR) using peripheral blood, which can be easily and safely obtained from patients to monitor EBV load. Therefore, the present study analyzed cell-free serum EBV DNA to identify its prognostic role in patients with newly diagnosed lymphomas.

MATERIALS AND METHODS

Patients

This study retrospectively reviewed 567 patients who were diagnosed with lymphoma between January 2014 and July 2020. Patients were enrolled according to the following criteria: i) pathologically confirmed lymphomas according to World Health Organization criteria [9], ii) age over 18 years, iii) serum EBV DNA measurement using PCR prior to first-line therapy, and iv) receipt of curative standard chemotherapy. A total of 263 patients met these criteria and were included in this study. Patient records on medical history, age, sex, pathological results, treatment method, response, and survival were reviewed using electronic medical records (EMR). We predicted the prognosis of the enrolled patients according to the Lugano modification of the Ann Arbor staging system and the International Prognostic Index (IPI) [10]. Treatment response was evaluated using the Lugano classification. This study was approved by the Institutional Review Board of KNUH.

Measurement of cell-free serum EBV DNA

To determine the clinical significance of serum EBV DNA, we retrospectively reviewed the data from the EMR. For the detection and quantification of serum EBV DNA at the time of diagnosis, EBV-specific RQ-PCR assays were conducted in the KNUH molecular microbiology laboratory using the Real-Q EBV DNA Quantification Kit (BioSewoom, Seoul, Korea) which was approved by the Korean Ministry of Food and Drug Safety. It targets the Epstein-Barr nuclear antigen 1 gene and utilizes the TaqMan probe-primer system. Nucleic acid was extracted using the MagNA Pure 96 system (Roche Diagnostics, Mannheim, Germany) and real-time PCR was carried out using the 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The analytical measurement range of the Real-Q EBV DNA

Quantification Kit ranges from 125 copies/mL to 1.0×10^{11} copies/mL, and a serum EBV DNA of ≥ 125 copies/mL was defined as positive (reference value < 125 copies/mL).

Statistical analyses

Categorical variables were summarized as counts with proportions, and continuous variables were reported as medians with ranges. Progression-free survival (PFS) was calculated from the date of diagnosis to either the date of relapse, progression of disease, death from any cause, or last follow-up. Overall survival (OS) was measured from the date of diagnosis to the date of death from any cause or the date of the last follow-up. The Kaplan-Meier method was used to analyze the PFS and OS. Survival curves were compared using log-rank tests. The Cox regression model was used to identify factors affecting long-term survival. Factors with a *P*-value of < 0.1 in the univariate analysis were then included in the multivariate analysis. The hazard ratio (HR) and 95% confidence interval (CI) were estimated for each factor. Statistical significance was set at $P < 0.05$. The data of this retrospective study were analyzed using R statistical

Table 1. Patient characteristics.

Variables	N (%)
N	263
Age, median years (range)	62 (18-87)
Sex	
Male	141 (53.6)
Female	122 (46.4)
Diagnosis	
Hodgkin lymphoma	11 (4.2)
Diffuse large B-cell lymphoma	179 (68.1)
Follicular lymphoma	13 (4.9)
Mantle cell lymphoma	8 (3.0)
NK/T-cell lymphoma	14 (5.3)
Angioimmunoblastic T-cell lymphoma	19 (7.2)
Peripheral T-cell lymphoma	13 (4.9)
Anaplastic large cell lymphoma	6 (2.3)
Ann Arbor staging	
I	41 (15.6)
II	62 (23.6)
III	74 (28.1)
IV	86 (32.7)
Prognostic risk group ^{a)}	
Low risk	97 (36.9)
Low-intermediate risk	71 (27.0)
High-intermediate risk	62 (23.6)
High risk	33 (12.5)
Positive serum EBV DNA	79 (30.0)
Relapse/refractory	67 (25.5)
Death	65 (24.7)

^{a)}The International Prognostic Index (IPI) was used for non-Hodgkin lymphoma. The International Prognostic Score (IPS) was adjusted for patients with Hodgkin lymphoma, and IPS 0 and 1 were classified as low risk, 2 as low-intermediate, 3 as high-intermediate, and ≥ 4 as high-risk group in this analysis. Abbreviations: EBV, Epstein-Barr virus; NK/T, natural killer T.

software program version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria; available at <http://www.r-project.org>).

RESULTS

Patient characteristics

In this study, 263 patients with lymphoma were analyzed. The patient characteristics are summarized in [Table 1](#). The median age was 62 years (range, 18–87 yr) at the time of diagnosis and 141 (53.6%) patients were men. DLBCL was the most frequently diagnosed lymphoma, accounting for 68.1% of the cases. A small number of other types of lymphomas were also included. The Ann Arbor staging system and IPI prognoses were well stratified ([Supplementary Fig. 1](#)). Serum EBV DNA was detected in 79 patients (30.0%). Forty-two patients with DLBCL (23.5%) were EBV positive. Among the serum EBV-positive cases, cases of mantle cell lymphoma (62.5%), natural killer/T-cell lymphoma (57.1%), angioimmunoblastic T-cell lymphoma (47.4%), and peripheral T-cell lymphoma (PTCL, 53.8%) tended to be relatively high ([Table 2](#)).

Relevance of serum EBV positivity with regard to clinicopathologic factors

Serum EBV-positive patients tended to be older than those who were negative ($P=0.090$), whereas sex was less relevant. The proportion of T-cell lineage lymphomas was higher in the EBV-positive group ($P=0.003$). Serum EBV positivity was significantly associated with a more advanced disease based on the Ann Arbor staging system ($P=0.008$) and IPI ($P=0.009$). Patients who were EBV positive at the time of diagnosis had higher rates of disease relapse ($P=0.038$) and death ($P=0.005$) ([Table 3](#)).

Clinical outcomes and impact of serum EBV positivity on survival

With a median follow-up duration of 23.3 months, 67 (25.5%) patients were primarily refractory to first-line therapy or had disease relapse, whereas 65 (24.7%) died. The

2-year PFS and OS rates were 67.5% and 76.9%, respectively. In the analysis of all enrolled patients, serum EBV-positive lymphoma at diagnosis showed inferior long-term survival outcomes in terms of PFS ($P=0.053$) and OS ($P=0.014$) ([Fig. 1](#)). In the subgroup analyses, serum EBV positivity was a significant prognostic factor for patients with B-cell lineage lymphomas in terms of PFS ($P=0.003$) and OS ($P=0.033$) ([Fig. 2](#)). In T-cell lymphomas, EBV-positive patients showed a more inferior OS; however, EBV positivity was not a significant factor affecting PFS ([Fig. 3](#)).

Table 3. Clinicopathological characteristics according to serum EBV positivity.

Variables	EBV-negative (%)	EBV-positive (%)	P
N	184	79	
Age, median years (range)	59.6 (19–81)	62.5 (18–87)	0.090
Sex			0.176
Male	97 (52.7)	44 (55.7)	
Female	87 (47.3)	35 (44.3)	
Diagnosis			0.003
B cell lineage lymphomas	157 (85.3)	54 (68.4)	
T-cell lineage lymphomas	27 (14.7)	25 (31.6)	
Ann Arbor staging			0.008
I	32 (17.4)	9 (11.4)	
II	52 (28.3)	10 (12.7)	
III	44 (23.9)	30 (38.0)	
IV	56 (30.4)	30 (38.0)	
Prognostic risk group ^{a)}			0.005
Low risk	79 (42.9)	18 (22.8)	
Low-intermediate risk	40 (21.7)	31 (39.2)	
High-intermediate risk	44 (23.9)	18 (22.8)	
High risk	21 (11.4)	12 (15.2)	
Relapse/refractory	39 (21.2)	27 (34.2)	0.038
Death	36 (19.6)	29 (36.7)	0.005

^{a)}The International Prognostic Index (IPI) was used for non-Hodgkin lymphoma. The International Prognostic Score (IPS) was adjusted for patients with Hodgkin lymphoma, and IPS 0 and 1 were classified as low risk, 2 as low-intermediate, 3 as high-intermediate, and ≥ 4 as high-risk group in this analysis. Abbreviation: EBV, Epstein–Barr virus.

Table 2. Proportion of positive serum EBV according to the lymphoma subtype and clinical outcomes of serum EBV-positive patients.

Diagnosis	Total patients (N=263)	EBV positive (N=79)	CR/PR (%)	Relapse/refractory (%)	Death (%)
Hodgkin lymphoma	11	4 (36.3)	2 (50.0)	2 (50.0)	2 (50.0)
Diffuse large B-cell lymphoma	179	42 (23.5)	40 (95.2)	11 (26.2)	12 (28.6)
Follicular lymphoma	13	3 (23.1)	3 (100)	2 (66.7)	2 (66.7)
Mantle cell lymphoma	8	5 (62.5)	5 (100)	4 (80.0)	1 (20.0)
NK/T-cell lymphoma	14	8 (57.1)	7 (87.5)	1 (12.5)	2 (25.0)
Angioimmunoblastic T-cell lymphoma	19	9 (47.4)	8 (88.9)	3 (33.3)	4 (44.4)
Peripheral T-cell lymphoma	13	7 (53.8)	6 (85.7)	3 (42.9)	5 (71.4)
Anaplastic large cell lymphoma	6	1 (16.7)	0	1 (100)	1 (100)

Abbreviations: CR/PR, complete response/partial response; EBV, Epstein–Barr virus; NK/T, natural killer T-cell.

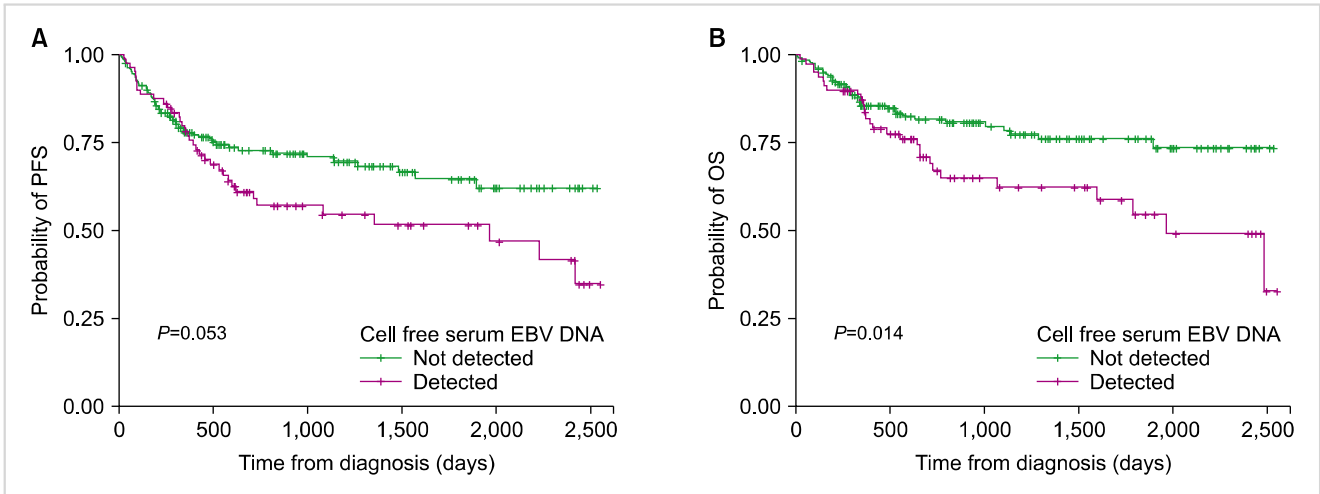


Fig. 1. Kaplan–Meier curves for long-term survival outcomes. Patients with positive serum EBV status showed inferior (A) PFS and (B) OS compared with EBV-negative patients.

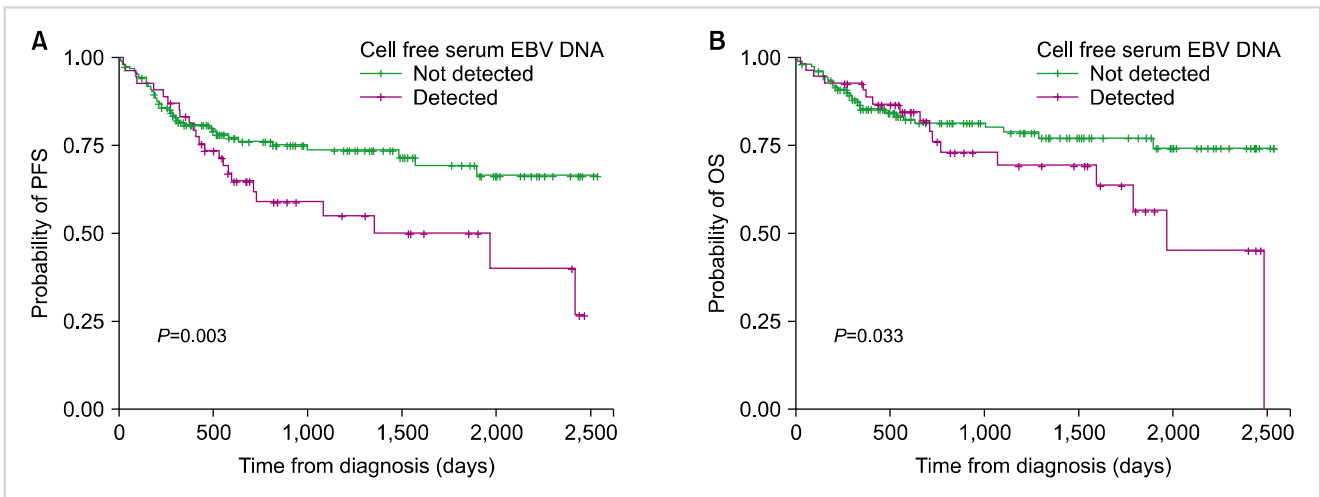


Fig. 2. Kaplan–Meier curves for long-term survival outcomes. In the B-cell lymphoma group, serum EBV-positive patients showed inferior (A) PFS and (B) OS compared with EBV-negative patients.

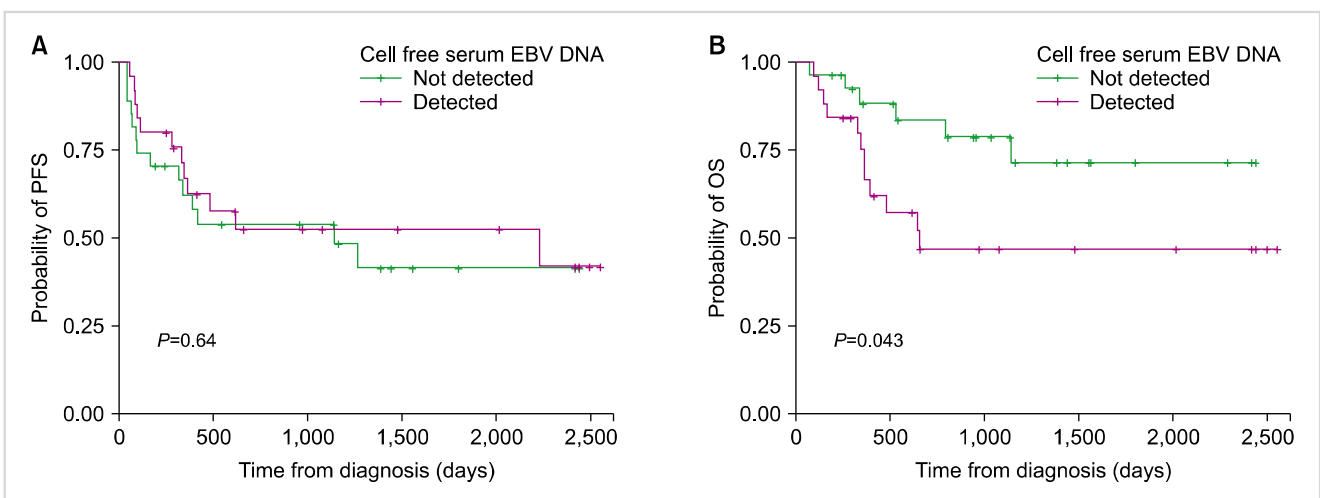


Fig. 3. Kaplan–Meier curves for long-term survival outcomes. In the T-cell lymphoma group, serum EBV positivity was not a significant factor affecting (A) PFS. However, EBV-positive patients showed inferior (B) OS.

Independent prognostic factors affecting long-term outcomes

In the univariate survival analysis, the subtypes of lymphoma (B-cell lymphomas vs. T-cell lymphomas), prognostic risk group (high-intermediate, high risk vs. low, low-intermediate risk), and serum EBV positivity were significantly associated with PFS (HR, 0.528; 95% CI, 0.326–0.853; $P=0.009$; HR, 3.018; 95% CI, 1.699–5.358; $P<0.001$; and HR, 1.354; 95% CI, 1.102–1.723; $P=0.045$; respectively) and OS (HR, 0.618; 95% CI, 0.350–1.092; $P=0.097$; HR, 4.389; 95% CI, 2.157–8.929; $P<0.001$; HR, 1.647; 95% CI, 0.990–2.738; $P=0.054$; respectively). Multivariate survival analysis revealed that B-cell lymphoma was significantly associated with better PFS (HR, 0.503; 95% CI, 0.318–0.795; $P=0.003$), while high- and high-intermediate-risk groups and positive serum EBV were independent poor prognostic factors for PFS (HR, 3.083; 95% CI, 2.022–4.699; $P<0.001$; HR, 1.211; 95% CI, 1.097–1.582; $P=0.048$; respectively) and OS (HR, 4.352; 95% CI, 2.612–7.252; $P<0.001$; HR, 1.643; 95% CI, 1.108–2.705; $P=0.050$; respectively) (Table 4).

DISCUSSION

Latent EBV infection is known to be associated with the pathogenesis of several lymphomas. Moreover, EBV positivity based on ISH to the EBER has shown potential as a clinically significant prognostic marker [6, 11]. Although cell-free serum EBV status also has the potential to predict the prognosis of patients with lymphomas, critical studies on clinical implications are still lacking. In this study, we

analyzed 263 newly diagnosed patients with lymphoma who had received curative standard treatment, and serum EBV DNA was detected in 79 patients. Although our data included various lymphoma subtypes, patients who were serum EBV DNA positive prior to the first treatment showed significantly poor prognosis regardless of age and sex. Moreover, serum EBV status was correlated with the Ann Arbor staging system and IPI, suggesting that serum EBV DNA status at the time of diagnosis might be an independent prognostic indicator.

Previous studies reported that approximately 10% of patients with DLBCL had a positive EBER ISH status [6, 12]. However, the frequency of serum EBV DNA positivity in pretreated patients with lymphoma has not been elucidated. A retrospective study reported that 25% of PTCL patients showed elevated serum EBV DNA load, which was associated with shorter survival outcomes [13]. In this study, 30% of the patients had a positive serum EBV status. However, except for DLBCL, which had a 23.5% positive rate, the proportion of patients with other lymphoma subtypes was too small to be statistically significant. Therefore, larger sample sizes for each lymphoma subtype are required. Meanwhile, serum EBV positivity is more prevalent than tissue-based positive EBER ISH status according to previous studies [13, 14]. The frequency of serum EBV positivity may be higher than that of lymphoma tissue because >90% of the population is EBV seropositive regardless of lymphoma diagnosis. Therefore, validation studies confirming the clinical usefulness and standardization of serum EBV measurements are warranted. Given that the EBV status of patients with lymphoma shows potential as a novel biomarker for prognosis, serum EBV DNA status may be more clinically valuable.

Table 4. Factors affecting long-term clinical outcomes.

	Univariate			Multivariate		
	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
(A) Factors affecting progression-free survival						
Age > 70 vs. ≤ 70	1.144	0.714–1.835	0.574			
Male vs. female	0.902	0.589–1.383	0.636			
B-cell vs. T-cell	0.528	0.326–0.853	0.009	0.503	0.318–0.795	0.003
Ann Arbor staging III, IV vs. I, II	0.984	0.525–1.843	0.958			
Prognostic risk group						
High-intermediate, high vs. Low, low-intermediate	3.018	1.699–5.358	<0.001	3.083	2.022–4.699	<0.001
EBV positive vs. negative	1.354	1.102–1.723	0.045	1.211	1.097–1.582	0.048
(B) Factors affecting the OS						
Age > 70 vs. ≤ 70	1.033	0.593–1.799	0.909			
Male vs. Female	0.953	0.575–1.578	0.851			
B-cell vs. T-cell	0.618	0.350–1.092	0.097	0.624	0.358–1.087	0.096
Ann Arbor staging III, IV vs. I, II	0.987	0.441–2.209	0.975			
Prognostic risk group						
High-intermediate, high vs. Low, low-intermediate	4.389	2.157–8.929	<0.001	4.352	2.612–7.252	<0.001
EBV positive vs. negative	1.647	0.990–2.738	0.054	1.643	0.108–2.705	0.050

Furthermore, serum biomarkers can be evaluated after continuous, easy, and safe treatment is established as a concept of minimal residual disease.

While the present study showed the potential role of serum EBV status in predicting long-term clinical outcomes, the results should be cautiously interpreted due to certain limitations. First, data in this study were retrospectively analyzed. Second, this study included heterogeneous histologic subtypes, and no detailed information was provided on the treatments. In addition, the sample size, except for DLBCL, was too small to identify clinical significance. Third, we could not clarify the association between tissue and serum EBV infection. Lastly, comparison survival outcomes based on the viral load in serum EBV DNA and cut-off values should be explored in future studies.

In conclusion, this study demonstrated cell-free serum EBV status at the time of diagnosis as a potential prognostic marker for newly diagnosed lymphomas. B-cell lymphomas were shown to be more relevant than T-cell lymphomas in this study; however, well-designed studies with large samples for each lymphoma subtype are warranted to clarify the clinical impact of serum EBV status.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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