ORIGINAL ARTICLE

Plasma EBV quantification is associated with the efficacy of immune checkpoint blockade and disease monitoring in patients with primary pulmonary lymphoepithelioma-like carcinoma

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Abstract

Objectives. Primary pulmonary lymphoepithelioma-like carcinoma (PLELC) is a subtype of lung carcinoma associated with the Epstein–Barr virus (EBV). The clinical predictive biomarkers of immune checkpoint blockade (ICB) in PLELC require further investigation. **Methods.** We prospectively analysed EBV levels in the blood and immune tumor biomarkers of 31 patients with ICB-treated PLELC. Viral *EBNA-1* and *BamHI-W* DNA fragments in the plasma were quantified in parallel using quantitative polymerase chain reaction. **Results.** Progression-free survival (PFS) was significantly longer in *EBNA-1* high or *BamHI-W* high groups. A longer PFS was also observed in patients with both high plasma *EBNA-1* or *BamHI-W* and PD-L1 $\geq$ 1%. Intriguingly, the tumor mutational burden was inversely correlated with *EBNA-1* and *BamHI-W*. Plasma EBV load was negatively associated with intratumoral CD8$^+$ immune cell infiltration. Dynamic changes in plasma EBV DNA level were in accordance with the changes in tumor volume. An increase in EBV DNA levels during treatment indicated molecular progression that preceded the imaging progression by several months. **Conclusions.** Plasma EBV DNA could be a useful and easy-to-use biomarker for predicting the clinical activity of ICB in PLELC and could serve to monitor disease progression earlier than computed tomography imaging.

Keywords: Epstein–Barr virus, PD-1/PD-L1 inhibitor, plasma EBV DNA, pulmonary lymphoepithelioma-like carcinoma, tumor mutational burden

INTRODUCTION

Lymphoepithelioma-like carcinoma (LELC) is a type of epithelium-derived undifferentiated carcinoma that has been reported to be present in many organs including the nasopharynx, lung, thymus and salivary gland.[1–3] Since its first publication in 1987, a close association has been established between pulmonary LELC (PLELC) and Epstein–Barr virus (EBV) infection, especially in Asian populations.[4,5] The standard treatment strategy for metastatic PLELC remains controversial and multidisciplinary management has been recommended for this disease.[6–8] Immune checkpoint blockade (ICB) has shown antitumor activity in the treatment of some virus-associated malignancies.[9,10] ICB could be a promising choice for treating PLELC. However, practical predictive biomarkers for liquid biopsy in patients with PLELC require further investigation.

Plasma EBV DNA has been established as a circulating tumor biomarker for EBV-associated nasopharyngeal carcinoma (NPC).[11–14] Real-time quantitative polymerase chain reaction (PCR) to detect plasma EBV DNA could be used to screen for early asymptomatic NPC during diagnosis in a population at risk.[11] Patients with persistently undetectable plasma EBV DNA had significantly better overall survival (OS) and relapse-free survival than those with detectable plasma EBV DNA in a large cohort.[12] In patients with PLELC, increasing evidence has shown that plasma EBV DNA could be potentially be used as a disease biomarker to predict chemotherapy efficacy and prognosis, as well as to monitor disease burden at serial time points.[15] The report by Xie *et al.* showed that patients with PLELC with high baseline EBV DNA concentrations had a shorter OS than those with low plasma EBV DNA.[16] Because of the rarity of this disease and the modern era of cancer immunotherapy, the utility of circulating EBV DNA needs further validation for its potential predictive and prognostic significance in specific clinical settings of patients treated with immunotherapy.

Therefore, this study aimed to explore the clinical significance of plasma EBV DNA quantification as a potential easy-to-use biomarker for immunotherapy in patients with PLELC.

RESULTS

Clinical characteristics of ICB-treated PLELC

Thirty-one patients with PLELC treated with ICB were analysed, of whom 27 had unresectable and four had resectable carcinoma. The dates at which sufficient plasma samples were collected to detect EBV DNA are shown in Figure 1a. Among them, four with available tissue samples were collected from primary tumor lesions or metastatic lymph nodes (Supplementary figure 1a). The clinicopathological characteristics of the patients are summarised in Table 1.

The median age of patients was 50 years (range: 13–73 years), and 41.9% (13/31) were females and 83.9% (26/31) were non-smokers. Patients with stage II, III and IV disease accounted for 3.2% (1/31), 12.9% (4/31) and 83.9% (26/31) of the patients, respectively (Table 1).
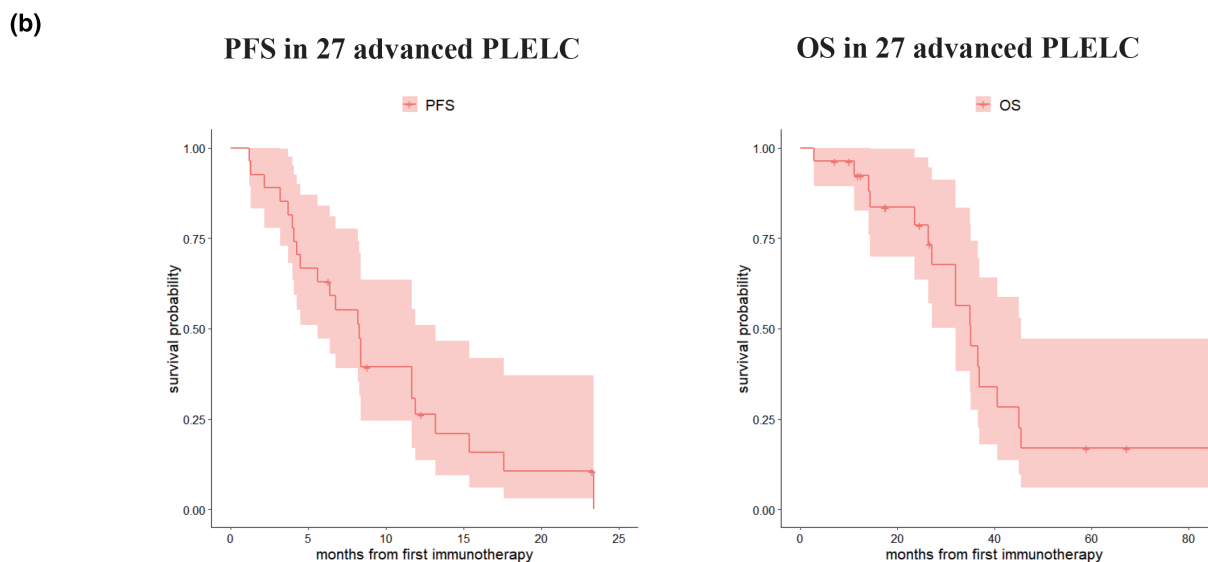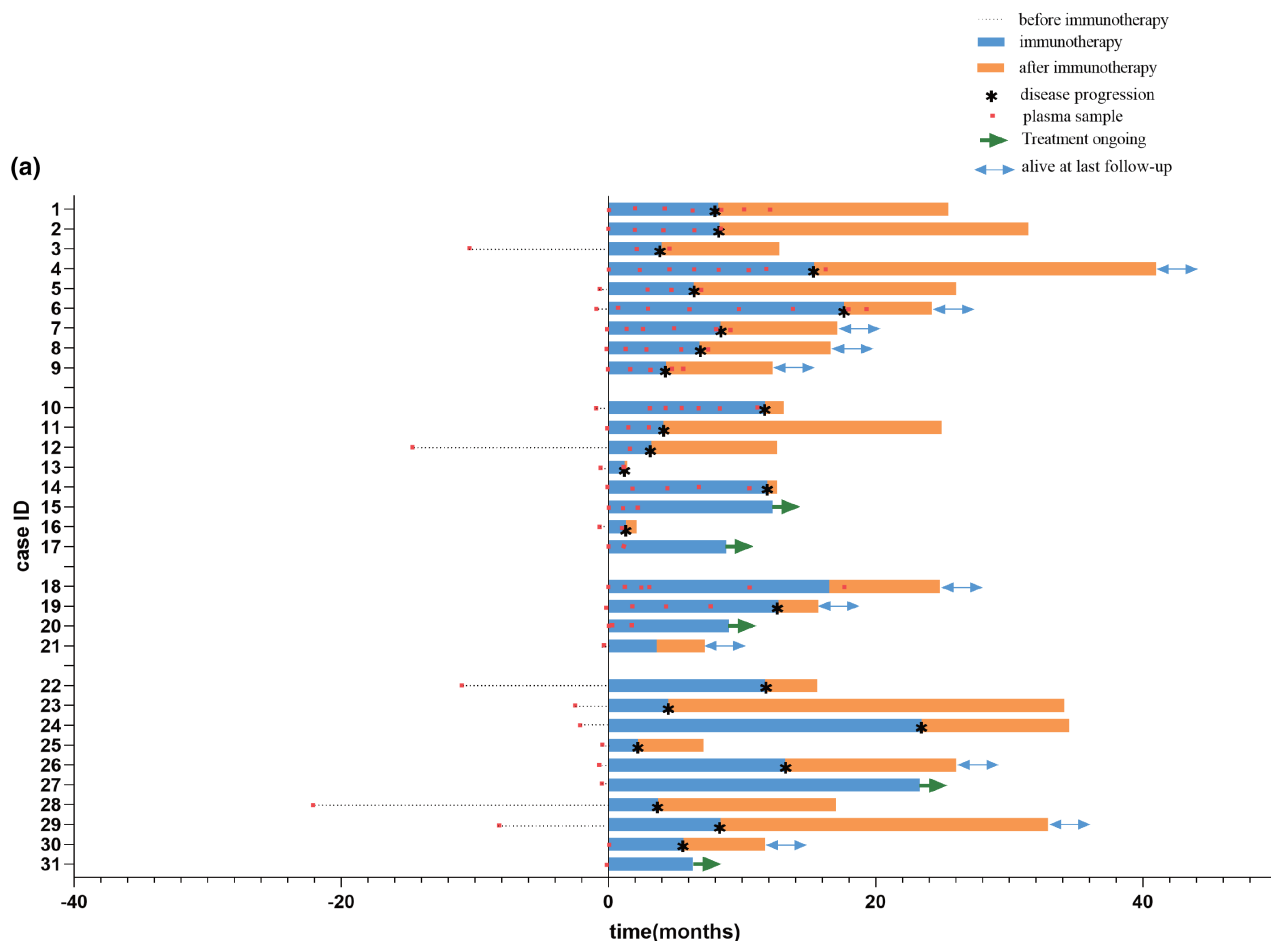
Efficacy of immunotherapy in PLELC

Among the 27 patients with advanced PLELC, 12 were treated with ICB as the first- line treatment. Fifteen patients were administered ICB as second-line or later-line therapy. ICB treatment was administered as neoadjuvant therapy to four patients with resectable tumors.

Twenty-one patients were treated with ICB monotherapy and 10 were treated with ICB in combination with chemotherapy. PD-1 inhibitors included pembrolizumab, nivolumab, sintilimab and tislelizumab, while PD-L1 inhibitors included atezolizumab and avelumab.

The objective response rate (ORR) was 22.6% (7/31) and disease control rate (DCR) was achieved in 90.3% (28/31). The median progression-free survival (PFS) and overall survival (OS) were 8.3 and 35.1 months, respectively (Table 1, Figure 1b).

Baseline plasma EBV DNA is associated with the efficacy of immunotherapy

To detect the exact number of copies of plasma EBV DNA, serial dilutions of Namalwa cell DNA (50 000, 25 000, 5000, 2500, 500, 50, 25 and 5 copies) were prepared (Supplementary figure 2a and b). Representative amplification plots are shown in Supplementary figure 2c and d. Plasma *BamHI-W* before immunotherapy was not associated with the ICB treatment lines ($P = 0.09$).

(a)

(b)

Figure 1. Dates of available plasma samples and efficacy of immunotherapy in 31 patients with PLELC treated with ICB. **(a)** During immunotherapy (blue bar) and after immunotherapy (orange bar) of PLELC treated with ICB at the initiation of the first immunotherapy. The period before immunotherapy is indicated by the dotted line. Patients with green arrows were still undergoing treatment at their follow-up. Patients with blue arrows were alive at the last follow-up. * indicates ICB progression. **(b)** PFS and OS in 27 patients with advanced PLELC.

Table 1. The clinicopathological characteristics of the 31 PLELC patients

Variable	No. of patients	Percentage (%)
Median age (range)-years	50 (13–73)	
Gender		
Male	13	41.9
Female	18	58.1
Smoking		
Ever	5	16.1
Never	26	83.9
Stage		
II	1	3.2
III	4	12.9
IV	26	83.9
EBERs		
1	10	32.3
2	8	25.8
3	11	35.5
NA	2	6.5
PD-L1 expression		
< 1%	5 (one resectable disease)	16.1
1–49%	15 (one resectable disease)	48.4
≥ 50%	8 (two resectable disease)	25.8
NA	3	9.7
Status		
Alive	15	51.6
Died	16	48.4
Lines of ICB treatment		
First line	12	38.7
Second or later line	15	48.4
Neoadjuvant	4	12.9
Monotherapy or Combination therapy		
ICB Monotherapy	21	67.7
ICB Combination	10	32.3
Objective response		
PR	7	22.6
SD	21	67.7
PD	3	9.7
ORR		22.6
DCR		90.3
Median TMB	23	2.0/MB
Median PFS	27	8.3 months
Median OS	27	35.1 months

DCR, disease control rate; ICB, immune checkpoint blockade; ORR, objective response rate; OS, overall survival disease; PD, progression disease; PFS, progression free disease; PR, partial response; SD, stable disease; TMB, tumor mutation burden.

First, the correlation between baseline plasma EBV DNA levels before ICB treatment and the clinical response to ICB were explored in 27 patients with unresectable PLELC. The optimal cut-off value of pretreatment *EBNA-1* concentration was found to be 11 231 copies mL^{-1} to distinguish those with better prognosis using the surv_cutpoint function of the R package survminer (Figure 2a). Using this optimal cutoff value, patients were divided into *EBNA-1* high (*EBNA-1* > 11 231 copies mL^{-1}) and *EBNA-1* low (*EBNA-1* ≤ 11 231 copies mL^{-1}) groups. PFS was longer in the EBV-high group (11.7 vs. 5.4 months, $P = 0.002$, Figure 2b) than in the EBV-low group. However, with different cut-off values, OS did not show statistically significant differences between *EBNA-1* high and low groups (Supplementary figure 3a–c).

Similar results were obtained using the *BamHI-W* PCR system. The optimal cut-off value of plasma *BamHI-W* before immunotherapy was 10 446 copies mL^{-1} (Figure 2c). Compared with the *BamHI-W* low group (*BamHI-W* ≤ 10 446 copies mL^{-1}), the *BamHI-W* high group (*BamHI-W* > 10 446 copies mL^{-1}) was associated with a longer PFS (11.9 vs. 6.4 months, $P = 0.006$, Figure 2d). Similarly, analysis of OS did not reach statistical significance between *BamHI-W* high and low groups (Supplementary figure 3d–f).

However, in resectable patients, patients with higher *BamHI-W* or *EBNA-1* levels before treatment were associated with a shorter disease-free survival after surgery (Figure 4e and f, Supplementary figure 4).

Subsequently, the correlation between plasma EBV DNA levels and the best response to ICB treatment was evaluated in 31 patients with PLELC. The best response to ICB was not significantly different between EBV-high and EBV-low patients (Supplementary table 2).

Correlation between PD-L1 expression and tumor mutational burden (TMB) and plasma EBV DNA in PLELC

Prior to treatment PD-L1 expression was detected in 24 patients with unresectable PLELC. The proportion of patients with PD-L1 expression < 1%, 1–49% and ≥ 50% did not differ between the *EBNA-1* high and *EBNA-1* low groups (Figure 3a). In addition, patients with both high plasma *EBNA-1* concentration and PD-L1 ≥ 1% were found to have a significantly longer PFS than the others (15.4 vs. 6.6 months, $P < 0.001$, Figure 3b). Similarly, a special subgroup of patients with both high plasma *BamHI-W* levels and PD-L1 ≥ 1% also had a significantly superior PFS (15.4 vs. 6.8 months, $P = 0.001$, Figure 3c).

To explore the correlation between plasma EBV DNA and TMB, next-generation sequencing (NGS)
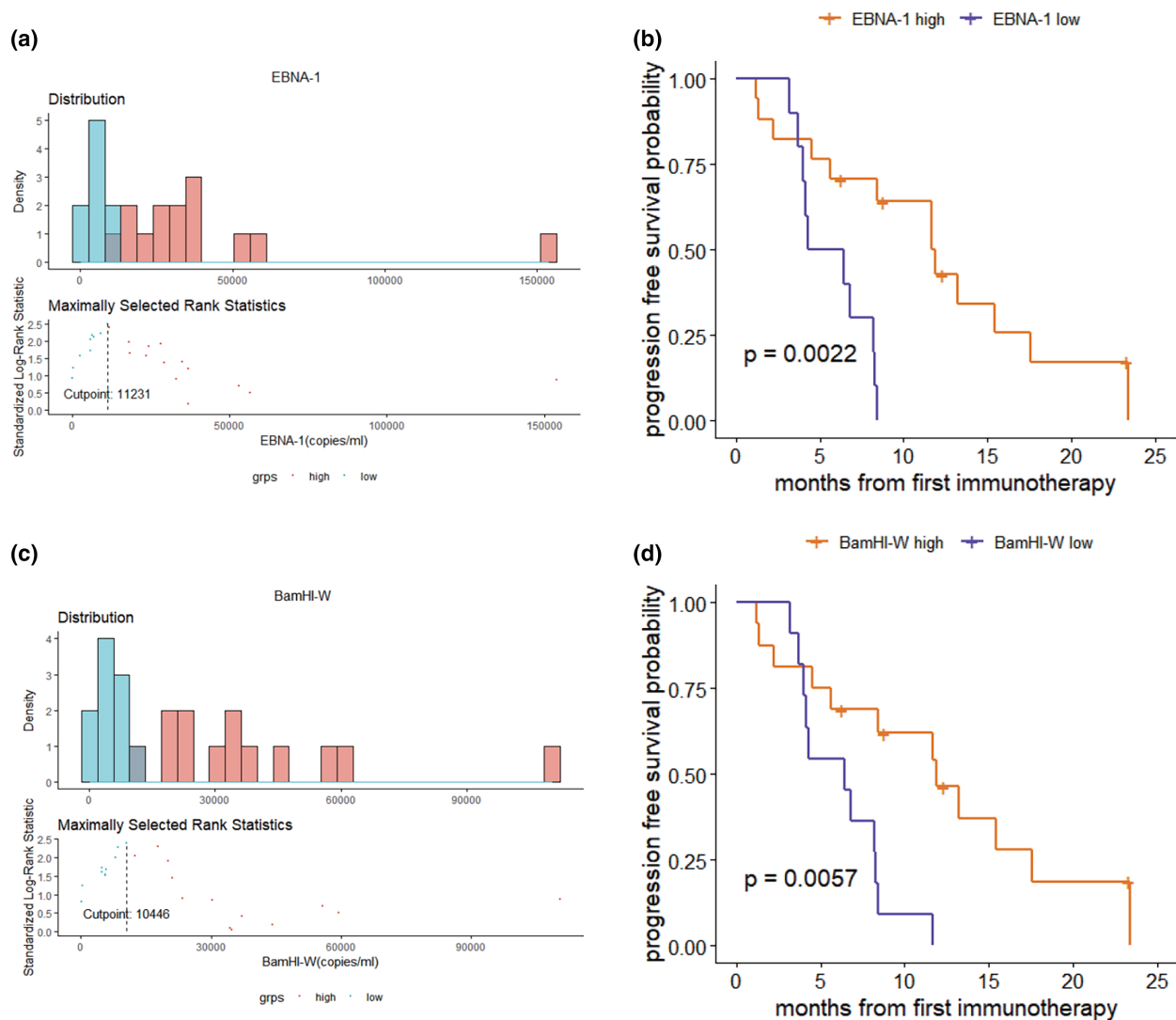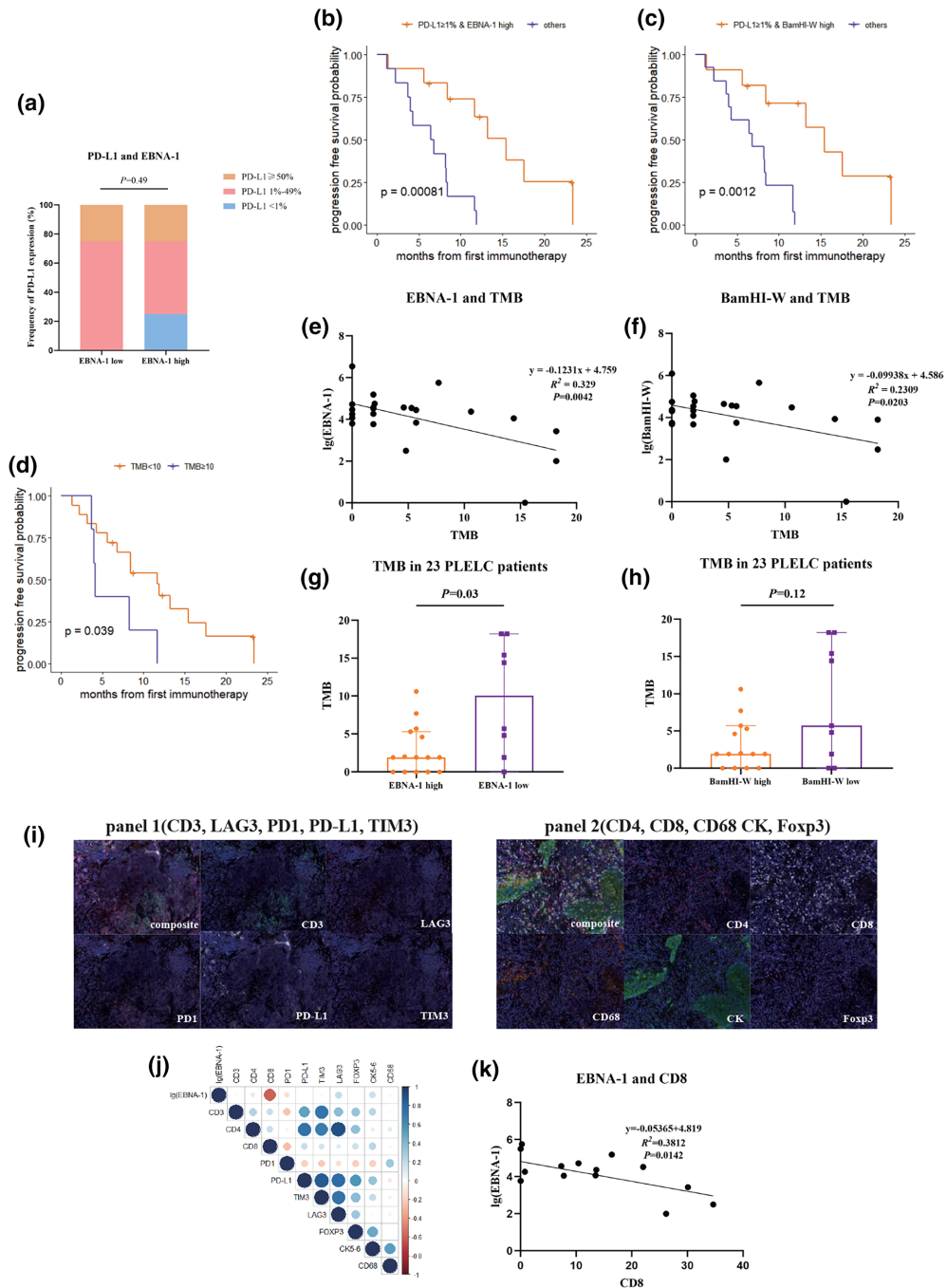
Figure 2. Correlation between plasma EBV DNA before immunotherapy and PFS in PLELC. **(a)** The histogram above describes the distribution of patients with different plasma *EBNA-1* concentration. The scatter plot shows the optimal cut-off value of plasma *EBNA-1* before immunotherapy to distinguish patients with longer PFS. **(b)** PFS of the *EBNA-1* high group (*EBNA-1* > 11 231 copies mL^{-1}) vs. the *EBNA-1* low group (*EBNA-1* $\leq$ 11 231 copies mL^{-1}). **(c)** The histogram describes the distribution of patients with different plasma *BamHI-W* concentration. The scatter plot shows the optimal cut-off value of plasma *BamHI-W* before immunotherapy to distinguish patients with longer PFS. **(d)** PFS of the *BamHI-W* high group (*BamHI-W* > 10 446 copies mL^{-1}) vs. the *BamHI-W* low group (*BamHI-W* $\leq$ 10 446 copies mL^{-1}).

data from 23 ICB-treated patients with unresectable PLELC were analysed. The median TMB among these patients was 2.0/MB (Table 1). Patients with TMB < 10/MB had superior PFS than those with TMB $\geq$ 10/MB (11.7 vs. 4.1 months, *P* = 0.039, Figure 3d). The scatter plot shows that TMB correlated with lg (*EBNA-1*) in these patients (R^2 = 0.329, *P* = 0.004, Figure 3e). TMB was also associated with lg (*BamHI-W*) (R^2 = 0.231, *P* = 0.020, Figure 3f). The median TMB was higher

in the *EBNA-1* low group than in the *EBNA-1* high group (*P* = 0.03, Figure 3g), and numerically higher in the *BamHI-W* low and high groups (*P* = 0.12, Figure 3h).

Multiple immunohistochemistry (mIHC) staining was conducted to explore the relationship between immune tumor markers and pretreatment plasma EBV DNA in 15 patients with PLELC (Figure 3i). There was no association between the positive percentages of CD3, CD4,

Figure 3. Correlation between PD-L1 expression and TMB and plasma EBV DNA before ICB treatment in PLELC. **(a)** Proportion of different PD-L1 expression in the *EBNA-1* high group (*EBNA-1* > 11 231 copies mL^{-1} before immunotherapy) vs. the *EBNA-1* low group (*EBNA-1* ≤ 11 231 copies mL^{-1}). **(b)** PFS of patients with ICB-treated PLELC with both high plasma *EBNA-1* before immunotherapy and PD-L1 ≥ 1% vs. others. **(c)** PFS of patients with ICB-treated PLELC with both high plasma BamHI-W before immunotherapy and PD-L1 ≥ 1% vs. others. **(d)** PFS of 23 patients with ICB-treated PLELC with TMB < 10/MB vs. ≥10/MB. **(e)** Scatter plot of TMB and lg (*EBNA-1*). **(f)** Scatter plot of TMB and lg (*BamHI-W*). **(g)** Median TMB of the *EBNA-1* high group (*EBNA-1* > 11 231 copies mL^{-1} before immunotherapy) vs. the *EBNA-1* low group (*EBNA-1* ≤ 11 231 copies mL^{-1}). **(h)** Median TMB of the *BamHI-W* high group (*BamHI-W* ≥ 10 446 copies mL^{-1} before immunotherapy) vs. the *BamHI-W* low group (*BamHI-W* < 10 446 copies mL^{-1}). **(i)** Representative mIHC staining for PD-1, PD-L1, LAG3, TIM3 and CD3 (panel 1; left), and CD4, CD8, CD68, FOXP3 and CK (panel 2; right). **(j)** Heatmap of correlation analysis of lg (EBNA-1) and CD3, CD4, CD8, PD1, PD-L1, TIM3, LAG3, FOXP3, CK5-6, CD68. **(k)** Scatter plot of positive percentage of lg (*EBNA-1*) and CD8 in 15 patients with advanced PLELC.
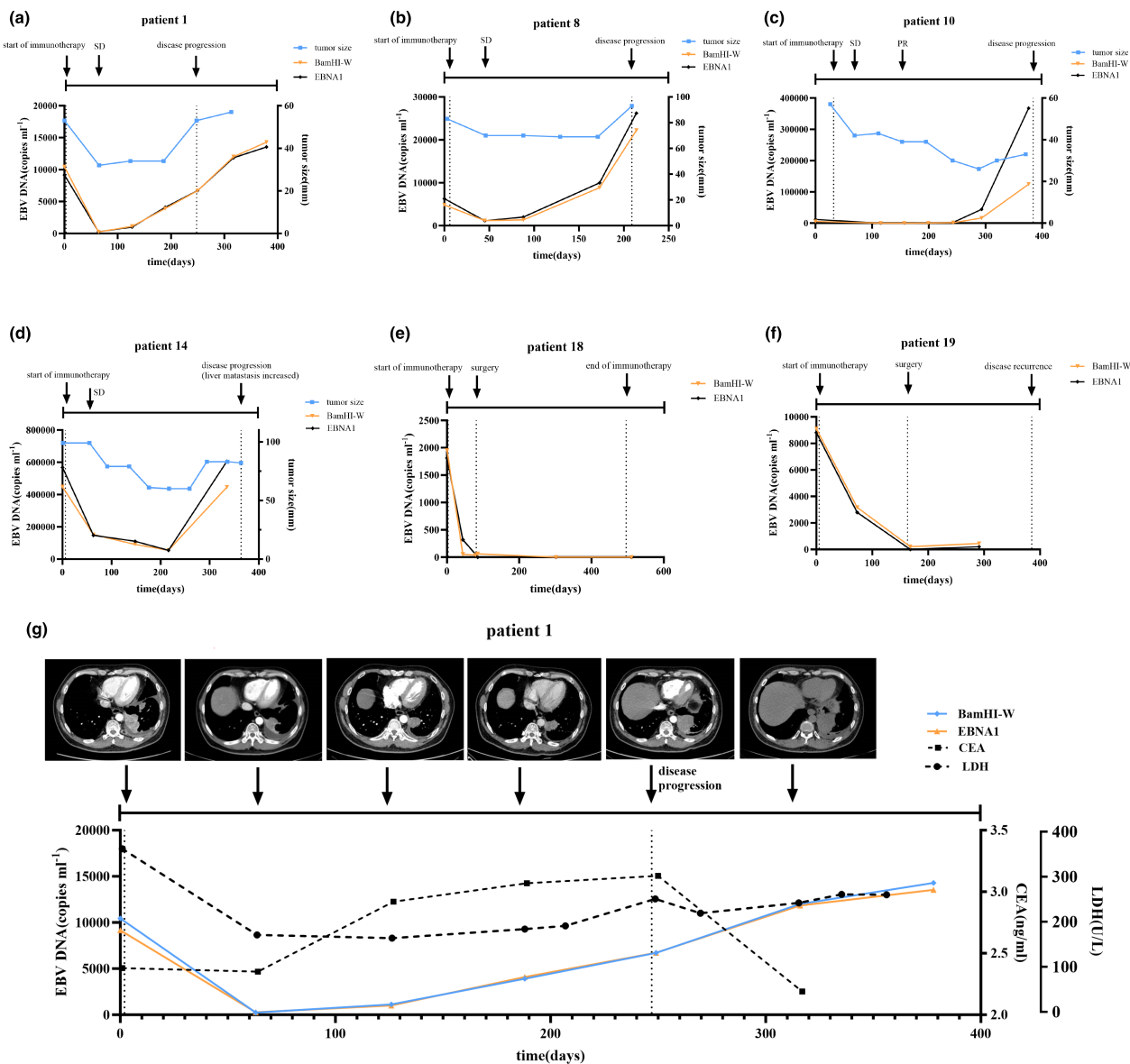
Figure 4. Plasma *BamHI-W* profile and tumor size in patients with ICB-treated PLELC. **(a–d)** Plasma *BamHI-W* (orange line), *EBNA-1* (black line) profile and tumor size in CT (blue line) of Patient 1, 8, 10 and 14. **(e, f)** Plasma *BamHI-W* (orange line) and *EBNA-1* (black line) profile of Patient 18 and 19. **(g)** Plasma *BamHI-W* profile (blue line), *EBNA-1* profile (orange line), CEA (black dot line, square), LDH (black dot line, circular) levels and representative images of CT of Patient 1. The dates of 'start of immunotherapy', 'end of immunotherapy' and 'disease progression' are marked in the panels.

PD1, PD-L1, T-cell immunoglobulin and mucin domain 3 (TIM3), lymphocyte activation gene 3 (LAG3), forkhead box P3 (FOXP3), CK5-6, or CD68 and baseline plasma Ig (*EBNA-1*) (Figure 3j). Plasma Ig (*EBNA-1*) was inversely associated with the tumoral positive percentage of CD8$^+$ cells before treatment ($R^2 = 0.381$, $P = 0.014$; Figure 3k).

Plasma EBV DNA could monitor clinical response to ICB in PLELC

Real-time quantitative PCR showed that dynamic changes in plasma EBV DNA levels could monitor the clinical response to ICB in PLELC (Figure 4). Generally, after treatment with ICB, plasma EBV DNA detected by the *BamHI-W* PCR system decreases in patients

Table 2. Days between timepoints of plasma EBV DNA increase and clinical PD

Case ID	Leading time
1	184
2	62
3	58
6	249
7	105
8	121
9	82
10	141
11	80
14	148
Range	58–249
Average	123
Median	113

Leading time: days between plasma EBV DNA had a significant tendency to increase and PD.

with unresectable PLELC, indicating a response to the immunotherapy. The number of days between significant EBV DNA elevation in plasma and clinical disease progression was defined as the leading time. The average and median of leading time were 123 and 113 days, respectively (Table 2). Dynamic changes in plasma EBV DNA kept pace with the alteration in the maximum diameter of the primary tumor on computed tomography (CT) (Figure 4a–d, Supplementary figure 4). Similar results were obtained with in the *EBNA-1* PCR system (Supplementary figure 4). Compared to carcinoembryonic antigen (CEA) and lactate dehydrogenase (LDH) in Patient 1, the change in plasma *EBNA-1* concentration more accurately reflected the alteration in primary tumor size on CT (Figure 4g).

Additionally, plasma EBV DNA levels play a potential role in monitoring disease relapse after neoadjuvant immunotherapy in patients with resectable PLELC. During ICB treatment as neoadjuvant therapy, the concentration of plasma *BamHI-W* decreased (Figure 4e and f). After surgery, the plasma EBV DNA level continued to decrease to below the detection limit (Figure 4e and f). During the follow-up period, elevated *BamHI-W* levels tended to lead to relapse (Figure 4f). The *EBNA-1* system showed a similar trend (Supplementary figure 4).

DISCUSSION

PLELC, an EBV-associated cancer, is a subtype of lung squamous cell carcinoma with unclear aetiology.[17,18] An increasing number of studies have shown that ICB could be a promising therapeutic choice for this rare disease.[19,20] However, circulating biomarkers in liquid biopsies predicting responses to immunotherapy in PLELC are still unknown and require further investigation. Our study shows that plasma EBV DNA can be used to monitor the clinical response to ICB in PLELC patients. EBV DNA elevation in the plasma occurs earlier than in clinical disease progression. Importantly, we found that plasma EBV DNA before immunotherapy could be a potential biomarker for predicting the clinical outcomes of ICB treatment for PLELC.

Virus-related DNA has already been detected in the serum or plasma of several kinds of virus-associated cancers.[21–24] In a previous study, by comparing the genotypes of EBV in plasma and tissue, Chan *et al.* demonstrated that most of plasma EBV DNA molecules in patients with NPC are tumor derived.[25] In our study, we also found that the concentration of *EBNA-1* in plasma correlated well with its concentration in tissue. Plasma EBV DNA is released by tumor cells at the primary tumor site in PLELC. Therefore, our study and previous studies suggest that EBV DNA in plasma could reflect the tumor burden, and monitoring its dynamic change may have the potential to predict disease progression in ICB-treated PLELC.

To date, few studies have investigated free circulating EBV DNA in PLELC.[16,26,27] Ngan *et al.*[26] detected the serum EBV DNA in 19 patients with advanced PLELC, revealing that serologic decrease was associated with clinical remission and the rise in serum EBV DNA preceded disease progression or recurrence in patients with PLELC in clinic. Xie *et al.*[16] measured plasma EBV DNA in 429 patients with PLELC, showing that *BamHI-W* concentration ≥ 4000 copies mL^{-1} was significantly associated with an increased risk of death both in early and advanced disease stages, suggesting EBV DNA as a negatively prognostic factor. Li *et al.*[27] found that baseline plasma *BamHI-W* copy number $> 10\,000$ copies mL^{-1} was a risk factor for PFS in stage I-III PLELC. These studies included both early stage and metastatic disease and showed that EBV DNA could predict the outcomes of surgery with or without radiotherapy and chemotherapy.

In our cohort, the lack of association between plasma EBV DNA and OS might have been

because of the heterogeneous subsequent treatment after immunotherapy. A previous study showed that, in patients with PLELC treated with chemotherapy and/or radiotherapy, higher baseline plasma EBV DNA levels might be associated with shorter OS. However, in this study we focused on patients with PLELC treated with immunotherapy and showed that patients with higher plasma EBV DNA levels might have longer PFS and benefit more from ICB treatment. Thus, the OS may be influenced by the differential effects of immunotherapy and chemotherapy in patients with high plasma EBV DNA levels. However, our results must be validated in larger and more rigorously designed clinical trials.

In contrast, in our study, all the patients had advanced disease and were treated with ICB-based regimens. Patients with high baseline plasma EBV DNA levels showed a better response and longer PFS than those with low EBV DNA levels. Similarly, ICB could produce a good response in some virus-associated cancers.[28,29] The detailed mechanism underlying why patients with PLELC with higher plasma EBV DNA levels were associated with a better response to ICB needs further investigation.

In our study, the plasma EBV DNA levels decreased after immunotherapy in some beneficiary cases, followed by an increase before disease progression in PLELC. The dynamic change in plasma EBV DNA was consistent with the alteration in primary tumor size on CT images. On average, the molecular increase of EBV DNA in the plasma occurred 123 days before disease progression, EBV-encoded small RNAs (EBERs) and EBV-encoded RNAs have already been used clinically for the tissue diagnosis of this virus-associated malignancy. In our study, EBERs were also used as diagnostic criteria for PLELC. Notably, the difference in EBERs scores did not reach statistical significance between patients with high and low plasma EBV DNA levels. The dynamic changes in plasma CEA and LDH levels did not correlate well with primary tumor size in PLELC. Thus, we suggest that plasma EBV DNA could be superior to EBERs, CEA and LDH in terms of serving as a potential real-time biomarker for monitoring therapeutic response in ICB-treated PLELC.

Notably, EBV can infect the pulmonary epithelial cells in a latent or lytic pattern and disturb innate and adaptive immunity.[30] At the moment, details of the interplay between EBV-infected epithelial cells and immune cells and the mechanisms underlying the landscape of the tumor immune microenvironment are scarce. Future studies on the EBV activation status and the details of the release of EBV DNA or virions into the circulation by tumor cells may help interpret the better response to ICB in patients with higher plasma EBV DNA.

Interestingly, in our study, patients with high plasma EBV DNA levels before ICB-based treatment had a lower TMB and better response to ICB treatment. Intratumoral infiltration of $CD8^+$ immune cells is inversely associated with plasma EBV DNA. This may be attributed to the interaction between EBV infection and the immune system in this rare disease. Wu *et al.*[31] showed that T-cell epitopes, most of which are located in EBV latent genes, were detected in PLELC. Mutations in the T-cell epitopes might help EBV escape immune recognition and avoid T cell infiltration. In some EBV-associated carcinomas, latent EBV or lytic proteins, rather than epithelial genetic alterations, may play an essential role in oncogenesis and tumor progression. However, the role of EBV-associated antigens in antitumor immunity and their potential clinical applications require further investigated.

A limitation of this study was the small number of cases. Randomised prospective clinical trials with larger cohorts are warranted to confirm these results. Additionally, not all patients had sufficient plasma samples at serial time points during immunotherapy or after disease progression. With more patients enrolled, further research will be conducted in the future to clarify the mechanism of interaction between EBV infection and immune lymphocytes in the tumor microenvironment of PLELC.

Taken together, our results suggest that plasma EBV DNA is a useful biomarker for predicting the clinical outcomes of ICB in PLELC and for monitoring therapy response and disease progression. Patients with higher plasma EBV DNA levels before immunotherapy showed a longer PFS in PLELC. EBV DNA elevation in plasma occurs earlier than disease progression in clinical imaging. There is an urgent need to prospectively validate the predictive role of plasma EBV DNA in a large cohort of patients with PLELC and elucidate the interplay between EBV and the immune microenvironment to provide new insights into PLELC management.

METHODS

Patient characteristics

We prospectively collected the clinical plasma samples from patients diagnosed with primary PLELC at Guangdong Lung Cancer Institute between January 2016 and November 2021. The electronic records of these patients treated with immunotherapy were reviewed, including their clinicopathological features, types of ICB treatment, primary tumor size on CT and ICB treatment outcomes.

Plasma and tissue DNA extraction and real-time quantitative PCR

Blood samples were prospectively collected from the patients with PLELC. Plasma was retrieved and stored at −80 °C until further processing. Genomic DNA was extracted from plasma samples, tissue samples and Namalwa cells using the QIAamp DNA Blood Mini Kit (Qiagen, Dusseldorf, Germany) or QIAamp DNA Mini Kit (Qiagen). For DNA extraction, 200 μL plasma was used, with a final elution volume of 50 μL. We developed two real-time quantitative PCR systems with minor groove binder probes for EBV DNA detection: one for the *BamHI-W* region and the other for the *EBNA-1* region. The primer and probe sequences are listed in Supplementary table 1. A reaction volume of 25 μL was set up for fluorogenic PCR reactions. Each reaction system contained 12.5 μL of TaqMan Universal Master Mix II, with UNG, 2× (Applied Biosystem, Waltham, USA), 300 nM of each of the amplification primers; 25 nM (for the EBV probes) or 100 nM (for the β-globin probe) of the corresponding fluorescent probe. Extracted plasma DNA (5 μL) or tissue DNA (5 ng) was used for amplification. Each sample was analysed in duplicate. The method used to calculate the EBV DNA copy number has been described previously.[32,33]

Multiple immunohistochemistry

Tissue samples from 15 patients with advanced PLELC were used to detect the lymphocytes markers CD3, LAG3, PD1, PD-L1, TIM3, CD4, CD8, CD68, CK and FOXP3 by mIHC staining (Genecast Biotechnology Co., Ltd, Wuxi, China). The detailed methods have been described previously.[34]

Genome sequencing and TMB

Genomic DNA was extracted from frozen tumor tissues, paired blood samples and normal tissues. Genomic alterations were determined using NGS prior to ICB treatment. Comprehensive genomic profiling of the 23 ICB-treated patients was performed using NGS with 425 (Geneseeq, Nanjing, China), 168, 520 (Burning Rock, Guangzhou, China), or 1021 (Geneplus, Beijing, China) cancer-related gene panels. Sequencing methods have been described in detail previously.[35–37] TMB was calculated using the NGS gene panels mentioned above.

Statistical analysis

PFS was defined to be the time from the initial ICB treatment to the date of disease progression or death from any cause or censored at the date of the last follow-up. The OS was calculated from the initial ICB treatment until death or the last follow-up date.

The two-sided Mann–Whitney *U*-test was used to compare TMB, PD-L1 distribution and best response to ICB between the high and low plasma EBV DNA groups. The surv_cutpoint function of the R package survminer was used to determine the optimal cut-off values for plasma EBV DNA before immunotherapy. Statistical analyses were performed using SPSS (27.0, International Business Machines Corporation, Chicago, America) and R software (version 4.1.2, Lucent Technologies, New Jersey, America).

CONFLICT OF INTEREST

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Yu-Min Zhong: Data curation, visualisation, writing-original draft and software. **Ji Chen:** Investigation, data curation and writing-original draft. **Jie Jiang:** Data curation. **Wen-Bin Zhou:** Visualisation, data curation, writing-reviewing and editing. **Ling-Ling Gao:** Data curation, writing-reviewing and editing. **Shui-Lian Zhang:** Supervision. **Wen-Qing Yan:** Supervision. **Yu Chen:** Supervision. **Dong-Kun Zhang:** Supervision. **Dan-Xia Lu:** Supervision. **Zhi-Yi Lv:** Supervision. **Zhi Xie:** Supervision. **Ying Huang:** Supervision. **Wei-Bang Guo:** Supervision. **Bin-Chao Wang:** Writing-reviewing and editing. **Jin-Ji Yang:** Writing-reviewing and editing. **Xue-Ning Yang:** Writing-reviewing and editing. **Yi-Long Wu:** Writing-reviewing and editing. **Xu-Chao Zhang:** Conceptualisation, methodology, validation and funding acquisition.

DATA AVAILABILITY STATEMENT

The raw data that supporting the findings of our study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.