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A proteome-wide analysis unveils a core Epstein–Barr virus antibody signature of classic Hodgkin lymphoma across ethnically diverse populations

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Abstract

Epstein–Barr virus (EBV) is an oncogenic virus associated with various malignancies, including classical Hodgkin lymphoma (cHL). Despite its known association, the specific role of humoral immune response to EBV remains poorly characterized in cHL. To address this, we conducted a study using a custom protein microarray to measure the antibody responses in cHL patients and matched healthy controls recruited from an East-Asian hospital-based case–control study. We identified 16 IgG antibodies significantly elevated in EBV-positive cHL compared with controls, defining an "East-Asian antibody signature of EBV-positive cHL." We evaluated responses against these 16 antibodies in a distinct European population, leveraging data from our previous European cHL case–control study from the UK, Denmark, and Sweden. A subset of antibodies (14/16, 87.5%) from the "East-Asian antibody signature of EBV-positive cHL" exhibited significant associations with cHL in the European population. Conversely, we assessed the "European antibody signature of EBV-positive cHL" identified in our prior study which consisted of 18 EBV antibodies (2 IgA, 16 IgG), in the East-Asian population. A subset of these antibodies (15/18, 83.3%) maintained significant associations with cHL in the East-Asian population. This cross-comparison of antibody signatures underscores the robust generalizability of EBV antibodies across populations. Five anti-EBV IgG antibodies (LMP-1, TK, BALF2, BDLF3, and BBLF1), found in both population-specific antibody signatures, represent a "core signature of EBV-positive cHL." Our findings suggest that the antibody responses

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targeting these core EBV proteins reflect a specific EBV gene expression pattern, serving as potential biomarkers for EBV-positive cHL independent of population-specific factors.

What's new?

Epstein–Barr virus (EBV) is associated with classical Hodgkin lymphoma and other cancers. Here, the authors measured antibody response to EBV using a custom protein microarray. Their results define an antibody signature of 16 IgG antibodies in a population from East Asia. By testing a population from Europe, they identified a "core signature" of five IgG antibodies consistently elevated in cHL patients, suggesting that these may be significant to the disease progression and could point to new therapeutic interventions.

1 | INTRODUCTION

Epstein–Barr virus (EBV) is an oncogenic virus associated with various malignancies, including carcinomas of the stomach and nasopharynx, as well as lymphomas such as natural killer/T-cell lymphoma (NKTCL), Burkitt lymphoma (BL), and a subset of classical Hodgkin lymphoma (cHL).[1] cHL is a type of B cell-derived malignancy characterized by bi- or multinucleated Reed-Sternberg cells from mononuclear Hodgkin cells in a background of reactive inflammatory cells.[2,3] Genomic evidence strongly suggests that EBV plays a crucial role in the pathogenesis of cHL.[4–6] Elevated antibody levels against EBV antigens further support the potential involvement of EBV in cHL pathogenesis.[7]

Genomic, serological, and epidemiological studies of cHL suggest that EBV-associated (EBV-positive) and nonassociated (EBV-negative) cases may represent two distinct etiological entities.[4,8,9] Epidemiological studies have revealed diverse incidence patterns of cHL globally, influenced by factors such as histological subtype, age, sex, ethnicity, geographical location, and socioeconomic status.[8,10] Moreover, there is a remarkable diversity in the prevalence of EBV-associated cases between ethnic groups and geographic locations.[11,12] Specifically, in North America and Western Europe, the proportion of EBV-positive cHL is about 20%–50%, whereas notably higher prevalences have been reported predominantly in Southern Chinese populations (~59%–61%), including Hong Kong (65%) and Taiwan (63.9%).[13–17]

The role of EBV in cHL pathogenesis remains poorly characterized, and our understanding of EBV-specific immune responses is limited.[18] A comprehensive analysis of antibody responses against all EBV proteins in individuals with cHL is necessary to deepen our understanding of the underlying pathology. To date, most serological studies have focused on major EBV-encoded antigen complexes, including the viral capsid antigen (VCA), early antigen (EA), EBV nuclear antigen 1 (EBNA-1), EBV nuclear antigen 2 (EBNA-2), and latent membrane protein 1 (LMP-1).[19,20] However, the complete EBV genome translates into nearly 100 EBV transcripts,[21] and investigations of the immune responses against most of these proteins are lacking.

To comprehensively investigate IgG and IgA antibody responses against the complete EBV proteome, we developed a custom EBV proteome microarray representing each open reading frame from all EBV proteins, and known and predicted splice variants (199 protein sequences and 3 synthetic peptides).[21] We have previously utilized this custom EBV protein microarray to characterize EBV antigen-specific responses in various EBV-associated cancers, including nasopharyngeal carcinoma,[21] endemic BL,[22] and NKTCL[23] and identified specific antibody signatures associated with these malignancies. Recently, we used this EBV proteome microarray to analyze the immune responses in cHL patients from European populations in the UK, Denmark, and Sweden[24] and identified a signature of 16 IgG and 2 IgA antibodies that were significantly elevated in EBV-positive cHL cases compared to controls.

In the current study, we applied this EBV proteome array to profile anti-EBV antibody responses in an East-Asian population from Hong Kong and Taiwan diagnosed with cHL and matched healthy controls as part of a hospital-based case–control AsiaLymph study with the aim to identify a specific EBV antibody signature of cHL in this East-Asian study population.

Furthermore, to identify a generalized antibody signature for EBV-positive cHL across ethnically and geographically diverse populations, we conducted a cross-population comparative analysis of the antibody profiles of EBV-positive cHL in the European and East-Asian study populations.

2 | MATERIALS AND METHODS

2.1 | Study samples

Subjects were selected from the AsiaLymph study, a multicenter, hospital-based case–control study conducted in Hong Kong and Taiwan from 2012 to 2017.[23] Eligible cases were aged 18–79 years at cHL diagnosis. Individuals with a history of lymphoma were excluded from this study. Blood and buccal cell collections were performed at the time of diagnosis and before cancer therapy. This study included 127 patients diagnosed with cHL via histological examination in Hong Kong ($N = 118$) and Taiwan ($N = 9$) (Table 1). The EBV status of cHL cases was determined by immunohistochemical staining of tumor

TABLE 1 Characteristics of study subjects by case–control status.

Characteristic	EBV-positive cHL ($n = 35$) *n* (%)	EBV-negative cHL ($n = 92$) *n* (%)	Controls ($n = 60$) *n* (%)	*p*-value test of proportion between EBV-positive cHL and Controls
Study area				
Taiwan	2 (5.7)	7 (7.6)	4 (6.7)	1
Hong Kong	33 (94.3)	85 (92.4)	56 (93.3)	1
Sex				
Female	8 (22.9)	51 (55.4)	27 (45.0)	.006
Male	27 (77.1)	41 (44.6)	33 (55.0)	.053
Age at diagnosis (years)				
0–39	3 (8.6)	63 (68.5)	22 (36.7)	.006
39–54	5 (14.3)	16 (17.4)	22 (36.7)	.036
54–100	27 (77.1)	13 (14.1)	16 (26.7)	5.26×10^{-6}

Abbreviation: cHL, classical Hodgkin lymphoma.

biopsies for EBV latent membrane antigen (LMP)-1 and in situ hybridization for EBV-encoded small RNAs (EBERs), or by using the EBV DNA viral load[25] (Figure S1 and Table S1). The analysis included 35 EBV-positive and 92 EBV-negative patients with cHL. In addition, 60 controls were selected from patients in the same participating hospital in the same region who were frequency-matched to the overall cHL cases by sex, age (±5 years), date of enrolment (within 3 months), and region (Hong Kong/Taiwan). Control subjects with diseases other than lymphoma, including injuries and selected diseases of the circulatory, digestive, genitourinary, and central nervous systems, were recruited.

2.2 | EBV custom proteome microarray

Our custom EBV proteome microarray encompassed 199 protein sequences representing nonredundant open reading frames and predicted splice variants from all known 86 EBV proteins derived from five prototypical EBV strains (AG876, Akata, B95-8, Mutu, and Raji) with 97% of the predicted sequences for the 86 proteins from each strain represented on the microarray at ≥99% homology.[21–24] Also, included on the array were three synthetic EBV peptides (VCAp18, EBNA-1, and EA p47) considered the current gold standard for detecting EBV-specific antibody responses and putative cancer biomarkers.[21–24] Four "no DNA" (no translated protein) spots were included in the array to correct for person-specific background (e.g., *Escherichia coli* reactivity). Each microarray slide was printed using 16 arrays.

For each study subject, plasma samples were tested blinded to the case–control status by laboratory personnel for both IgA and IgG antibody responses, as described previously.[21–24] Briefly, antibody responses were detected with biotin-conjugated goat anti-human IgG (1:1000 dilution) or IgA (1:500 dilution) secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and visualized with streptavidin-conjugated SureLight® P3 (Columbia Biosciences, Columbia, MD, USA) antibody (1:200 dilution). After testing, air-dried probed slides were scanned using an Axon GenePix 4300 B (Molecular Devices). Raw fluorescence intensities were corrected for spot-specific background using Axon GenePix Pro 7 software, and data were variant-log-transformed using variance stabilizing normalization (VSN) transformation in Gmine.[26] The array output was standardized to the person-specific background by dividing VSN values with the individual's cutoff (mean ± 1.5 standard deviation of the four "no DNA" spots), and the transformed data were referred to as the standardized signal intensity (SSI).

2.3 | Statistical analysis

All statistical analyses were performed using the R statistical software (version 1.4.1103). The Benjamini and Hochberg false discovery rate (FDR) method was applied to adjust the *p*-values for multiple tests, with a significance threshold set at FDR <0.05 for testing the 199 protein sequences and 3 synthetic peptides included in our protein microarray. Nominal *p*-values were used for validation purpose in the cross-comparative analyses. Disparities between the case and control groups were identified by evaluating the distribution of EBV-positive cHL, EBV-negative cHL, and controls using a *p*-value test for proportions.

The mean SSI differences for IgG and IgA responses to all the EBV proteins and peptide sequences were compared between (i) overall cHL cases and controls, (ii) EBV-positive cHL cases and controls, and (iii) EBV-negative cHL cases and controls using unpaired *t*-tests. Linear regression analyses were performed to test the association between anti-EBV antibody responses for case–control status after adjusting for sex, age, and study area.

To extend our findings across populations, we conducted a cross-population comparative analysis using antibody data we previously obtained using the same EBV protein array from a European cohort,

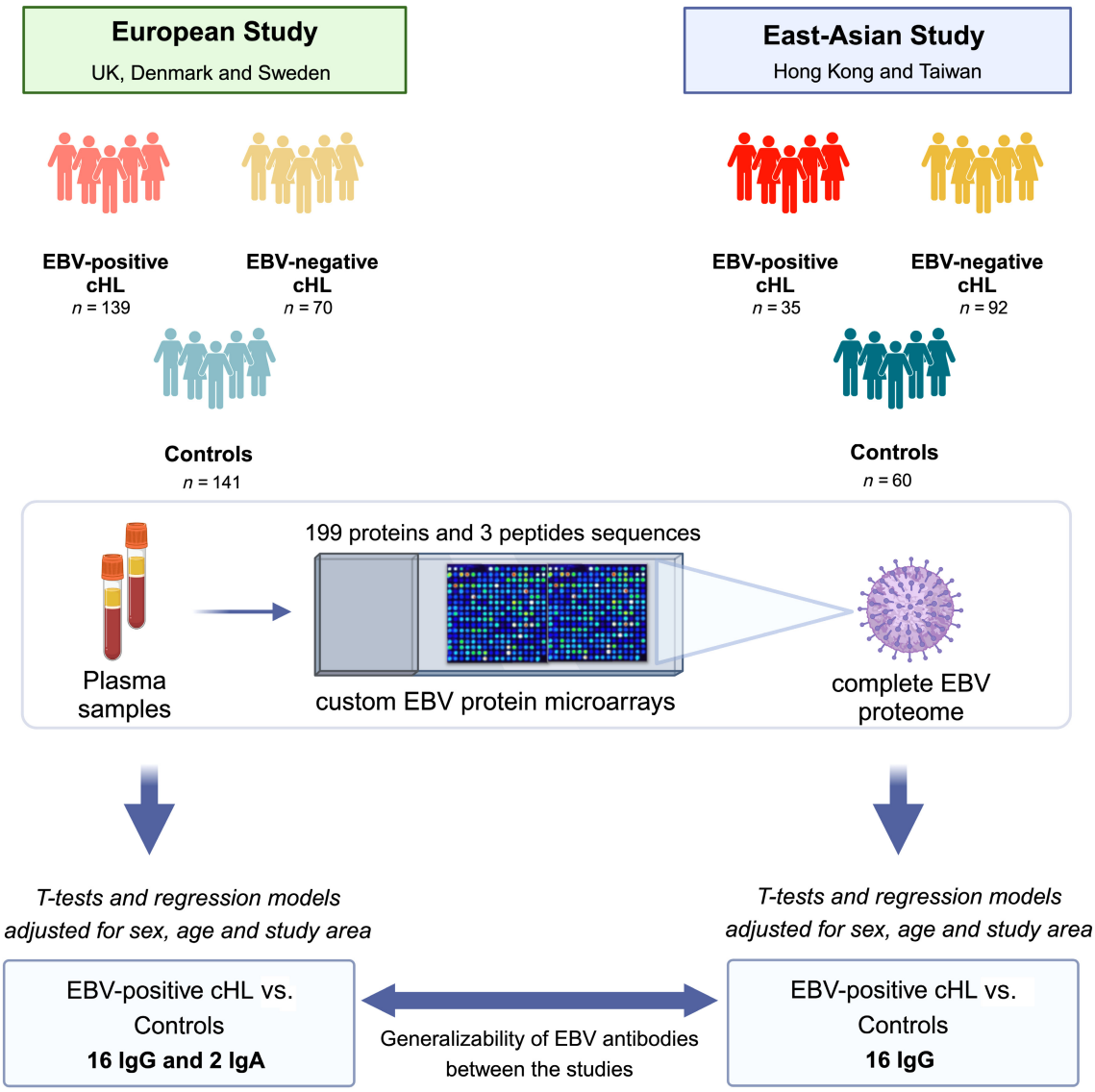

FIGURE 1 Study design. The European study population from the UK, Denmark, and Sweden and the East-Asian study population from Hong Kong and Taiwan consisted of EBV-positive cHL, EBV-negative cHL, and controls. Our custom EBV proteome microarray screened plasma samples from these individuals to evaluate IgG and IgA antibody responses against the entire EBV proteome. Differences in antibody responses were evaluated between cHL cases and control groups using unpaired *t*-tests. In addition, polytomous logistic regression models (in the European study) and linear regression analyses (in the current AsiaLymph study) were carried out to test the association between anti-EBV antibody responses (i.e., SSI) and case–control status while adjusting for sex, age, and study area. Created with BioRender.com.

which included 139 EBV-positive cHL cases, 70 EBV-negative cHL cases, and 141 controls (Figure 1).[24] This analysis aimed to assess the consistency of elevated antibodies in EBV-positive cHL cases compared to controls in European and East-Asian populations. Unpaired *t*-tests and linear regression analyses, adjusted for sex, age, and study area, were used to determine differential antibody responses. Statistical significance was set at $p < .05$. Additionally, a more rigorous comparative analysis directly compared the antibodies in East-Asian and European EBV-positive cHL signatures. This analysis aimed to identify common anti-EBV antibodies associated with cHL in both populations and establish a universal "core antibody signature of EBV-positive cHL."

To define the significance of the overlap within the East-Asian and European antibody signatures of EBV-positive cHL, we computed the statistical distributions of the intersection between the two sets and determined the exact probabilities of the significance of this intersection using the SuperExactTest package in R.[27] This method predicts the number of antibodies expected to overlap between two population-specific signatures if antibodies are randomly chosen. It then compares this theoretical 'expected intersection' to the actual 'observed intersection' to calculate a *p*-value and fold enrichment. Fold enrichment was calculated as the ratio of the observed intersection size to the expected intersection size, providing insights into the significance of the observed intersection.

3 | RESULTS

3.1 | Population characteristics

The demographic characteristics of 35 EBV-positive patients with cHL, 92 EBV-negative patients with cHL, and 60 healthy controls are presented in Table 1. Control samples ($n = 60$) were matched by age ($p = .60$) and sex ($p = .29$) to all cHL cases ($n = 140$), regardless of the EBV status. However, when we categorized cHL cases according to their EBV status for analysis (EBV-positive cHL cases), we observed a smaller number of EBV-positive cHL cases ($n = 35$ [28%]) than EBV-negative cHL cases ($n = 92$ [72%]) in the overall study population.

3.2 | Proteome-wide EBV analysis identified "East-Asian antibody signature of EBV-positive cHL"

We employed our custom EBV proteome microarray to comprehensively evaluate IgG and IgA antibody responses against all EBV proteins in individuals enrolled in the East-Asian study.

No significant differences were observed in the antibody profiles for IgG or IgA between the cHL cases and controls (Figure S2). However, when comparing EBV-positive cHL cases with controls, we identified 14 IgG antibodies (Figure 2A, Table 2, and Table S2), but no IgA antibodies (Table S3), that were significantly higher in EBV-positive cHL cases than in controls (FDR $p < .05$, t-test). Of these 14 IgG antibodies, 10 were significantly associated with EBV-positive cHL when adjusted for demographic characteristics, including sex, age, and study area (odds ratio [OR] > 1.3, FDR $p < .05$, linear regression). Moreover, this linear regression analysis identified two additional IgG antibodies (EBNA3A and BDLF3) that were significantly associated with EBV-positive cHL, although this association was not significant in the t-test analysis (ORs > 1.5, FDR $p < .05$, linear regression) (Table 2). Thus, we defined a population-specific signature for EBV-positive cHL in the East-Asian population of 16 IgG antibodies, comprising 14 IgG antibodies identified by the t-test and two IgG antibodies identified by linear regression. This signature was designated as the "East-Asian antibody signature of EBV-positive cHL."

The most pronounced SSI differences between EBV-positive cHL cases and controls within this signature were observed for IgG antibodies against sequences representing LMP1 (FDR $p = .037$, t-test), Epstein–Barr nuclear antigen 3A (EBNA3A) (FDR $p = .037$, t-test), EBV nuclear antigen leader protein (EBNA-LP) (FDR $p = .037$, t-test), and thymidine kinase (TK) (FDR $p = .037$, t-test) (Figure S3). All antigens showed an OR >1.4 (Table 2).

In addition, we evaluated the differences in the mean SSI for IgG and IgA antibodies against each of the 199 proteins and 3 synthetic peptide sequences between EBV-negative cHL cases and

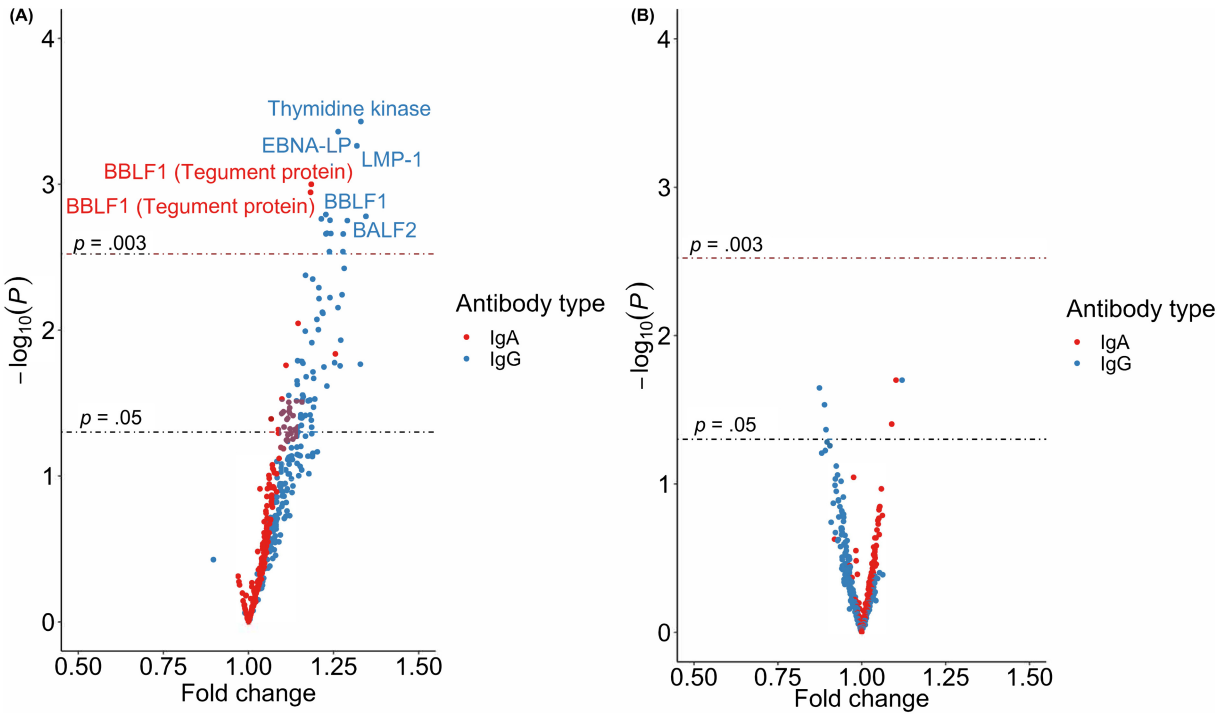

FIGURE 2 Case–control differences in mean antibody responses for IgA and IgG. Case–control differences in mean antibody responses for (A) EBV-positive cHL cases vs. controls and (B) EBV-negative cHL cases vs. controls. The x-axis of the volcano plot displays the fold change (case vs. control ratio of standardized signal intensity) for 199 proteins and 3 synthetic peptide sequences for each antibody type (red, IgA; blue, IgG). The y-axis illustrates the p-value corresponding to the t-test for a difference in standardized signal intensity (SSI) between groups. (A) 14 IgG antibodies were significantly higher in EBV-positive cHL cases than controls (FDR <0.05). Seven antibodies with significant p-values are highlighted. (B) No anti-EBV antibodies were significantly higher in EBV-negative cHL cases compared to controls. Dashed lines represent the statistically significant p-value thresholds (FDR <0.05). FDR <0.05 = nominal $p < .003$.

TABLE 2 EBV proteins (name and microarray sequence) for the IgG antibody responses significantly higher in EBV-positive cHL cases compared with controls in the East-Asian population of the AsiaLymph study samples.

Protein name	Array sequence	EBV life cycle	*t*-test *p*	*t*-test FDR	95% CI	OR	L95	U95	Lin. reg *p*-value	Lin .reg FDR
THY.KINASE	YP_001129497.1-133399-131576	Early lytic	3.72×10^{-4}	**0.037**	0.734–0.908	1.56	1.21	2.01	.001	**0.041**
EBNA-LP	YP_001129440.1-20824-20955	Latent	4.36×10^{-4}	**0.037**	0.727–0.899	1.35	1.14	1.61	.001	**0.041**
LMP1	YP_401722.1-168507-167702	Latent	.001	**0.037**	0.768–0.928	1.43	1.21	1.69	.000	**0.012**
BBLF1	AFY97956.1-108555-108328	Early lytic	.002	**0.037**	0.719–0.899	1.46	1.12	1.9	.006	0.061
BALF2 (EA(D)_p138)	YP_001129510.1-165796-162410-1	Early lytic	.002	**0.037**	0.743–0.918	1.49	1.17	1.89	.001	**0.042**
LMP2A	YP_001129436.1-167587-167942	Latent	.002	**0.037**	0.715–0.891	1.23	1.07	1.42	.006	0.061
LF2	YP_001129504.1-151808-150519	Early lytic	.002	**0.037**	0.717–0.893	1.32	1.11	1.57	.002	**0.043**
EBNA3A	YP_001129463.1-80447-82888	Latent	.002	**0.037**	0.734–0.908	1.74	1.28	2.38	.001	**0.041**
BPFL1	YP_001129449.1-59370-49906-2	Late lytic	.002	**0.037**	0.726–0.909	1.39	1.13	1.7	.002	**0.043**
BDLF2	YP_001129491.1-120928-119666	Glycoprotein	.002	**0.037**	0.721–0.899	1.43	1.14	1.8	.003	**0.046**
BPFL1	YP_001129449.1-59370-49906-3	Late lytic	.002	**0.037**	0.706–0.893	1.32	1.1	1.58	.004	0.051
BLLF1 (gp350/220)	YP_001129462.1-79936-7727. r	Glycoprotein	.002	**0.037**	0.737–0.909	1.45	1.17	1.81	.001	**0.042**
LMP2B	AFY97910.1-1026-1196	Latent	.003	**0.042**	0.718–0.903	1.30	1.09	1.54	.004	0.051
EBNA3A	YP_401669.1-80382-82877	Latent	.003	**0.042**	0.727–0.904	1.67	1.24	2.27	.001	**0.042**
EBNA3A	AFY97915.1-80252-82747	Latent	.004	0.051	0.721–0.901	1.63	1.2	2.2	.002	**0.043**
BDLF3	AFY97964.1-118644-117940	Glycoprotein	.008	0.065	0.724–0.901	1.57	1.18	2.09	.003	**0.046**

Note: FDR correction method: Benjamini and Hochberg. The table is ordered by significant *t*-test *p*-value (lowest to highest). Odd ratios (ORs) were adjusted for age (continuous), sex (M/F), and study area (Hong Kong and Taiwan) using a linear regression model. FDR *p*-value obtained for *t*-tests, Adj *p* FDR. Antibodies met with an FDR $p < .05$ are highlighted in bold.
Abbreviations: CI: confidence interval, L95: lower 95% confidence interval, OR: odds ratio; U95: upper 95% confidence interval.

control samples. Similar to our previous findings in the European population,[24] no significance was identified for either IgG or IgA in our EBV proteome-wide analysis of the East-Asian study population (Figure 2B, Table S4, and Table S5).

3.3 | Generalizability of EBV antibody signature of EBV-positive cHL across populations

To ensure the broad relevance of our findings, we conducted a cross-population comparative analysis to assess the consistency of the antibody signatures identified in the East-Asian population when applied to a distinct European population (Figure 3). For this comparison, we employed data from our previous European study using the same custom EBV protein microarray.[24]

Of the 16 antibodies differentially elevated in EBV-positive cHL in the East-Asian population, 14 (all IgG; 87% of antibodies tested) consistently showed significant elevation in EBV-positive cHL compared to the control groups in the European population (nominal $p < .05$, t-test) (Figure 3 and Table S6). All 14 antibodies remained significantly associated with the disease after adjusting for demographic factors, including sex, age, and study area (nominal $p < .05$, linear regression) (Table S6), indicating the high generalizability of the East-Asian antibody signature of EBV-positive cHL in the European population.

In our previous European study,[24] we identified a "European Antibody signature of EBV-positive cHL" comprising 18 EBV antibodies (16 IgG and 2 IgA). In this study, we investigated whether these 18 antibodies consistently exhibit elevated levels in an ethnically and geographically distinct East-Asian population.

Among the 18 EBV antibodies, 15 (2 IgA, 13 IgG) (83% of the antibodies tested) consistently exhibited higher levels in EBV-positive cHL than in controls (nominal $p < .05$, t-test) (Figure 3 and Table S7). Notably, even after adjusting for sex, age, and residential area, 14 of the 15 antibodies remained significantly associated with EBV-positive cHL (nominal $p < .05$, linear regression), indicating the high generalizability of the European antibody signature for EBV-positive cHL.

3.4 | "Generalized" and "Core" signature of EBV-positive cHL in ethnically diverse populations

To define a "Generalized EBV antibody signature of cHL" that transcends population-specific factors, we pooled together all antibodies from the "East-Asian signature" that maintained significant elevation in the European population ($n = 14/16$, $p < .05$) and all the antibodies in the "European signature" that maintained significant elevation in the East-Asian population ($n = 15/18$, $p < .05$). Additionally, we established a more robust core EBV antibody signature of EBV-positive cHL across both populations by testing the intersection between antibodies in the "East-Asian signature" and the "European signature" (Table 3 and Figure 3).

The combination of the two population-specific antibody signatures comprised 29 antibodies, representing 85.3% of the 34 antibodies tested from both the East-Asian ($n = 16$) and the European ($n = 18$) antibody signatures. This subset of 29 antibodies constitutes a "Generalized signature of EBV-positive cHL," transcending population-specific factors, such as host genetic diversity, geographical, and ethnic variations (Figure 3). Five IgG antibodies (LMP-1, TK, BALF2, BDLF3, and BBLF1) were found in both population-specific signatures and representing a more

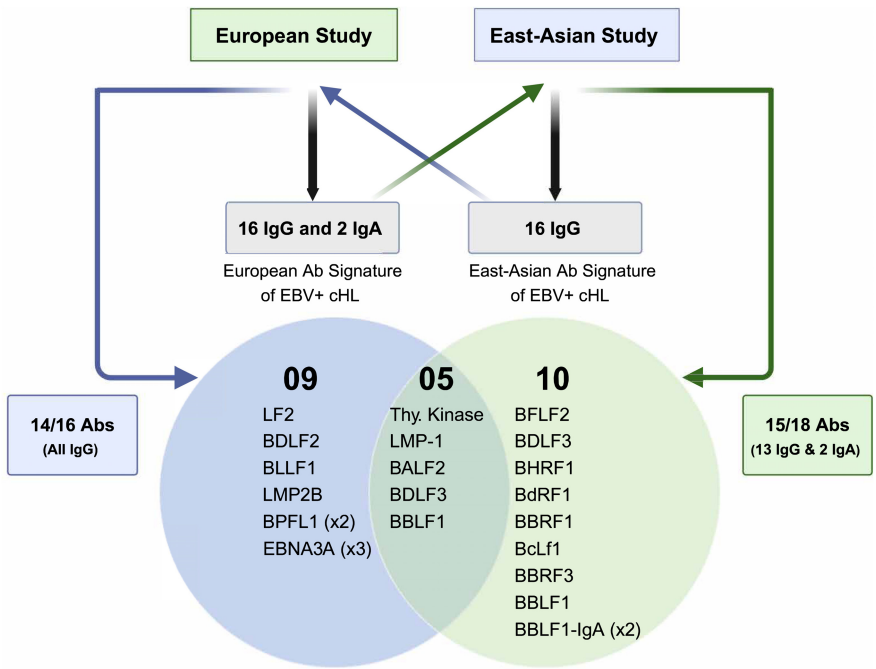

FIGURE 3 Cross-population comparative analysis of antibody signatures. The cross-population comparative analysis testing the 16 IgG antibodies from the "East-Asian antibody signature of EBV-positive cHL" in the European population revealed 87% consistency (14 out of 16 antibodies). Conversely, testing the 18 antibodies (16 IgG and 2 IgA) from the "'European antibody signature of EBV-positive cHL" in the East-Asian population showed 83% consistency (15 out of 18 antibodies). Overall, we identified a total of 29 antibodies that consistently exhibited significant elevation in EBV-positive cHL cases in both populations and represented the "Generalized signature of EBV-positive cHL." Five of these anti-EBV IgG antibodies targeting LMP-1, TK, BALF2, BDLF3, and BBLF1 were identified in both cross-population analyses and form a universal and robust "Core antibody signature for EBV-positive cHL." Created with BioRender.com.

TABLE 3 The list of anti-EBV antibodies targeting EBV proteins as the "Generalized" and "Core" antibody signatures of EBV-positive cHL from European and East-Asian study populations.

	Protein name	Description	Array sequence	IgG/IgA	Generalized signature of EBV+ cHL	Core signature of EBV+ cHL	*t*-test cross-population *p*-value
East-Asian signature	THY.KINASE	Early lytic	YP_001129497.1-133399-131576	IgG	x	y	**6.85×10^{-7}**
	BBLF1	Early lytic	AFY97956.1-108555-108328	IgG	x	y	**9.19×10^{-7}**
	BDLF3	Glycoprotein	AFY97964.1-118644-117940	IgG	x	y	**3.40×10^{-5}**
	BALF2 (EA(D) p138)	Early lytic	YP_001129510.1-165796-162410-1	IgG	x	y	**1.17×10^{-4}**
	LMP1	Latent	YP_401722.1-168507-167702	IgG	x	y	**1.46×10^{-4}**
	BPFL1	Late lytic	YP_001129449.1-59370-49906-2	IgG	x		**2.18×10^{-4}**
	BPFL1	Late lytic	YP_001129449.1-59370-49906-3	IgG	x		**4.23×10^{-4}**
	BLLF1 (gp350/220)	Glycoprotein	YP_001129462.1-79936-7727. r	IgG	x		**.001**
	LF2	Early lytic	YP_001129504.1-151808-150519	IgG	x		**.002**
	LMP2B	Latent	AFY97910.1-1026-1196	IgG	x		**.002**
	EBNA3A	Latent	AFY97915.1-80252-82747	IgG	x		**.004**
	EBNA3A	Latent	YP_401669.1-80382-82877	IgG	x		**.010**
	BDLF2	Glycoprotein	YP_001129491.1-120928-119666	IgG	x		**.017**
	EBNA3A	Latent	YP_001129463.1-80447-82888	IgG	x		**.034**
	EBNA-LP	Latent	YP_001129440.1-20824-20955	IgG			.066
	LMP2A	Latent	YP_001129436.1-167587-167942	IgG			.106
European signature	THY.KINASE	Early lytic	YP_001129497.1-133399-131576	IgG	x	y	**3.72×10^{-4}**
	LMP1	Latent	YP_401722.1-168507-167702	IgG	x	y	**.001**
	BBLF1	Tegument protein	YP_001129480.1-109516-109289	IgA	x		**.001**
	BBLF1	Tegument protein	AFY97956.1-108555-108328	IgA	x	y	**.001**
	BBLF1	Tegument protein	AFY97956.1-108555-108328	IgG	x		**.002**
	BALF2 (EA(D)_p138)	Early lytic	YP_001129510.1-165796-162410-1	IgG	x	y	**.002**
	BFLF2	Late lytic)	YP_001129443.1-44763-43807	IgG	x		**.006**
	BDLF3	glycoprotein 150	AFY97964.1-118644-117940	IgG	x	y	**.008**
	BDLF3	glycoprotein 150	YP_001129490.1-119605-118901	IgG	x		**.01**
	BHRF1)	Bcl-2 homolog)	YP_001129442.1-42204-42779	IgG	x		**.016**
	BdRF1	VCA_p40	AFY97974.1-136284-137321_US	IgG	x		**.018**
	BBRF1	Late lytic	YP_001129476.1-102746-104587	IgG	x		**.024**
	BcLF1	VCA_p160)	CAA24794.1-137466-133321-1	IgG	x		**.03**
	BBLF1	Tegument protein	YP_001129480.1-109516-109289	IgG	x		**.04**
	BBRF3	glycoprotein M	YP_001129479.1-107679-108896	IgG	x		**.049**
	BcLF1	VCA_p160	AFY97965.1-125044-120899-1	IgG			.068
	BcLF1	VCA_p160	YP_001129493.1-126005-121860-1	IgG			.082
	BARF1	Oncogene	YP_001129453.1-66746-67654	IgG			.100

Note: The table is ordered by significant *t*-test *p*-value (lowest to highest). The 29 antibodies consistently exhibited significant elevation in EBV-positive cHL cases in both populations and representing the "Generalized signature of EBV-positive cHL" are marked in "x." The anti-EBV IgG antibodies targeting the five EBV proteins LMP-1, TK, BALF2, BDLF3, and BBLF1 representing "core antibody signature for EBV-positive cHL" are marked in "y." Significant *p*-values at $p < 0.05$ are in bold.

robust "Core antibody signature for EBV-positive cHL" (Table 3, Figure 3 and Figure S4).

To assess whether the overlap between these two antibody signatures was higher than expected by chance, we implemented a two-set intersection analysis using SuperExactTest developed by Wang et al[27] This test demonstrated that the intersection between the two signatures (five IgG antibodies) significantly exceeded the expected number of antibodies that would be obtained if the two population-

specific signatures were randomly selected from the original list of 199 proteins and 3 peptide sequences ($n = 1.26$) (SuperExactTest, $p < .004$, 3.9-fold enrichment).

Overall, our results demonstrated a high similarity between the EBV antibody profile of East-Asian and European EBV-positive cHL patients, and that antibody responses against a core of five core proteins, namely, LMP-1, TK, BALF2, BDLF3, and BBLF1, reflect a specific gene expression pattern of EBV in cHL, independent of population-specific effects such as ethnicity, host genetic variation, and EBV pathogen dynamics.

4 | DISCUSSION

Multiple lines of evidence indicate that EBV plays a critical etiological role in cHL,[4] with EBV-positive and EBV-negative cHL mostly considered two distinct diseases. Aberrant levels of EBV-specific antibodies and the expression of several viral antigens (i.e., EBNA1, LMP-1, and LMP-2) have been reported in malignant cHL tumor cells with potential pathogenic functions.[4,7] However, the biological mechanisms underlying the contribution of EBV to cHL pathogenesis remain elusive.

In a previous European study, we examined antibody responses to the complete EBV proteome in cHL patients and matched controls and identified 18 antibodies (16 IgG and two IgA) that were significantly elevated in EBV-positive cHL cases that formed the "European antibody signature of EBV-positive cHL."[24] To the best of our knowledge, that was the first study measuring the EBV proteome-wide antibody responses in cHL patients. Our current research extends that study by analyzing antibodies against the entire EBV proteome in an independent case–control study involving an East-Asian population.

In this study, we observed more robust IgG antibody responses against EBV than IgA. IgA antibodies typically indicate ongoing or recent EBV reactivation in the oral epithelium, whereas IgG antibodies are the predominant type produced in response to cumulative exposure to EBV.[28] These findings suggest systemic exposure of circulating B cells to EBV infection rather than recent reactivation at mucosal surfaces.[29] Consequently, these IgG antibodies could serve as relevant markers for lymphoid tumors. We have previously reported broader and stronger IgG responses than IgA responses against EBV in individuals diagnosed with cHL in the European population, consistent with findings in other studies of EBV-associated diseases, such as endemic Burkitt's lymphoma and NKTCL.[30]

When we applied our computational pipeline used in the European study to our data from the East-Asian Asia Lymph Study, we identified 16 IgG antibodies but no IgA antibodies that were significantly associated with cHL, representing the "East-Asian antibody signature of EBV-positive cHL ". The most significant IgG antibody responses associated with EBV-positive cHL were against latent proteins such as LMP-1, EBNA3A, and EBNA-LP, which is consistent with the possible roles of these latent proteins in disease pathogenesis.[31] The presence of LMP-1 has historically been considered a defining feature of Reed-Sternberg cells in EBV-associated Hodgkin's lymphoma.[32] LMP-1 expression, determined by immunohistochemistry, is a surrogate marker of EBV-positive cHL. LMP-1 mimics CD40, directing constitutive activation of NF-κB signaling, which is essential for EBV-induced transformation and enhanced B-cell survival. However, antibody responses against EBNA-LP and EBNA-3A have not been previously reported to be associated with EBV-positive cHL. EBNA-LP is recognized as the first latent protein in resting B cells after EBV infection.[33] It facilitates EBNA2-mediated transcriptional activation and is essential for EBV-mediated B-cell immortalization. EBNA-3A typically functions as a transcriptional regulator involved in B-cell transformation and immortalization. Recently, we reported significantly higher IgG antibodies against EBNA3A in NKTCL.[24]

Glycoproteins (BLLF1 [gp350/220], BDLF2, and BDLF3) and early lytic proteins, including TK, BALF2, BBLF1, and LF2, were significantly associated with EBV-positive cHL in the East-Asian population. Glycoprotein 150, encoded by BDLF3, functions as a novel viral immune evasion molecule.[34] BDLF2, a glycoprotein that interacts with BMRF2, has been implicated in epithelial cell infection.[35] BLLF1 (gp350/220) is abundantly expressed in the viral envelope and plays a critical role in viral entry and infection of target cells, contributing to the generation of neutralizing antibodies in vivo. Elevated responses to early lytic proteins suggest increased exposure to lytic viral replication and virion maturation in EBV-positive cHL patients,[36] although the timing of this lytic activity in relation to cHL diagnosis remains uncertain.

Consistent with our findings in the European population,[24] we observed no differences in the anti-EBV antibody profile between EBV-negative cHL cases and controls in the East-Asian population in the Asia Lymph study.

A crucial aspect of disease biomarkers is their generalizability, which refers to how effectively the antibody signature identified in one population can be applied to another population independent of potential population-specific effects due to ethnicity, host genetics, pathogen dynamics, etc. To assess this, we tested the applicability of the European signature of EBV-positive cHL in the East-Asian population, and vice versa. This involved cross-population comparisons to determine whether the antibodies identified in one study population were consistently elevated in another, ensuring the robustness and transferability of the biomarker signatures.

We identified 29 antibodies, representing 85.3% of the 34 antibodies present in both the East-Asian ($n = 16$) and European ($n = 18$) antibody signatures, which maintained a significant elevation in cHL patients when tested in the other population. This subset of 29 antibodies consistently elevated in EBV-positive cHL subjects in cross-population comparisons represents a "Generalized signature of EBV-positive cHL." The broad reproducibility of these antibodies across diverse populations and their robust association with cHL in EBV-positive individuals imply a potential role in cHL pathogenesis, independent of genetic or population-specific factors.

Notably, our investigation identified LMP-1, TK, BALF2, BDLF3, and BBLF1 as overlapping "core signatures of EBV-positive cHL" in both populations. This overlap is significantly greater than expected by chance, suggesting a shared etiological basis and reflecting a

specific gene expression pattern of EBV in cHL across diverse populations.

Our study was limited by its retrospective nature and the small number of samples analyzed using a proteome array. A prospective study design is required to explore the anti-EBV antibody profiles before disease onset. However, the rarity of this disease in the overall population makes it challenging to conduct such studies. The study samples from the East-Asian populations in Hong Kong and Taiwan may not fully represent the overall population-wide prevalence of EBV-positive cHL. Furthermore, the lower sensitivity of the EBV DNA viral load in distinguishing EBV-positive from EBV-negative cHL could lead to missed cases, potentially resulting in a lower expected proportion of EBV-positive cHL. Additional information on the history of infectious mononucleosis, education level, cigarette smoking, and other potential confounding factors for the study subjects was not available in the current study. A technical limitation includes the inability of proteomic arrays to detect antibody responses to conformational epitopes, thus limiting information on the posttranslational processing associated with cHL. However, our previous findings were validated using multiplex serology[37] and enzyme-linked immunosorbent assay specifically tested for synthetic VCAp18, EBNA1, and EAdp47 peptides, which are designed to detect conformational epitopes.[21,23]

In summary, this study identified a generalized anti-EBV signature of cHL in EBV-positive individuals comprising 24 consistently elevated anti-EBV IgG antibodies across diverse populations. This robust association transcends population-specific variation. Additionally, the identified core EBV proteins, including LMP-1, TK, BALF2, BDLF3, and BBLF1, constitute overlapping "core signatures" in both populations, suggesting a shared etiological basis in cHL pathogenesis and as potential biomarkers of EBV-positive cHL. This knowledge could refine the diagnostic strategies for cHL and present an opportunity to identify novel therapeutic interventions for individuals with EBV-positive cHL.

AUTHOR CONTRIBUTIONS

Yomani D. Sarathkumara: Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Rena R. Xian:** Data curation; investigation; resources; writing – review and editing. **Zhiwei Liu:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Kelly J. Yu:** Investigation; project administration; resources; writing – review and editing. **John K. C. Chan:** Data curation; project administration; resources; writing – review and editing. **Yok-Lam Kwong:** Data curation; project administration; resources; writing – review and editing. **Tai Hing Lam:** Data curation; project administration; resources; writing – review and editing. **Raymond Liang:** Data curation; methodology; project administration; resources; writing – review and editing. **Brian Chiu:** Data curation; project administration; resources; writing – review and editing. **Jun Xu:** Data curation; project administration; resources; writing – review and editing. **Wei Hu:** Data curation; project administration; resources; writing – review and editing. **Bu-Tian Ji:** Data curation; project administration; resources; writing – review and editing. **Anna E. Coghill:** Investigation; resources; writing – review and editing. **Ashton M. Kelly:** Visualization; writing – review and editing. **Ruth M. Pfeiffer:** Formal analysis; writing – review and editing. **Nathaniel Rothman:** Data curation; investigation; methodology; project administration; resources; writing – review and editing. **Richard F. Ambinder:** Methodology; project administration; resources; writing – review and editing. **Allan Hildesheim:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; writing – original draft; writing – review and editing. **Qing Lan:** Conceptualization; data curation; project administration; resources; writing – review and editing. **Carla Proietti:** Conceptualization; formal analysis; investigation; methodology; supervision; validation; writing – original draft; writing – review and editing. **Denise L. Doolan:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author upon request.

ETHICS STATEMENT

This study was approved by the Institutional Review Board (IRB) of the US National Institutes of Health and the US National Cancer Institute (#11CN206). Written informed consent was obtained from all participants. All laboratory tests reported in this study were conducted according to a protocol approved by the James Cook University Human Research Ethics Committee (#H7696).

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SUPPORTING INFORMATION

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

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