ORIGINAL ARTICLE

Identification of anti-Epstein-Barr virus (EBV) antibody signature in EBV-associated gastric carcinoma

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Abstract

Background Around 10% of gastric carcinomas (GC) contain Epstein–Barr virus (EBV) DNA. We characterized the GC-specific antibody response to this common infection, which may provide a noninvasive method to detect EBV-positive GC and elucidate its contribution to carcinogenesis.

Methods Plasma samples from EBV-positive ($n = 28$) and EBV-negative ($n = 34$) Latvian GC patients were immune-profiled against 85 EBV proteins on a multi-microbial Nucleic Acid Programmable Protein Array (EBV-NAPPA). Antibody responses were normalized for each sample as ratios to the median signal intensity (MNI) across all antigens, with seropositivity defined as MNI ≥ 2. Antibodies with $\geq 20\%$ sensitivity at 95% specificity for tumor EBV status were verified by enzyme-linked immunosorbent assay (ELISA) and validated in independent samples from Korea and Poland ($n = 24$ EBV-positive, $n = 65$ EBV-negative).

Results Forty anti-EBV IgG and eight IgA antibodies were detected by EBV-NAPPA in $\geq 10\%$ of EBV-positive or EBV-negative GC patients, of which nine IgG antibodies were discriminative for tumor EBV status. Eight of these nine were verified and seven were validated by ELISA: anti-LF2 (odds ratio = 110.0), anti-BORF2 (54.2), anti-BALF2 (44.1), anti-BaRF1 (26.7), anti-BXLF1 (12.8), anti-BRLF1 (8.3), and anti-BLLF3 (5.4). The top three had areas under receiver operating characteristics curves of 0.81–0.85 for distinguishing tumor EBV status.

Conclusions The EBV-associated GC-specific humoral response was exclusively directed against lytic cycle immediate-early and early antigens, unlike other EBV-associated malignancies such as nasopharyngeal carcinoma and lymphoma where humoral response is primarily directed against late lytic antigens. Specific anti-EBV antibodies could have utility for clinical diagnosis, epidemiologic studies, and immune-based precision treatment of EBV-positive GC.

Keywords Molecular subtyping · EBV-positive tumors · Noninvasive biomarkers · Viral reactivation

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Introduction

Gastric carcinoma (GC) is the third leading cause of cancer mortality worldwide, with an estimated 783,000 deaths and more than 1 million new diagnoses in 2018 [1]. While *Helicobacter pylori* infection is considered the primary etiology, around 8–10% of GC tumors also have evidence of Epstein–Barr virus (EBV). As a ubiquitous infection found in over 95% of adults, the implications of EBV positivity in GC are still not fully understood [2–4]. Reactivation of latent EBV infection may increase cell turnover and DNA replication errors contributing to malignant transformation.

EBV-positive GC is a distinct subtype of GC that has overall lower mortality [4–7], occurs more frequently in cases among males than females, and displays significantly greater intra- or peritumoral immune cell infiltration compared to EBV-negative GC [4, 7]. In The Cancer Genome Atlas studies of upper and lower gastrointestinal tract adenocarcinoma, EBV-positive tumors were found only in the stomach and recognized as a separate molecular subtype based on multiple unique features [8]. Current research on EBV-positive GC has focused on epigenetic and genetic aberrance [3, 4, 6, 9]. EBV-positive GC displays unique molecular characteristics including recurrent *PIK3CA* mutations, extreme DNA hypermethylation, and amplification of *JAK2, PD-L1* and *PD-L2* [6, 9, 10]. Given the biologic implications of these abnormalities, distinguishing EBV-positive GC from EBV-negative GC could potentially improve clinical management of GC patients through targeted therapies such as immune checkpoint inhibitors. Already, EBV-positive tumor status has been associated with favorable clinical response to PD-1 inhibition in metastatic GC [11].

EBV-positivity of tumor tissue may be determined through in situ hybridization for EBV-encoded small RNA (EBER), a sensitive and specific marker for viral presence [12]. However, tumor EBV status is not routinely assessed in pathologic practice. Thus, a blood-based assay would allow for tumor classification in research or clinical settings where tissue diagnosis is unavailable, with utility for etiologic studies as well as the guidance of targeted therapy.

Anti-EBV antibodies have been found in patients with EBV-associated cancers but their performance in GC remains controversial [12–17]. We previously reported associations of GC tumor EBV status with higher levels of antibodies against EBNA1, BFRF3/ VCAp18, BMRF1/ EA-D, and BZLF1/ZEBRA [18]. Other than these four anti-EBV antibodies, circulating EBER has also been investigated for noninvasive identification of EBV-positive GC, with limited success [19]. Currently, no serologic markers have sufficient discriminative power to be used clinically. Comprehensive profiling of immune response against all proteins encoded by EBV could lead to novel biomarkers for noninvasive identification of EBV-positive GC, as has been successfully applied for the diagnosis of two other EBV associated cancers, nasopharyngeal carcinoma (NPC) and Hodgkin lymphoma (HL) [20, 21].

Nucleic acid programmable protein array (NAPPA) is an innovative protein microarray technology that substitutes a complex protein production, purification, and fabrication process with the printing of plasmid DNA, in vitro cell-free expression and in situ capturing [22, 23]. We have developed and applied NAPPA to study disease-related antibodies against proteins from various bacterial and viral agents, including EBV [24].

Here we report an application of this technology to investigate the anti-EBV immunoproteome in GC cases from the NCI's International EBV-Gastric Cancer Consortium (https://dceg.cancer.gov/research/who-we-study/cohorts/ebv-gastric-cancer-consortium).

Materials and methods

Subjects

EBV-positive and EBV-negative GC patients were identified from three participating centers in Latvia, Korea, and Poland. For all three series, in situ EBER hybridization testing of GC tissue was utilized to distinguish EBV tumor status [18, 25]. Ethylenediaminetetraacetic acid (EDTA)-plasma samples for marker discovery were obtained from 28 Latvian EBV-positive GC patients frequency-matched to 34 with EBV-negative tumors by age at diagnosis (overall mean, 63 years), sex (89% males), Lauren histological type (24% diffuse, 61% intestinal, 15% mixed/unspecified) and anatomical subsite (5% cardia, 95% non-cardia). Blood samples for marker validation were collected from 24 EBV-positive and 65 EBV-negative GC patients from Korea (plasma) and Poland (serum) with comparable clinical characteristics (mean age 57 years, 78% males, 56% diffuse-type, 26% intestinal-type, and 54% noncardia; Supplementary Table 1) as well as 50 population-based cancer-free controls identified by the center in Poland. Laboratory personnel performing biospecimen assays were blinded to patient characteristics and tumor EBV status. All subjects provided informed consent and the original studies were approved by Institutional Review Boards in Latvia, Korea, Poland, and NCI (Bethesda, MD, USA).

EBV-positive GC-associated antibody discovery on NAPPA

The NAPPA was fabricated with the same procedure as previously reported [26, 27]. We included 89 EBV open reading frames (ORFs) from 85 total unique proteins for Type-1 EBV (B95-8) and 1527 ORFs from *H. pylori*, along with 104 ORFs from several other microbes [28]. By stage of expression in the EBV replication cycle, there were 2 ORFs from immediate-early lytic phase, 31 from early lytic phase, 32 from late lytic phase, 12 from latent phase, and 12 of unknown phase (Supplementary Table 2). All clones were obtained from DNASU (https://dnasu.org/DNASU/Home.do; Tempe, AZ, USA) in a NAPPA compatible pANT7-cGST expression vector [24, 29].

Proteins were expressed by in vitro transcription and translation. Expression levels of all microbial proteins exceeded no DNA wells (mean + 3 standard deviations), as confirmed by a monoclonal mouse anti-GST antibody (Supplementary Fig. 1A). Arrays were probed with 1:100 diluted plasma from the discovery sample set, followed by incubation with 1:200 diluted Alex647 labeled Goat anti-human IgG (H + L) and 1:200 diluted Cy3 labeled Goat anti-human IgA (Jackson ImmunoResearch Labs, PA, USA), to evaluate specific anti-EBV IgG and IgA antibodies. IgG and IgA antibody binding signals were detected with a bi-color Tecan PowerScanner (Tecan Group Ltd., Männedorf, Switzerland) at 635 nm and 532 nm as two separate images, which were further analyzed with ArrayPro Analyzer Software (Media Cybernetics, Inc., MD, USA) to generate raw fluorescence intensity data. A pooled plasma that combined all samples was probed along with individual samples on each run day to determine array reproducibility. The inter-slide correlation coefficient r for pooled samples was 0.95 (Supplementary Fig. 1B).

Antibody responses on NAPPA were analyzed as Median Normalized Intensity (MNI) via dividing by the median signal intensity of all proteins within each array. Seropositive responses were defined as MNI ≥ 2.0. Antibodies that showed at least 10% seropositivity in either EBV-positive or EBV-negative GC were assessed for discrimination between these groups. Using an MNI cutoff at 95% specificity for EBV-negative GC with minimum cutoff 2.0, anti-EBV antibodies with more than 20% sensitivity for EBV-positive GC on NAPPA were selected as candidates for further evaluation. Furthermore, the ten most prevalent anti-*H. pylori* antibodies were compared between EBV-positive and -negative GC.

Verification and validation of anti-EBV antibodies on RAPID ELISA

Candidate biomarkers were verified in the discovery sample set by Rapid Antigenic Protein In Situ Display (RAPID) ELISA following a previously reported protocol [30]. In brief, 96-well ELISA plates (Corning, NY, USA) were first coated with goat anti-GST antibody (GE Healthcare Bio-Sciences, PA, USA) and incubated with candidate GST tag fusion antigen expressed with IVTT. After washing, 1:500 diluted plasma/serum samples were added, followed by incubation with HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch Labs, PA, USA). Plates were developed using TMB substrate (Thermo Fisher Scientific, MA, USA), and optical density at 450 nm (OD450) was measured on a PerkinElmer Envision plate reader (Waltham, MA, USA). GST tag alone was set as a blank control, and ELISA readings were normalized by subtracting the OD450 of GST alone from OD450 of the target protein. Using an OD cutoff at 95% specificity for EBV-negative GC with minimum cutoff 0.1, markers verified as more than 20% sensitive were further evaluated in the validation cases and controls. Sensitivity and specificity in the validation sample ELISAs were calculated using the same cutoffs that were generated with the discovery sample set. p values were calculated based on chi-square tests and antibodies with $p < 0.05$ were designated to be validated.

Statistical analyses

The difference in quantitative antibody response on NAPPA and differences between numbers of seropositive antibodies in EBV-positive and -negative GC and healthy controls were assessed by the Mann–Whitney U (MW) test. Odds ratios (OR) were analyzed for statistical significance by chi-square tests to select and validate markers in discovery and validation sample sets, respectively. The discriminatory power of selected markers was further evaluated in the validation sample set by the area under the receiver operating characteristics (ROC) curve (AUC). Lasso logistic regression model was used to construct antibody panel models using the validation data set, and classification performance was evaluated by AUC 95% confidence intervals (95% CI). Pearson correlation coefficients were used to assess pair-wise correlations between antibody responses in the validation sample set. All statistical tests were two-sided and p-values < 0.05 were considered statistically significant. The significance level was corrected for the number of examined markers with the Bonferroni procedure. Statistical analyses were conducted with Stata version 15 (Stata Corp, College Station, TX, USA), GraphPad Prism 8.0.2 (GraphPad Software, Inc., CA, USA) and R version 3.6 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Results

Antibody discovery by NAPPA

Immunoprofiling of discovery samples by NAPPA identified a total of 41 antibodies seropositive in at least 10% of either EBV-positive or EBV-negative GC patients, including 7 with both IgG and IgA, 33 with IgG only, and 1 with IgA only antibody (Fig. 1). Twenty-six of the 40 IgG antibodies and 2 of the 8 IgA antibodies were common to both EBV-positive and EBV-negative GC. Anti-EBNA1 and anti-BFRF3 were the most prevalent IgG antibodies, present in more than 90% of both EBV-positive GC and EBV-negative GC. Fourteen IgG and five IgA antibodies were only present in EBV-positive GC, whereas one IgA but no IgG antibody was found only in EBV-negative GC.

Median signal intensity for individual antibodies ranged up to 4.3-fold higher in EBV-positive as compared to EBV-negative GC (Fig. 2). Notably, there was no difference in signal intensity between EBV-positive and EBV-negative GC for anti-EBNA1 (median MNIs of 4.2 *vs.* 4.7, respectively, $p = 0.348$) or anti-BFRF3 (25.0 *vs.* 27.1, $p = 0.850$) by Mann–Whitney tests.

Out of the 85 EBV proteins displayed on NAPPA, the EBV-positive GC samples had a median of 20 seropositive IgG antibodies while the EBV-negative GC samples had a median of 14 (MW = 284.5, $p = 0.006$). EBV-positive GC had significantly more IgG antibodies than EBV-negative GC to immediate-early lytic, early lytic, and unknown
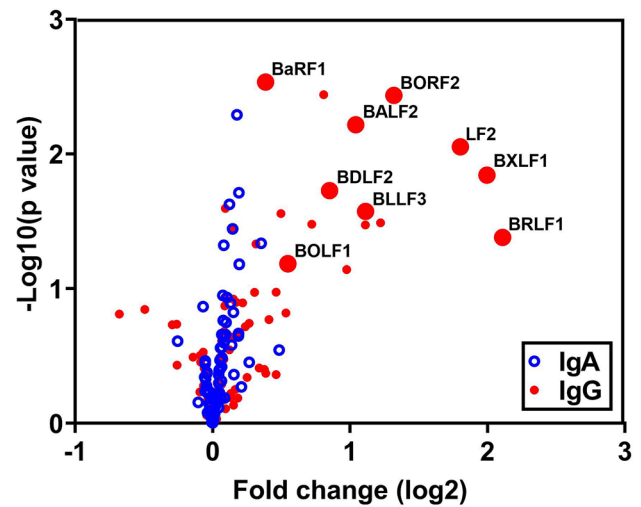
Fig. 2 Magnitude and statistical significance of differential antibody responses by NAPPA between EBV-positive and EBV-negative GC. Labels indicate viral antibodies that have ≥ 20% sensitivity at 95% specificity for EBV-positive GC. *IgA* immunoglobulin A, *IgG* immunoglobulin G, *GC* gastric carcinoma, *NAPPA* nucleic acid programmable protein array

phase proteins (Fig. 3). Both sample groups had medians of 1.0 seropositive IgA antibody ($p = 0.737$).

Nine IgG antibodies were elevated in EBV-positive GC with greater than 20% sensitivity at 95% specificity: anti-BALF2, anti-LF2, anti-BORF2, anti-BaRF1, anti-BRLF1, anti-BLLF3, anti-BXLF1, anti-BDLF2 and anti-BOLF1 (Table 1). None of the IgA antibodies were 20% sensitive and 95% specific for EBV-positive GC, and no IgG or IgA
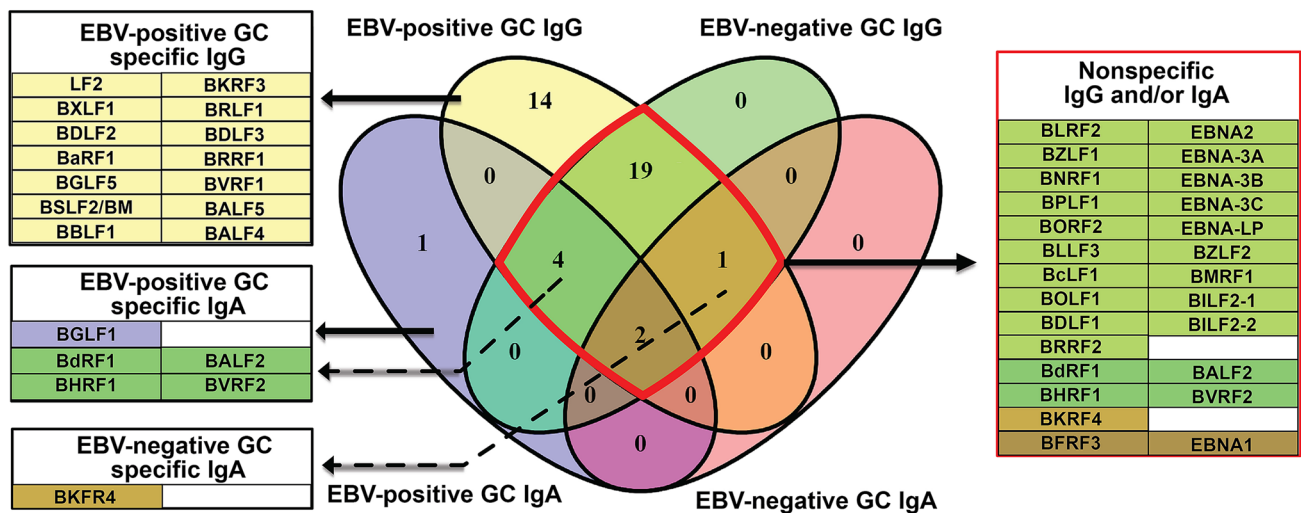
Fig. 1 Anti-EBV IgG and IgA antibodies with > 10% seropositivity by NAPPA in either EBV-positive or EBV-negative GC. Antibodies specific for either EBV-positive ($n = 19$) or EBV-negative GC ($n = 1$) are listed on the left and nonspecific antibodies ($n = 26$) are listed on the right. Table colors correspond to groups in the Venn diagram. *EBV* Epstein–Barr virus, *GC* gastric carcinoma, *IgA* immunoglobulin A, *IgG* immunoglobulin G, *NAPPA* nucleic acid programmable protein array

Fig. 3 Numbers of positive IgG NAPPA antibody responses of EBV-positive and EBV-negative GC to EBV proteins expressed at different stages of the viral replication cycle, classified as latent ($n=12$), immediate-early lytic ($n=2$), early lytic ($n=31$), late lytic ($n=32$), and other/unknown ($n=11$). p values represent differences between patient groups with $p > 0.05$ not statistically significant. *EBV* Epstein–Barr virus, *GC* gastric carcinoma, *IgG* immunoglobulin G, *NAPPA* nucleic acid programmable protein array, *NS* not statistically significant
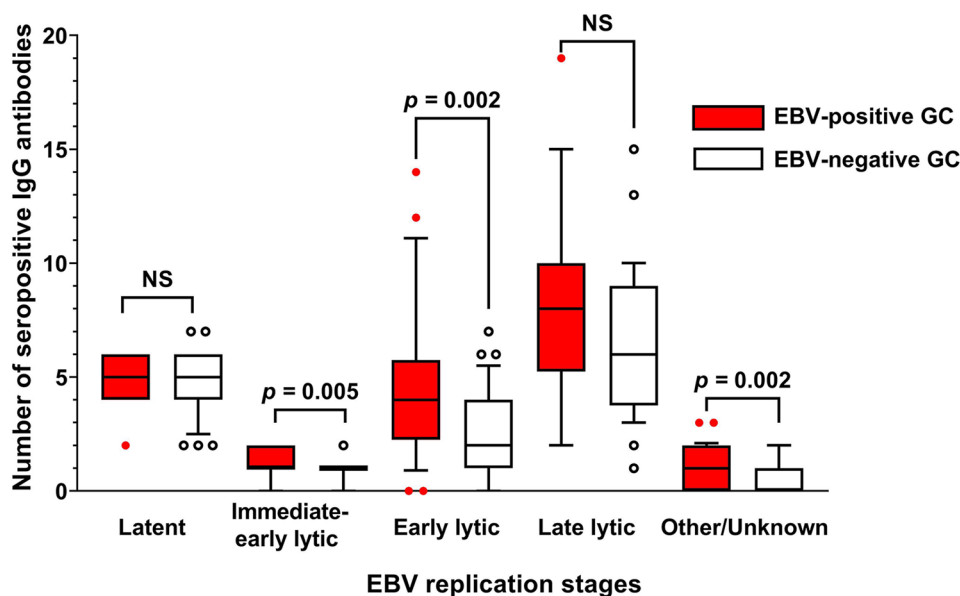
antibodies met these criteria for EBV-negative GC. Reactivity of all nine antibodies was also significantly higher as compared to the cancer-free controls for EBV-positive GC but not for EBV-negative GC.

The ten anti-*H. pylori* IgG antibodies with highest seropositivity in the discovery sample set are listed in Supplementary Table 3. For all ten antibodies, seroprevalence did not significantly differ between EBV-positive and -negative GC samples.

Antibody verification and validation by RAPID ELISA

Using the same discovery sample set (Supplementary Table 1), eight of the nine differential anti-EBV IgG antibodies by NAPPA were verified to have greater than 20% sensitivity at 95% specificity by RAPID ELISA, except for anti-BOLF1 (Table 1). Seven of these eight antibodies were blindly validated to differ at $p < 0.05$ between EBV-positive and EBV-negative GC in an independent validation sample set, except anti-BDLF2. These seven markers were either early lytic or immediate-early lytic phase in EBV life cycle, except anti-LF2 of which the cycle is unknown. Six of the seven validated markers still showed significant differences after Bonferroni correction, satisfying our alternative significance level of $0.05/8 = 0.00625$, except for anti-BLLF3 ($p = 0.018$).

ELISA reactivity was markedly stronger for EBV-positive GC than EBV-negative GC (Fig. 4a). Using the cutoffs for 95% specificity of EBV-negative GC in the discovery samples, the seven validated markers all had ORs exceeding 5.0 for distinguishing EBV status in the validation samples, ranging up to 111 for anti-LF2 (Fig. 4b).

All pair-wise correlations among the nine anti-EBV antibodies were statistically significant with p values < 0.01 in the validation samples (Supplementary Fig. 2). For EBV-positive and EBV-negative GC groups combined, correlation coefficients ranged from 0.35 (anti-BLLF3 *vs.* anti-BXLF1) to 0.79 (anti-BXLF1 *vs.* anti-LF2).

Anti-BALF2 had the greatest discriminatory power among individual antibodies with an AUC of 0.85 (95% CI, 0.75–0.96). AUCs of the other validated antibodies ranged from 0.58 to 0.84 (Table 1). LASSO logistic regression identified a maximal AUC of 0.88 (95% CI, 0.78–0.98) for the three-marker combination of anti-BALF2, anti-BORF2, and anti-LF2.

Discussion

We report the first comprehensive proteome-level study to identify anti-EBV antibodies for EBV-positive GC, identifying seven highly discriminatory IgG biomarkers. Our findings indicate that the EBV-associated GC-specific humoral response is primarily restricted to the lytic cycle immediate-early and early antigens.

The functions of the seven proteins targeted by the identified antibodies include roles in DNA replication, virus maturation, gene transcription, and protein–protein interaction.

Of note, antibodies to BRLF1, BALF2 and BXLF1 were also increased in patients with the epithelial cell tumor NPC [31, 32], but not in those with lymphoma [20, 31, 33, 34]. BRLF1, also known as Rta, encodes one of the two immediate-early EBV lytic proteins that control the initiation of viral lytic gene expression and viral reactivation from latency. BRLF1 expression is specific for viral reactivation

Table 1 Identification of EBV-positive GC discriminatory IgG antibodies

Antibody	EBV protein or activity	Stage of EBV life cycle	Discovery sample NAPPA			Discovery sample ELISA		Validation sample ELISA				
			Se (%) at MNI≥2.0	Sp (%) at MNI≥2.0	Se (%) at 95% Sp	Se (%) at 95% Sp	Verified[a]	Se (%) at discovery cutoff[b]	Sp (%) at discovery cutoff[b]	p value[c]	Validated[d]	AUC
Anti-BALF2	Single-stranded DNA binding protein	Early lytic	89	32	21	43	Yes	58	97	<0.001	Yes	0.85
Anti-LF2	Protein that binds Rta	Unknown	43	94	36	43	Yes	46	100	<0.001	Yes	0.81
Anti-BORF2	Ribonucleotide reductase, large subunit	Early lytic	68	59	36	39	Yes	46	99	<0.001	Yes	0.84
Anti-BaRF1	Ribonucleotide reductase, small subunit	Early lytic	21	100	21[e]	39	Yes	46	97	<0.001	Yes	0.79
Anti-BRLF1	Encodes lytic genes transactivator Rta	Immediate-early lytic	32	97	32[e]	25	Yes	21	97	0.006	Yes	0.58
Anti-BLLF3	dUTPase	Early lytic	36	88	21	39	Yes	21	95	0.018	Yes	0.71
Anti-BXLF1	Thymidine kinase	Early lytic	32	91	32	25	Yes	17	99	0.006	Yes	0.72
Anti-BDLF2	Glycoprotein that binds BMRF2, an important factor for EBV attachment to epithelial cells	Late lytic	29	97	29[e]	32	Yes	13	94	0.324	No	
Anti-BOLF1	Tegument protein binding phospho-protein	Unknown	32	79	25	7	No	(Not tested)				

AUC area under curve, *EBV* Epstein–Barr virus, *ELISA* enzyme-linked immunosorbent assay, *GC* gastric cancer, *IgG* immunoglobulin G, *Se* sensitivity, *Sp* specificity, *MNI* median normalized intensity, *NAPPA* Nucleic Acid-Programmable Protein Array, *OD450* optical density at 450 nm

[a] Defined as sensitivity ≥20%

[b] Using OD450 cutoff for 95% specificity in discovery samples

[c] p value calculated by chi-square test

[d] Defined as p value < 0.05, chi-square test

[e] Based on minimum MNI cutoff 2.0 instead of 95% specificity

Fig. 4 a Heatmaps of IgG antibody responses by ELISA in discovery (top) and validation (bottom) sample sets of EBV-positive GC and -negative GC. Optical density measurements were normalized according to the highest value for each antibody across all samples. Each vertical bar represents a different serum sample. **b** Odds ratio (OR) and 95% confidence intervals (CI) for discovery (top) and validation (bottom) samples based on cutoffs at 95% specificity for EBV-negative GC in the discovery samples. *EBV* Epstein–Barr virus, *ELISA* enzyme-linked immunosorbent assay, *GC* gastric carcinoma
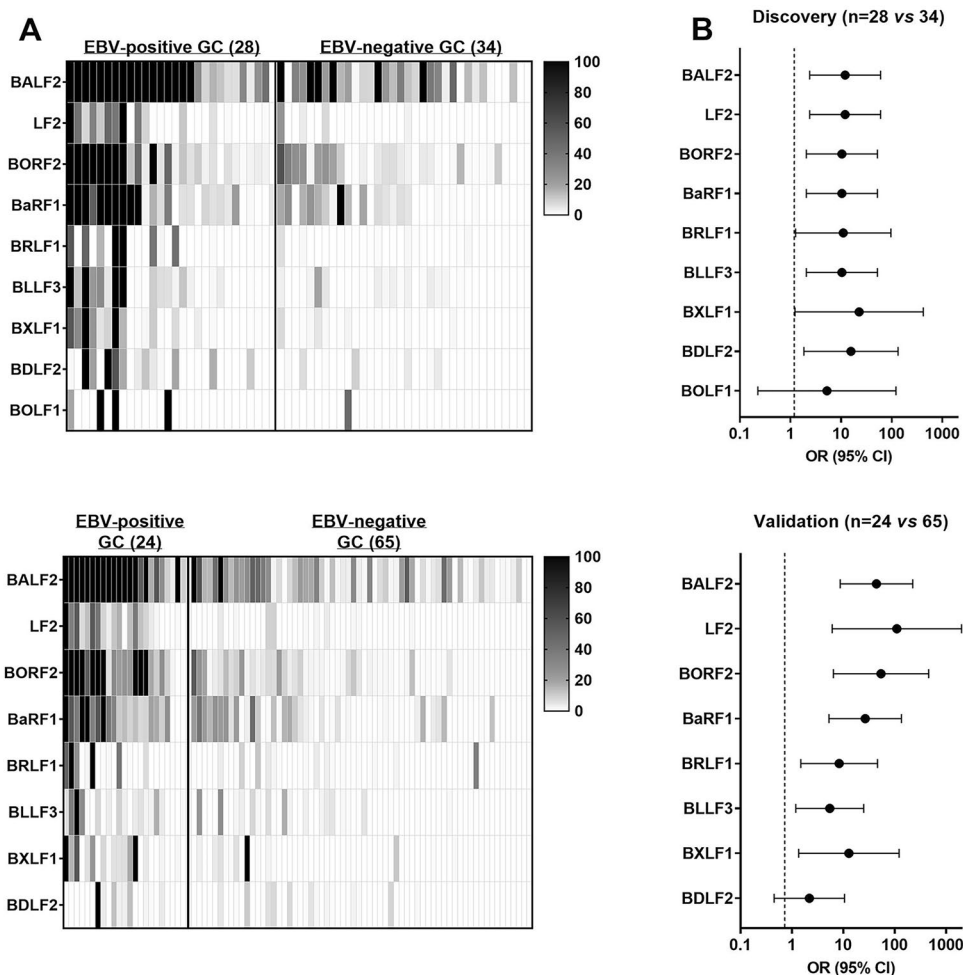
in epithelial cells, while the other immediate-early EBV lytic protein is needed in B cells [35–37]. BALF2 is the major single-stranded DNA binding protein and is required for viral DNA replication [38]. BXLF1 encodes the viral thymidine kinase that catalyzes the phosphorylation of deoxythymidine to deoxythymidine monophosphate, which is important for viral DNA replication [39]. BaRF1 and BORF2 are the ribonucleoside-diphosphate reductase small subunit and large subunit, respectively, and provide precursors necessary for viral DNA synthesis. Their presence enhances virus replication and assists in reactivation of virus from latency in NPC and BL [40, 41]. BORF2 can induce p53 expression to regulate G1/S transition arrest in the cell cycle [38]. This protein also binds with the cellular apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like protein APOBEC3B, inhibiting its DNA cytosine deaminase activity to preserve viral genome integrity [42, 43]. LF2 is a type I interferon antagonist that prevents establishment of an antiviral response by blocking cellular IRF7-mediated innate immunity [44]. It also inhibits viral replication by modulating BRLF1 (Rta) activity [45, 46]. BLLF3 is the viral deoxyuridine 5′-triphosphate nucleotidohydrolase

which modulates innate and adaptive immune responses by engaging toll-like receptor 2 to activate NF- κB and proinflammatory cytokines [33].

EBV has a life cycle alternating between latency and lytic replication [32]. Latency is manifested by persistence in host cells maintained with cell division, while lytic replication results in cell death and virus dissemination [47]. With respect to timing of expression in the EBV replication cycle, one of our EBV-positive GC-specific target antigens are present in immediate-early lytic phase (BRLF1) and five in early lytic phase (BALF2, BXLF1, BLLF3, BaRF1, BORF2). The stage for LF2 expression is unknown. While EBV-positive GC cells are known to express latency proteins with transforming activities, there is increasing evidence suggesting that lytic replication proteins also have an important role in tumor development and progression [47, 48]. Our discovery that antibodies to immediate-early and early lytic proteins are elevated in EBV-positive GC vs. EBV-negative GC is consistent with these concepts. An abortive lytic cycle, where full virus replication does not occur, may result in limited expression of lytic genes that promote tumorigenesis without complete lytic replication

that would lead to cell death; co-expression of these genes along with EBV latency proteins may together be important for induction of EBV-positive GC and may provide new targets for treatment of the disease [47].

Notably, LF2 is highly expressed in EBV-positive GC as reported in the TCGA study [10]. Our remaining six targets represent proteins expressed in the lytic cycle whereas the most highly expressed mRNAs were found to derive from latent transcription. Taken together, these findings suggest that viral replication in non-cancerous tissues may contribute to the pattern of antibody response in circulation.

The links between GC and the candidate markers anti-EA-D (BMRF1) IgG, anti-ZEBRA (BZLF1) IgG, anti-VCA (BFRF3/p18, BdRF1/p40) IgA have been previously reported [31, 49]. Although these antibodies did not pass the stringent discovery criteria in our agnostic screen of the entire immunoproteome, two (anti-BZLF1, anti-BMRF1) had moderately higher median reactivity in EBV-associated GC.

We found no significant difference in seropositivity for the ten most prevalent anti-*H. pylori* IgG antibodies comparing EBV-positive and -negative GC. Using a different platform, a 15-plex Luminex fluorescent bead-based immunoassay, we previously found no overall difference in anti-*H. pylori* seroprevalence between EBV-positive and -negative GC patients, although one antibody, anti-Catalase, had a borderline association [18].

EBV is also implicated in the etiologies of NPC, Burkitt lymphoma, HL, and non-Hodgkin lymphoma [2]. Anti-EBV antibodies in these epithelial and non-epithelial tumors have been assessed by multiplex platforms similar to the current study. In contrast to our findings for EBV-positive GC, the serologic response characterizing other EBV-associated tumors often includes proteins expressed in late lytic and latent phases of viral replication [20, 31–34]. Furthermore, unlike NPC's where IgA antibodies are frequently expressed, EBV-positive GCs largely elicited IgG responses.

For clinical diagnosis of EBV-positive GC tumors, tissue-based approaches such as EBER-ISH and next-generation sequencing have superior sensitivity and specificity. However, EBV status is not routinely assessed in pathologic practice, so EBV serology has particular utility for GC research in settings where tumor tissue is unavailable, such as large-scale epidemiologic cohorts. Further study and technological development of these newly identified markers may also yield useful diagnostics in the future. Given the limited options for identifying EBV-positive GC, apart from antibody profiles, other blood markers of the virus itself and/or host response warrant consideration as biomarkers. Conceivably, multiple markers in combination could be pathognomonic for tumor EBV-positivity.

Our serology study has several strengths. Covering 85 full-length EBV proteins, it is to our knowledge the most comprehensive evaluation of EBV-positive GC immunoproteomics. This approach enabled us to evaluate more viral proteins and the interplay among them, extending previous targeted studies. Second, our findings were consistent across two different assay platforms, increasing the technical validity of the markers. Third, results were replicated in two independent populations of different racial backgrounds. However, our study used post-diagnosis samples that may be reflective of the disease status, limiting interpretation regarding etiologic significance. To investigate causal pathways, well-designed prospective studies will be needed. In addition, our sample size would not have had enough statistical power for detecting associations with small effects, warranting a larger-scale study in the future.

Despite the near-universal infection of adults with EBV, we found seven novel IgG antibodies to discriminate EBV-positive from EBV-negative GC. Unlike nasopharyngeal carcinoma, EBV-specific IgA response does not seem to play an important role in GC. A noninvasive blood test for EBV-positive GC based on the IgG antibodies could have a potential translation to noninvasive detection, preventive screening, precision therapy, and etiologic understanding. Furthermore, the proteins bound by these antibodies, primarily expressed during the early lytic stage of virus replication, may be important for the development, maintenance, or progression of EBV-positive malignancies and represent potential new targets for precision therapeutics.

Compliance with ethical standards

Human rights statement All procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1964 and later versions. The study was excluded from Institutional Review Board Review (OHSR #4337) by the Office of Human Subjects Research Protection, National Institutes of Health.

Informed consent All participants provided written informed consent.

Conflict of interest The authors declare no conflict of interest.

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