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(54) NOVEL ANTIBODIES FOR DETECTING EPSTEIN BARR VIRUS-POSITIVE GASTRIC CANCER

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(57) **ABSTRACT**

Provided herein are methods, compositions, kits, and systems for detecting Epstein Barr virus infection (EBV) in gastric cancer (GC) patients. In particular, provided herein are methods, composition, kits, and systems for diagnosing and treating EBV-positive gastric cancer (EBV^+ GC) in a biological sample of an individual based on the presence and level of antibodies against particular Epstein Barr virus proteins. EBV^+ GC is a distinct subtype of gastric cancer and is associated with unique molecular profiles. Also provided herein are methods for providing more personalized therapy for each of these distinct cancer subtypes and methods for determining an Epstein Barr virus-positive gastric cancer antibody signature, and kits comprising components and protocols for performing the methods of this disclosure.

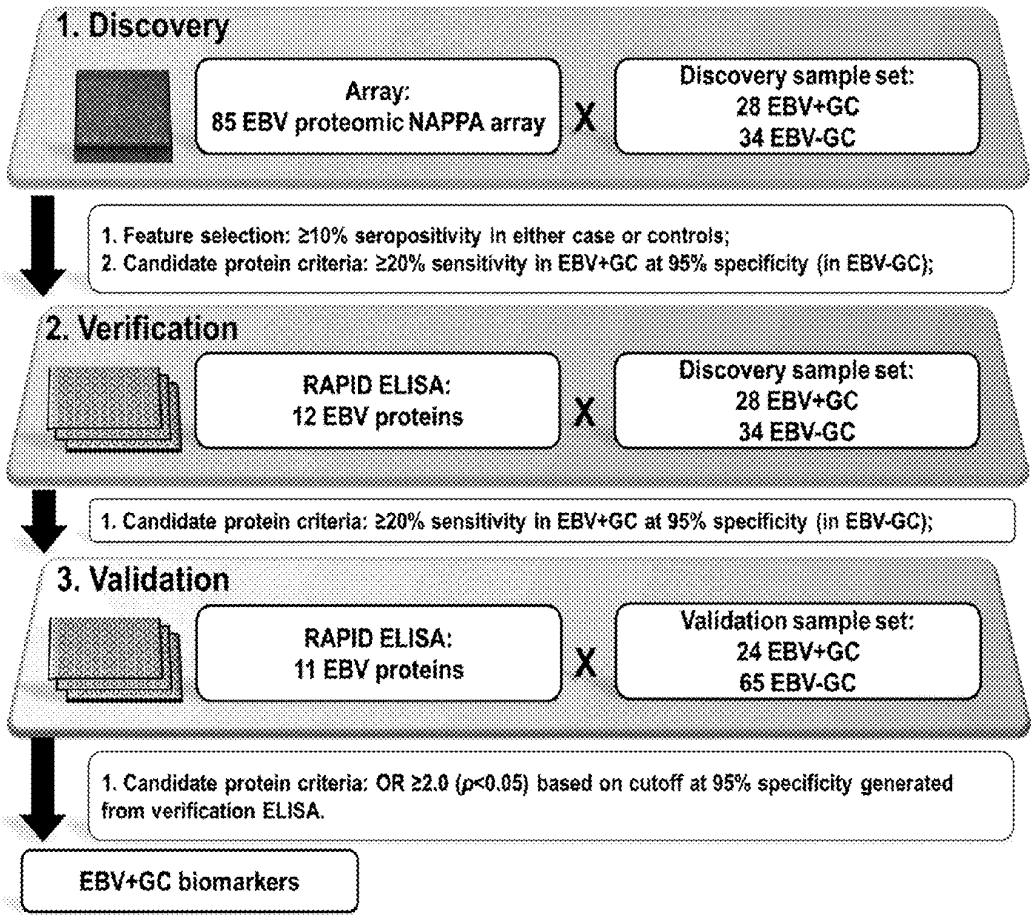


FIG. 1

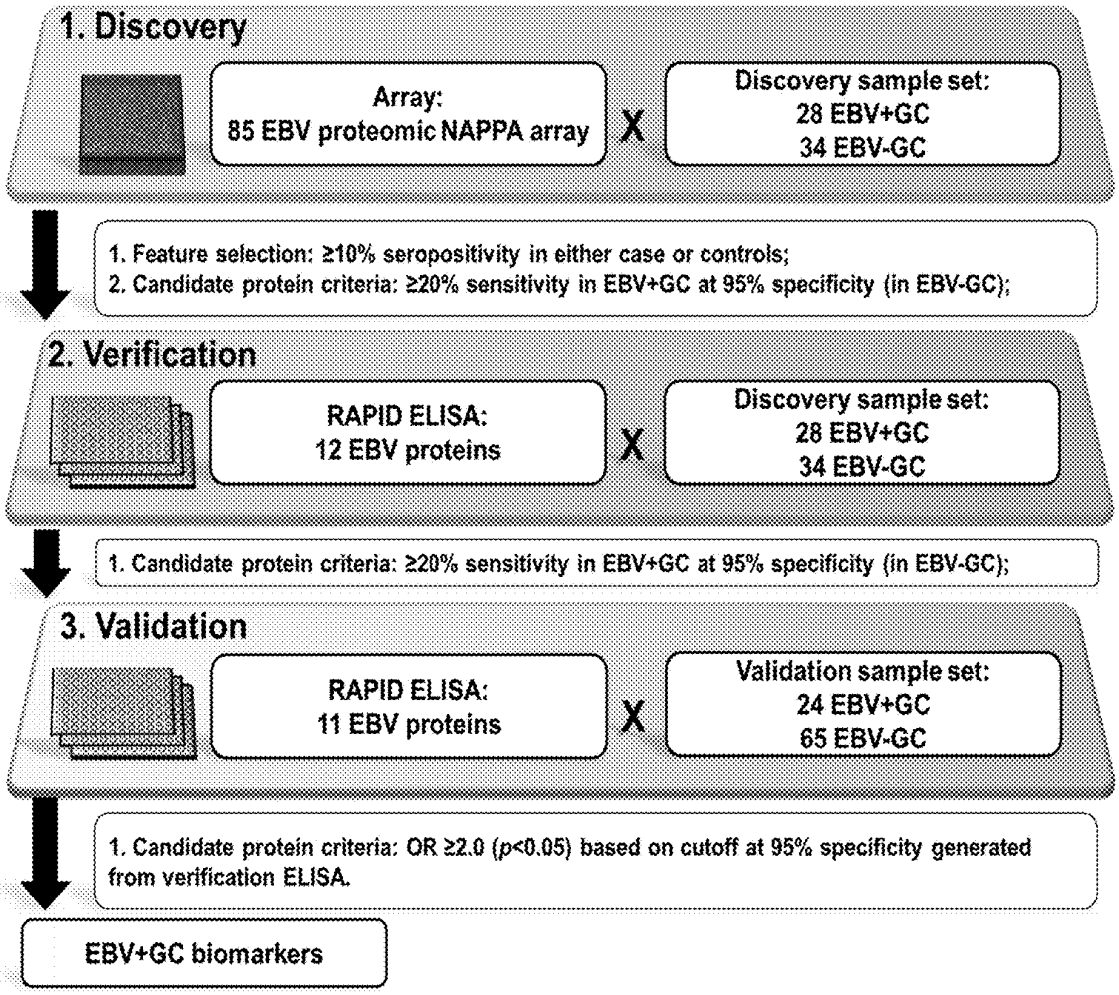

1. Discovery
Array:
85 EBV proteomic NAPPA array
X
Discovery sample set:
28 EBV+GC
34 EBV-GC
1. Feature selection: ≥10% seropositivity in either case or controls;
2. Candidate protein criteria: ≥20% sensitivity in EBV+GC at 95% specificity (in EBV-GC);
2. Verification
RAPID ELISA:
12 EBV proteins
X
Discovery sample set:
28 EBV+GC
34 EBV-GC
1. Candidate protein criteria: ≥20% sensitivity in EBV+GC at 95% specificity (in EBV-GC);
3. Validation
RAPID ELISA:
11 EBV proteins
X
Validation sample set:
24 EBV+GC
65 EBV-GC
1. Candidate protein criteria: OR ≥2.0 (p<0.05) based on cutoff at 95% specificity generated from verification ELISA.
EBV+GC biomarkers

FIG. 2

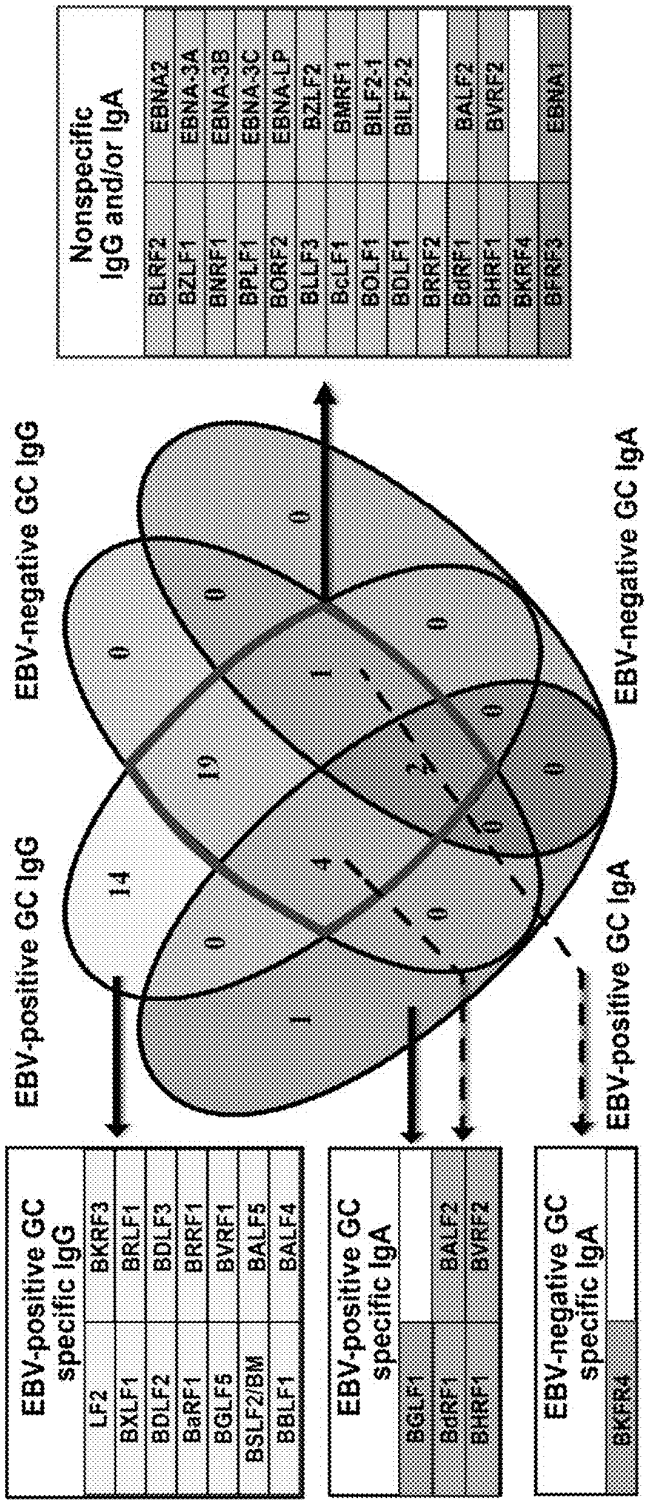

Nonspecific IgG and/or IgA
BLRF2
BZLF1
BNRF1
BPLF1
BORF2
BLLF3
BcLF1
BOLF1
BDLF1
BRRF2
BdRF1
BHRF1
BKRF4
BFRF3
EBNA2
EBNA-3A
EBNA-3B
EBNA-3C
EBNA-LP
BZLF2
BMRF1
BILF2-1
BILF2-2
BALF2
BVRF2
EBNA1
EBV-negative GC IgG
EBV-positive GC IgG
EBV-negative GC IgA
EBV-positive GC IgA
0
14
19
1
4
2
EBV-positive GC specific IgG
LF2
BXLF1
BDLF2
BaRF1
BGLF5
BSLF2/BM
BBLF1
BKRF3
BRLF1
BDLF3
BRRF1
BVRF1
BALF5
BALF4
EBV-positive GC specific IgA
BGLF1
BdRF1
BHRF1
BALF2
BVRF2
EBV-negative GC specific IgA
BKFR4

FIG. 3

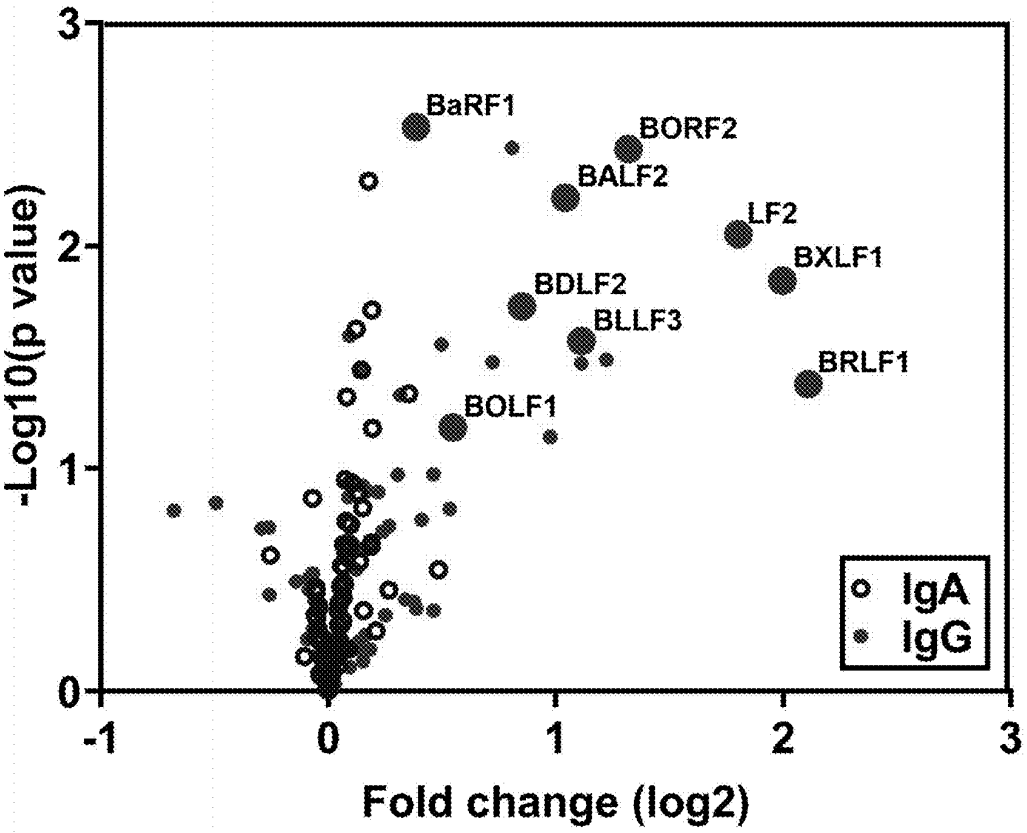

3
2
1
0
-Log10(p value)
-1
0
1
2
3
Fold change (log2)
BaRF1
BORF2
BALF2
LF2
BXLF1
BDLF2
BLLF3
BRLF1
BOLF1
IgA
IgG

FIG. 4

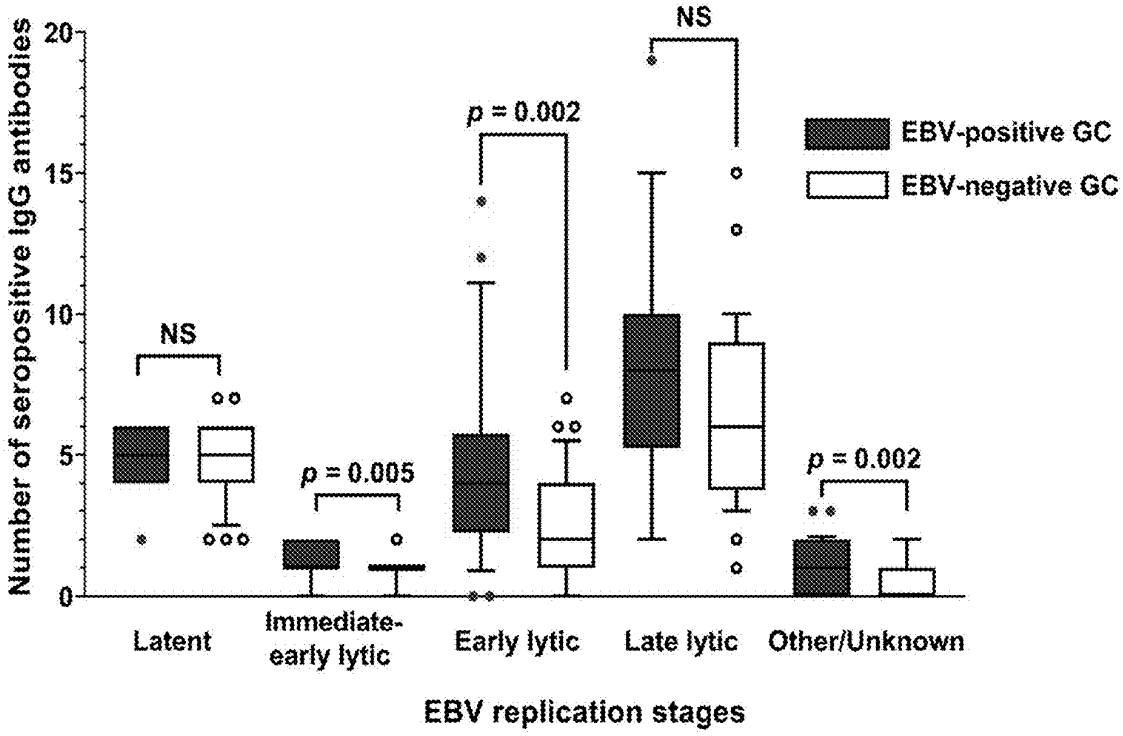

Number of seropositive IgG antibodies
20
15
10
5
0
NS
p = 0.005
p = 0.002
NS
p = 0.002
EBV-positive GC
EBV-negative GC
Latent
Immediate-early lytic
Early lytic
Late lytic
Other/Unknown
EBV replication stages

FIG. 5A

A.

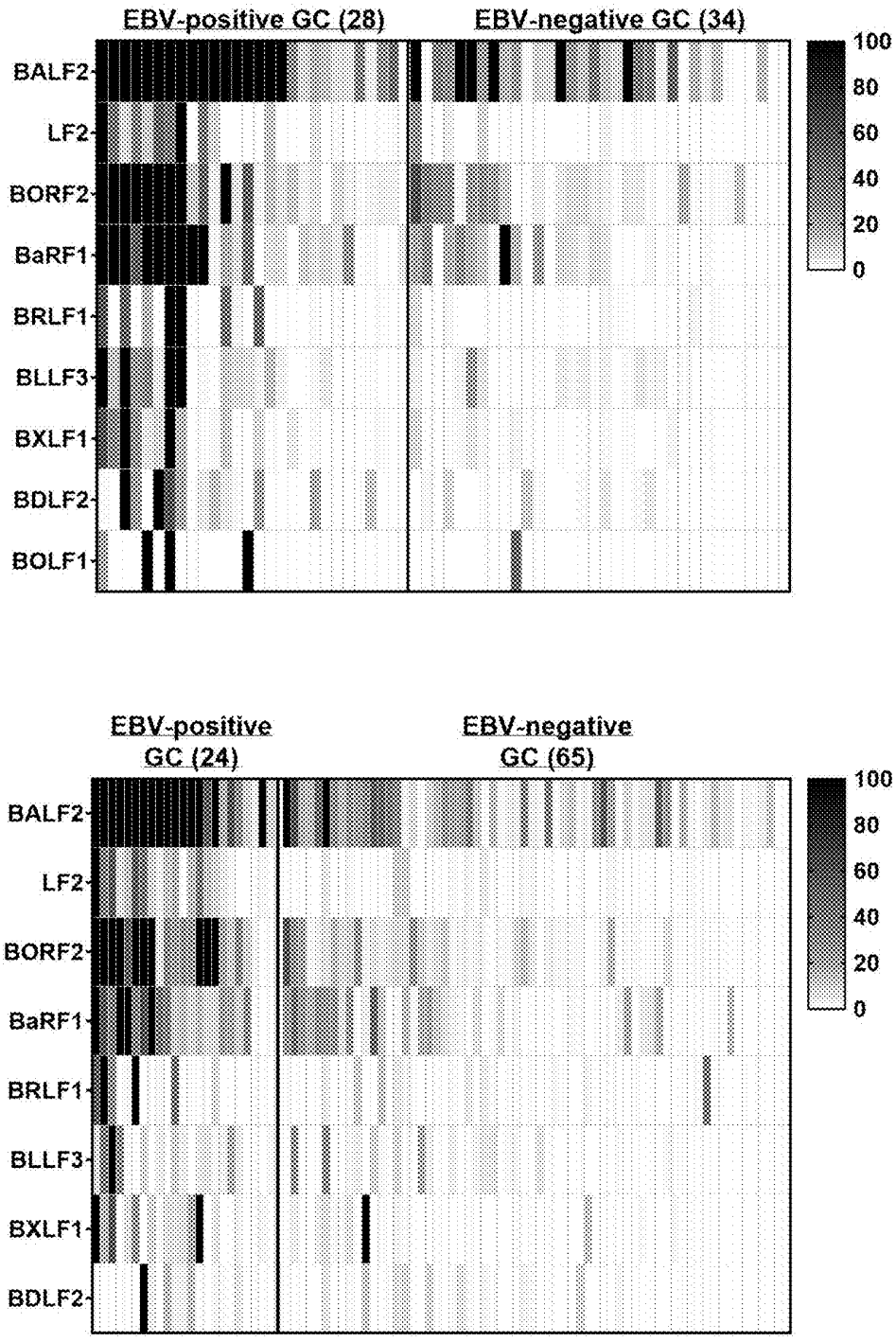

EBV-positive GC (28)
EBV-negative GC (34)
BALF2
LF2
BORF2
BaRF1
BRLF1
BLLF3
BXLF1
BDLF2
BOLF1
100
80
60
40
20
0
EBV-positive GC (24)
EBV-negative GC (65)
BALF2
LF2
BORF2
BaRF1
BRLF1
BLLF3
BXLF1
BDLF2
100
80
60
40
20
0

FIG. 5B

B.

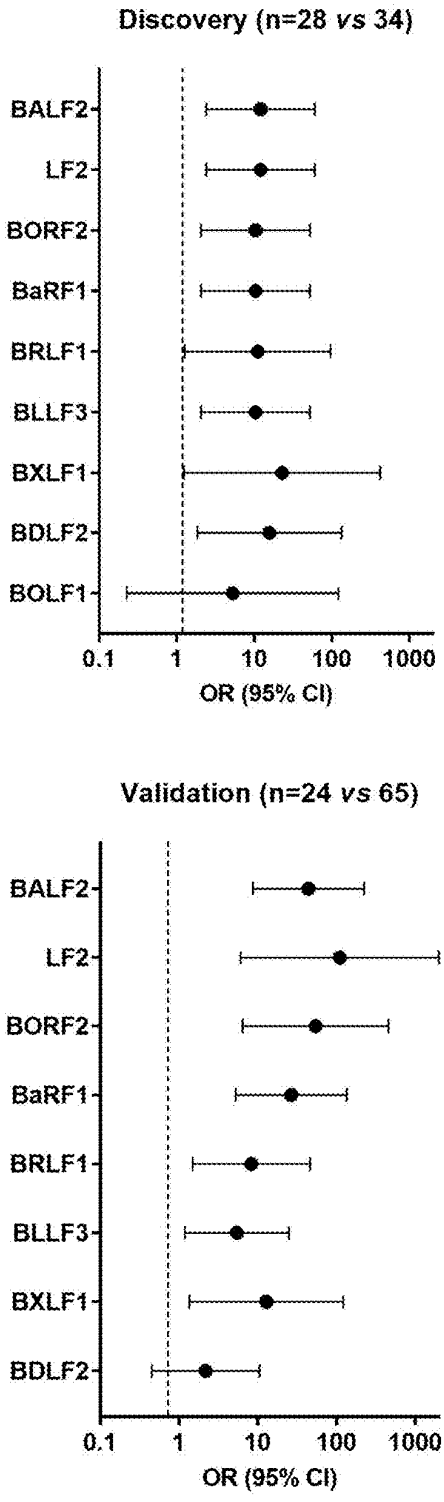

Discovery (n=28 vs 34)
BALF2
LF2
BORF2
BaRF1
BRLF1
BLLF3
BXLF1
BDLF2
BOLF1
0.1
1
10
100
1000
OR (95% CI)
Validation (n=24 vs 65)
BALF2
LF2
BORF2
BaRF1
BRLF1
BLLF3
BXLF1
BDLF2
0.1
1
10
100
1000
OR (95% CI)

FIG. 6

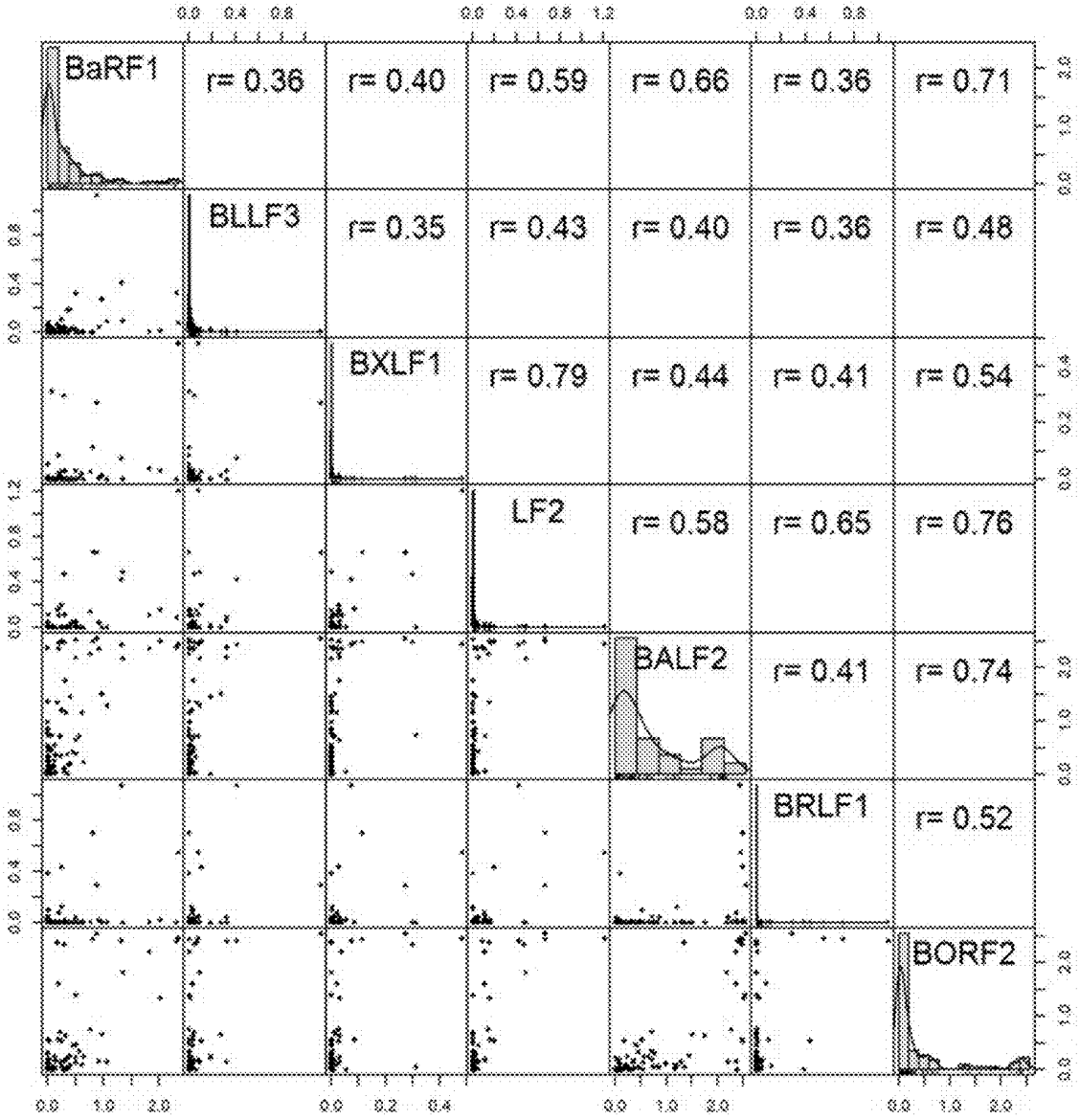

BaRF1
r= 0.36
r= 0.40
r= 0.59
r= 0.66
r= 0.36
r= 0.71
BLLF3
r= 0.35
r= 0.43
r= 0.40
r= 0.36
r= 0.48
BXLF1
r= 0.79
r= 0.44
r= 0.41
r= 0.54
LF2
r= 0.58
r= 0.65
r= 0.76
BALF2
r= 0.41
r= 0.74
BRLF1
r= 0.52
BORF2

FIG. 7A

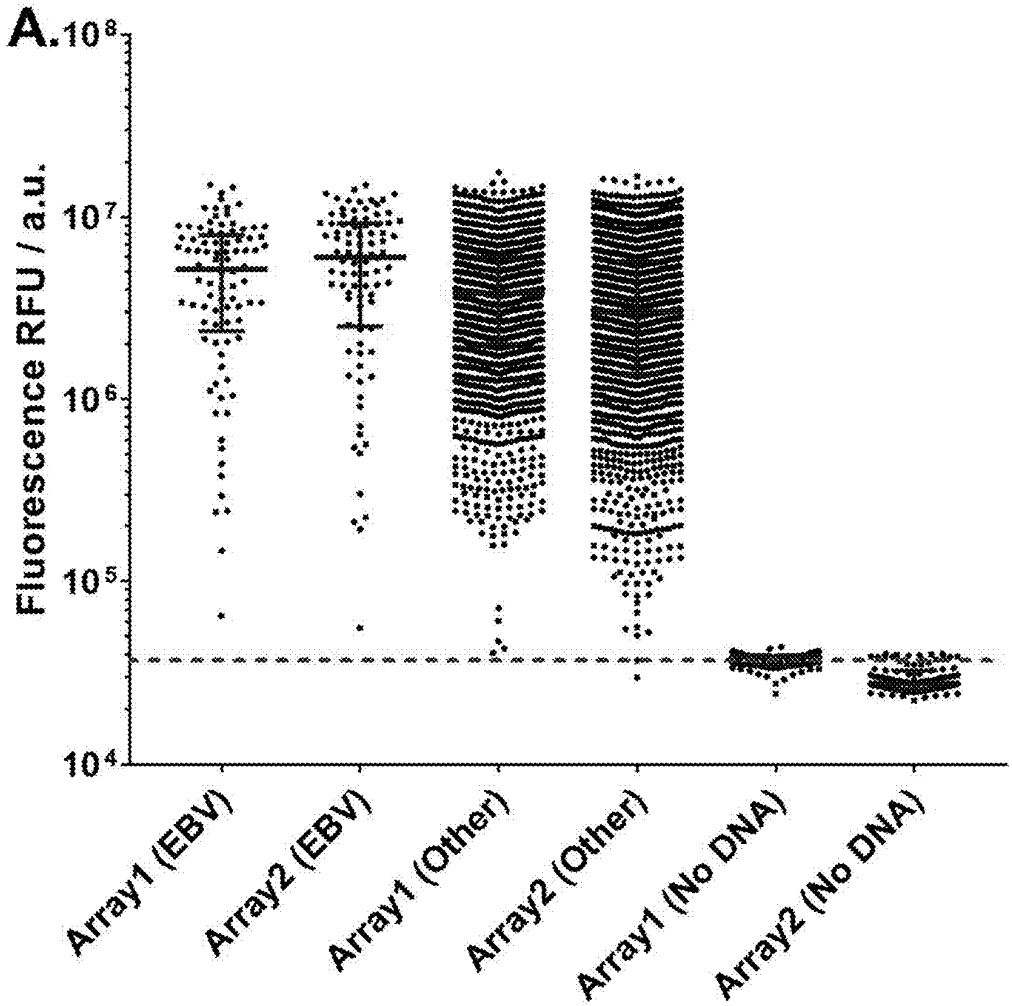
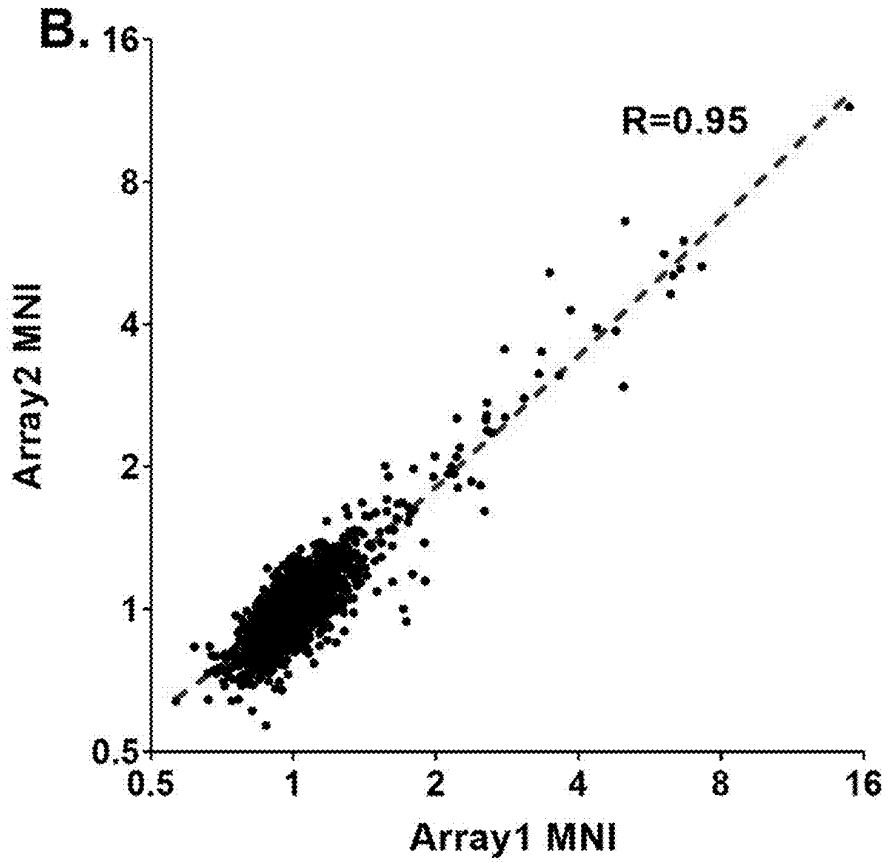

A.
10^8
10^7
10^6
10^5
10^4
Fluorescence RFU / a.u.
Array1 (EBV)
Array2 (EBV)
Array1 (Other)
Array2 (Other)
Array1 (No DNA)
Array2 (No DNA)

FIG. 7B



NOVEL ANTIBODIES FOR DETECTING EPSTEIN BARR VIRUS-POSITIVE GASTRIC CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/925,584, filed Oct. 24, 2019, which is hereby incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under R01 CA199948 and U01 CA214201 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] Gastric cancer (GC) is a major public health problem. GC represents the third leading cause of cancer mortality in the world, with approximately 1,000,000 new diagnoses and over 783,000 deaths in 2018 (equal to 1 in every 12 deaths globally). Besides gastric cancer induced by *Helicobacter pylori* (*H. pylori*) infection, around 10% of gastric cancer present the evidence of Epstein Barr Virus (EBV) involvement. As a ubiquitous virus that infects over 90% of adults, the association of EBV infection and gastric cancer is still unclear.

[0004] Like other cancers associated with EBV (e.g., nasopharyngeal carcinoma (NPC), Burkitt lymphoma (BL), Hodgkin lymphoma (HL), and non-Hodgkin lymphoma (NHL)), EBV-associated gastric cancer (EBV^+ GC) is conventionally detected by in situ hybridization of EBV-encoded small RNA (EBER), a specific marker for EBV presence. EBV^+ GC is a distinct subtype of gastric cancer classified by The Cancer Genome Atlas (TCGA) with overall lower mortality, occurs more frequently in male than female, is non-cardiac gastric carcinoma type with 90% prevalence of lymphoepithelioma-like gastric carcinoma, and displays significant intra- or peritumoral immune cell infiltration compared to EBV^- GC. Current knowledge of EBV^+ GC has been focused on epigenetic and genetic aberrance. EBV^+ GC displays unique molecular characteristics, recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2, PD-L1 and PD-L2.

[0005] Consideration of its unique molecular characterization and distinct gastric cancer subtype, distinguishing EBV^+ GC from EBV^- GC would benefit a following targeted therapy and precision medicine. Previously, anti-VCAp18 IgG/IgA, anti-EBNA1 IgG/IgA, and anti-Early protein (EA) IgG/IgA, which were used to detect EBV induced cancers, NPC, BL, and HL, as serological biomarkers, had been applied for EBV^+ GC detection. However, their presence in EBV^+ GC has been controversial. Some groups reported elevated anti-VCA IgA and anti-EA IgG in EBV^+ GC as compared to EBV− GC or healthy controls. Other groups have studied these antibodies but did not find a significant association between them and EBV^+ GC. Other than anti-EBV antibodies, Chung et al. had reported the present of circulating EBER in serum for limited EBV^+ GC cases (n=5) but not in controls (n=197). However, to date, none of these proposed markers have demonstrated enough discriminative power to be used for EBV^+ GC diagnosis. Accordingly, there remains a need in the art for improved reagents and methods for detecting EBV-associated gastric cancer, assessing risk of developing EBV^+ gastric cancer, and identifying subjects in need of treatment for this is unique subtype of gastric cancer, meaning reagents and methods that are more reliable and have sufficient discriminatory power for risk stratification and for early detection in asymptomatic individuals.

SUMMARY

[0006] In a first aspect, provided herein is a method for identifying a subject having increased risk of developing EBV-positive gastric cancer (EBV^+ GC). The method can comprise or consist essentially of (a) reacting a biological sample obtained from a subject with a reagent composition that comprises components for detecting in the biological sample the presence of one or more antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2; and (b) detecting the presence of the antibodies in the sample, wherein increased seroreactivity relative to a control for one or more of the antibodies is indicative of at least a four-fold increased risk of EBV^+ GC. The detected antibodies can comprise anti-BALF2, anti-BORF2, and anti-BRRF1. The biological sample can be one or more of a whole blood sample, a serum sample, and a plasma sample. The method can detect EBV^+ GC gastric cancer prior to symptom onset. The determining step can be carried out using an ELISA assay or a Western Blot assay. The method can further comprise administering a vaccine-based gastric cancer treatment to the subject if identified as having an increased risk of EBV^+ GC gastric cancer.

[0007] In another aspect, provided herein is a method to detect EBV-positive gastric cancer (EBV^+ GC) in a subject at risk of having EBV^+ GC, the method comprising: (a) contacting a biological sample obtained from the subject with a set of reagents, wherein the set of reagents specifically binds to at least three biomarkers in the biological sample, wherein the biomarkers are selected from the group consisting of BORF2, LF2, BDLF2, BXLF1, BRLF1, BaRF1, BGLF5, BRRF1, BALF2, BLLF3, and BSLF2; (b) measuring the level of the at least three biomarkers in the biological sample; and (c) detecting that the level of the at least three biomarkers is increased in the biological sample relative to a control sample from a subject without EBV^+ GC, thereby detecting the presence of EBV^+ GC in the subject. The at least three biomarkers can comprise anti-BALF2, anti-BORF2, and anti-BRRF1. The method can further comprise (d) administering an EBV^+ gastric cancer therapy to the subject, wherein the EBV^+ gastric cancer therapy is selected from the group consisting of chemotherapy, hormonal therapy, radiotherapy, immunotherapy, and surgical removal of stomach tissue. The biological sample can be one or more of a whole blood sample, a serum sample, and a plasma sample. The determining step can be carried out using an immunoassay.

[0008] In another aspect, provided herein is a method of determining an Epstein Barr Virus (EBV) gastric cancer antibody signature comprising antibodies, contained in a biological sample from an individual, that specifically bind to immobilized EBV antigens, the method comprising: (a) contacting the sample to a panel of immobilized EBV antigens under conditions that promote formation of anti-

gen-antibody complexes; and (b) identifying complexes formed by immobilized EBV antigens and antibody in the sample, to determine an EBV antibody signature. The antibody signature can be expressed as a level of antibody specifically binding to each immobilized antigen. The method can further comprise comparing an antibody signature from one individual to the antibody signature from another individual. In some cases, one individual has a disease process, and one individual is a healthy individual and the method allows comparison of the antibody signature in the healthy individual and the individual with a disease. The disease process can comprise EBV+ gastric cancer. The immobilized EBV antigens comprise one or more of BORF2, LF2, BDLF2, BXLF1, BRLF1, BaRF1, BGLF5, BOLF1, BRRF1, BALF2, BLLF3, and BSLF2.

[0009] In a further aspect, provided herein is a kit for determining and/or detecting at least one biomarker associated with EBV^+ gastric cancer, the kit comprising a reagent composition that comprises components for detecting in a biological sample the presence of one or more antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2.

[0010] In another aspect, provided herein is a kit for diagnosing a EBV^+ gastric cancer in a subject, the kit comprising a reagent composition that comprises components for detecting in a biological sample obtained from the subject the presence of one or more antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The present invention will be better understood and features, aspects, and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

[0012] FIG. **1** is a schematic flow-chart of EBV^+ GC biomarker discovery on NAPPA array, and verification and validation by ELISA.

[0013] FIG. **2** demonstrates anti-EBV IgG and IgA antibodies with >10% seropositivity by NAPPA in either EBV-positive or EBV-negative GC ("EBV^- GC"). Antibodies specific for either EBV-positive (n=19) or EBV-negative GC (n=1) antibodies are listed on the left and nonspecific antibodies (n=26) are listed on the right. Table colors correspond to groups in the Venn diagram. Abbreviations: EBV=Epstein-Barr virus; GC=gastric cancer; IgA=immunoglobulin A; IgG=immunoglobulin G; NAPPA=Nucleic Acid-Programmable Protein Array.

[0014] FIG. **3** demonstrates magnitude and statistical significance of differential antibody responses by NAPPA between EBV^+ GC and EBV^- GC. Labels indicate viral antibodies that have >20% sensitivity at 95% specificity for EBV^+ GC.

[0015] FIG. **4** demonstrates numbers of positive IgG NAPPA antibody responses of EBV-positive and EBV^- 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. NS=not statistically significant.

[0016] FIGS. **5**A-**5**B. (A) Heatmaps of IgG antibody responses by ELISA in discovery (top) and validation (bottom) sample sets of EBV^+ GC and EBV^- GC. Optical density measurements were normalized according to the highest value for each antibody across all samples. Each vertical bar represents a different serum. (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. ELISA=enzyme-linked immunosorbent assay.

[0017] FIG. **6** presents two-way scatterplots and pair-wise Pearson correlations among IgG antibody responses by ELISA for all validation samples. Four coefficients of correlation were high (>0.7), 6 were moderate (0.5-0.7) and 11 were low (<0.5); all correlations were statistically significant with p-values<0.01. Frequency histograms and density plots on diagonal represent individual antibody responses. Abbreviation: OD450=optical density 450.

[0018] FIGS. **7**A-**7**B. (A) Expression of EBV and other microbial proteins on multi-microbial NAPPA array compared to no DNA negative control spots. Red dotted line indicates cutoff based on no DNA sample spots from array 1 (mean+3 standard deviations). (B) Inter-array reproducibility (R=0.95) of median normalized intensities (MNI) for a pooled plasma sample. MNI=median normalized intensities; RFU=relative fluorescence units.

[0019] While the present invention is susceptible to various modifications and alternative forms, exemplary embodiments thereof are shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the description of exemplary embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0020] All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though set forth in their entirety in the present application.

[0021] With few biomarkers for non-invasive diagnostic testing, conventional methods of gastric cancer detection were based on endoscopy, which is a highly invasive technique, and histology to verify disease. Unfortunately, endoscopy is often performed after the patient has exhibited symptoms associated with gastric cancer, and the cancer is already in late stages, which lowers overall survival rate. The methods, devices, combinations, kits, and systems for diagnosing, predicting the risk of, and treating gastric cancer provided herein are based at least in part on the inventors' comprehensive anti-EBV protein immune profiling of EBV^+ GC and EBV^- GC patients and their development and validation of a panel of protein biomarkers useful for non-invasive identification of Epstein Barr Virus-associated gastric cancer patients from healthy controls in discovery samples and validation samples. In particular, this disclosure relates to the development and validation of unique EBV immunoproteomic profiles useful for identifying subjects having an increased risk of EBV-associated gastric cancer

relative to subject-matched controls. An interesting and unexpected finding of this investigation was that, while EBV infections are quite ubiquitous among adult humans, certain EBV antibodies possess high discriminatory power to distinguish EBV^+ GC and EBV^- GC samples. A three-antibody panel provides discriminatory power of AUC=0.87 with 79.2% sensitivity at 95% specificity, making non-invasive diagnosis of EBV^+ GC possible. Interestingly, the target proteins of these EBV antibodies are primarily expressed in during the early lytic stages of the EBV life cycle. Without being bound to any particular mechanism or mode of action, the early lytic stage proteins may be involved in cancer development or progression and, thus provide new targets for precision medicine and targeted therapies.

[0022] Accordingly, in a first aspect, this disclosure provides methods for identifying a subject as having increased risk of developing EBV-associated gastric cancer ("EBV^+ GC"). In some cases, the method comprises (a) reacting a biological sample obtained from a subject with a reagent composition that comprises components for detecting in the serum sample the presence of antibodies specific to EBV proteins; and detecting the presence of the antibodies in the sample. As used herein, the term "EBV-positive gastric cancer" or "EBV^+ GC" (also known as "EBV+ stomach cancer") refers to a type of cancer of the stomach or of stomach cells. EBV^+ GC is a distinct subtype of gastric cancer classified by The Cancer Genome Atlas (TCGA) with overall lower mortality, occurs more frequently in male than female, is non-cardiac gastric carcinoma type with 90% prevalence of lymphoepithelioma-like gastric carcinoma, and displays significant intra- or peritumoral immune cell infiltration compared to EBV^- GC. Previous studies of EBV^+ GC focused on epigenetic and genetic aberrance. EBV^+ GC displays unique molecular characteristics, recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2, PD-L1 and PD-L2, but the utility of detecting circulating nucleic acids (e.g., by EBER) for reliable, reproducible diagnostic purposes has not been established.

[0023] In preferred embodiments, the method comprises detecting and/or measuring a level of a biomarker such as, for example, an IgG or IgA antibody having specificity for Epstein Barr Virus (EBV) proteins. As used herein, the term "biomarker" means a distinctive biological or biologically derived indicator of a process, event, or condition. Protein or antibody biomarkers can be used in methods of diagnosis, e.g. clinical screening, and prognosis assessment and in monitoring the results of therapy. In some cases, biomarkers are useful for identifying patients most likely to respond to a particular therapeutic treatment and for drug screening and development. In some cases, biomarkers include IgG specific antibodies having specificity for EBV proteins that show a statistically significant difference in EBV^+ GC diagnosis. In some cases, therefore, the methods comprise detecting IgG and IgA antibodies having specificity for EBV proteins in a sample obtained from a subject. Data is provided herein which demonstrates that specific panels of biomarkers described herein, contains statistically significant biomarkers for the diagnosis of EBV^+ GC. For example, data provided herein demonstrates that increase seroreactivity for antibodies specific to particular EBV proteins is associated with increased risk of EBV-positive gastric cancer relative to a reference sample (e.g., in some cases obtained from healthy (e.g., free of EBV^+ GC) subject or pool of subjects; in other cases obtained from a EBV^- GC subject or pool of subjects). The antibodies include those listed in Table 1. Preferably, the antibodies are anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2 antibodies. Increased seroreactivity for these antibodies, relative to the reference sample, is associated with increased risk of EBV^+ GC. As shown in Table 1 and FIG. **3**, the following ten antibodies were validated (provided here with odds ratio (OR) and p-value): anti-LF2 (110.0, $p<0.01$), anti-BORF2 (54.2, $p<0.01$), anti-BALF2 (44.1, $p<0.01$), anti-BaRF1 (26.7, $p<0.01$), anti-BGLF5 (16.8, $p<0.01$), anti-BXLF1 (12.8, $p=0.01$), anti-BRRF1 (10.2, $p<0.01$), anti-BRLF1 (8.3, $p<0.01$), anti-BLLF3 (5.4, $p=0.02$), and anti-BSLF2 (4.0, $p=0.04$). In various embodiments, diagnostic tests that use these biomarkers alone or in combination show a sensitivity and specificity of at least 85%, at least 90%, at least 95%, at least 98% and at least 99%.

[0024] As used herein, the term "seroreactivity" refers to a level and/or presence of reactivity to specific antibodies in a sample (e.g., biological sample of a subject or a pooled sample from multiple subjects) as determined using with techniques known in the art, such as ELISA. As described in this disclosure, it was determined that increased seroreactivity to particular antigen-specific antibodies relative to a control (e.g., in some cases, a control sample obtained from a subject that does not have gastric cancer) is indicative of an increased risk of EBV^+ GC. The increased level of seroreactivity may be at least or about a 5% increase, at least or about a 10% increase, at least or about a 15% increase, at least or about a 20% increase, at least or about a 25% increase, at least or about a 30% increase, at least or about a 35% increase, at least or about a 40% increase, at least or about a 45% increase, at least or about a 50% increase, at least or about a 55% increase, at least or about a 60% increase, at least or about a 65% increase, at least or about a 70% increase, at least or about a 75% increase, at least or about a 80% increase, at least or about a 85% increase, at least or about a 90% increase, at least or about a 95% increase, at least or about at 100% increase, at least or about at 200% increase, or more.

[0025] As used herein, the terms "seropositivity" and "seropositive" refer to a positive result (or a subject having a positive result) in a test of blood serum, e.g., obtaining a positive result for the presence of an antigen-specific antibody. The term seropositive can encompass patients for whom blood tests reveal the presence of particular antibodies. As used herein, the terms "seronegativity" and "seronegative" refer to a reduced or a negative result (or a subject having a negative result) in a test of blood serum, e.g., obtaining a negative result for the presence of an antigen-specific antibody. The term seronegative can encompass patients for whom blood tests do not reveal the presence of particular antibodies, which can mean the patient does not possess the antibodies, or the patient possesses low levels of the antibodies that cannot be detected by a particular assay.

[0026] In some cases, the levels of one or more EBV^+ GC biomarkers or the levels of a specific panel of EBV^+ GC biomarkers in a sample are compared to a reference standard ("reference standard" or "reference level") in order to direct treatment decisions. The reference standard used for any embodiment disclosed herein may comprise average, mean, or median levels of the one or more EBV^+ GC biomarkers

or the levels of the specific panel of EBV+ GC biomarkers in a control population. The reference standard may additionally comprise cutoff values or any other statistical attribute of the control population, such as a standard deviation from the mean levels of the one or more EBV+ GC biomarkers or the levels of the specific panel of EBV+ GC biomarkers.

[0027] In some embodiments, comparing the level of the one or more EBV+ GC biomarkers is performed using a cutoff value. In related embodiments, if the level of the one or more EBV+ GC biomarkers is greater than the cutoff value, the individual may be diagnosed as having, or being at risk of developing EBV+ GC. In other distinct embodiments, if the level of the one or more EBV+ GC biomarkers is less than the cutoff value, the individual may be diagnosed as having, or being at risk of developing EBV+ GC. Cutoff values may be determined by statistical analysis of the control population to determine which levels represent a high likelihood that an individual does or does not belong to the control population. In some embodiments, comparing the level of the one or more EBV+ GC biomarkers is performed using other statistical methods. In related embodiments, comparing comprises logistic or linear regression. In other embodiments, comparing comprises computing an odds ratio.

[0028] In a further aspect, provided herein are methods for detecting Epstein Barr Virus-associated gastric cancer in a subject. In some cases, the method comprises (a) reacting a biological sample obtained from a subject with a reagent composition that comprises components for determining a level of antibodies to one or more of the EBV proteins listed in Table 2 are present in the sample; (b) determining levels of the antibodies in the biological sample; and (c) comparing the levels to predetermined values indicative of EBV+ GC, wherein if the level of antibodies in the biological sample falls within the predetermined values indicative of EBV+ GC, the level in the biological sample indicates that the subject has EBV+ GC. The predetermined values can be obtained from a reference sample obtained from an individual or a group of individuals (e.g., a cohort) having EBV+ GC.

[0029] In preferred embodiments, the antibodies comprise one or more of anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2. In some cases, the antibodies are a panel that comprises or consists essentially of anti-BALF2, anti-BORF2, and anti-BRRF1 antibodies.

[0030] In another aspect, provided herein are methods to determine risk of EBV+ GC. In some cases, the method comprises (a) reacting a biological sample obtained from a subject with a reagent composition that comprises components for determining the level of antibodies to one or more EBV proteins present in the sample; (b) determining levels of the antibodies in the sample; and (c) comparing the levels of antibodies to predetermined values indicative of high risk of EBV+ GC, wherein if the level of antibodies in the sample falls within the levels antibodies of a subject with high risk of EBV+ GC, the level in the sample of the subject is predictive for the risk of EBV+ GC in the subject. Preferably, the EBV proteins are selected from those listed in Table 1.

[0031] In another aspect, provided herein is a method to detect EBV-positive gastric cancer (EBV+ GC) in a subject at risk of having EBV+ GC, where the method comprises: (a) contacting a biological sample obtained from the subject with a set of reagents, wherein the set of reagents specifically binds to at least three biomarkers in the biological sample, wherein the biomarkers are selected from the group consisting of BORF2, LF2, BDLF2, BXLF1, BRLF1, BaRF1, BGLF5, BOLF1, BRRF1, BALF2, BLLF3, and BSLF2; (b) measuring the level of the at least three biomarkers in the biological sample; and (c) detecting that the level of the at least three biomarkers is increased in the biological sample relative to a control sample from a subject without EBV+ GC, thereby detecting the presence of EBV+ GC in the subject. In some cases, the at least three biomarkers comprise anti-BALF2, anti-BORF2, and anti-BRRF1.

[0032] In some cases, the method further comprises (d) administering an EBV+ GC therapy to the subject, wherein the EBV+ GC therapy is selected from the group consisting of chemotherapy, hormonal therapy, radiotherapy, immunotherapy, and surgical removal of stomach tissue.

[0033] In some cases, the method further comprises administering an effective amount of a treatment regimen to treat EBV+ GC. In some cases, the treatment regimen comprises one or more of a vaccine-based therapy, chemotherapy, hormonal therapy, radiotherapy, surgery, and immunotherapy.

[0034] As used herein, the term "individual," which may be used interchangeably with the terms "patient" or "subject," refers to one who receives medical care, attention or treatment and may encompass a human patient. As used herein, the term "individual" is meant to encompass a person who has EBV+ GC, is suspected of having EBV+ GC, or is at risk of EBV+ GC. As used herein, "at risk of gastric cancer" means that the subject may be asymptomatic or suffering from one or more symptoms of gastric cancer such as discomfort in the upper abdomen, a feeling of fullness, and the like, but has not been diagnosed with EBV+ GC.

[0035] In preferred embodiments, the biological sample is a blood sample. Any suitable blood sample obtained from the subject may be used, including but not limited to whole blood, serum, and blood plasma. In a preferred embodiment, a blood plasma sample is used. Methods for obtaining and preparing blood samples are well known in the art; such methods include those described herein. In one embodiment, plasma is prepared by centrifuging a blood sample under conditions suitable for pelleting of the cellular component of the blood.

[0036] The methods for detecting gastric cancer of this disclosure can be used as methods for diagnosing gastric cancer, and are effective for detecting EBV+ GC at an early stage and/or prior to symptom onset. As used herein, the term "symptom onset" refers to the time point where the subject presents one or more symptoms characteristic of gastric cancer. Exemplary symptoms of gastric cancer include but are not limited to stomach pain, fatigue, feeling bloated after eating, feeling full after eating small amounts of food, severe persistent heartburn, severe indigestion, unexplained persistent nausea, persistent vomiting, and unintentional weight loss. In another aspect, provided herein is a method for assessing the risk for gastric cancer in a subject, i.e., the likelihood of gastric cancer being present in the subject and/or the likelihood of the subject developing the disease at a later time.

[0037] As used herein, the terms "detect" and "detection" refer to identifying the presence, absence, or amount of the object to be detected. Standard detection methods include,

for example, radioisotope immunoassay, an enzyme-linked immunosorbent assay (ELISA), SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies, mass spectrometry, immunofluorescence assays, Western blot, affinity chromatography (affinity ligand bound to a solid phase), fluorescent antibody assays, immunochromatography, and in situ detection with labeled antibodies. Although any appropriate method can be selected, taking various factors into consideration, ELISA methods are particularly sensitive.

[0038] The terms "biomolecular marker," "biomarker," or "marker" (also sometimes referred to herein as a "target analyte") are used interchangeably and refer to a molecule whose measurement provides information as to the state of a subject. In various exemplary embodiments, the biomarker is used to assess a pathological state. Measurements of the biomarker may be used alone or combined with other data obtained regarding a subject in order to determine the state of the subject. In one embodiment, the biomarker is "differentially present" in a sample taken from a subject of one phenotypic status (e.g., having EBV^+ GC) as compared with another phenotypic status (e.g., not having EBV^+ GC). In one embodiment, the biomarker is "differentially present" in a sample taken from a subject undergoing no therapy or one type of therapy as compared with another type of therapy. Alternatively, the biomarker may be "differentially present" even if there is no phenotypic difference, e.g. the biomarkers may allow the detection of asymptomatic risk. A biomarker may be determined to be "differentially present" in a variety of ways, for example, between different phenotypic statuses if the mean or median level (particularly the expression level of antibodies specific to the EBV proteins described herein) of the biomarker in the different groups is calculated to be statistically significant. Common tests for statistical significance include, among others, t-test, ANOVA, Kruskal-Wallis, Wilcoxon, Mann-Whitney and odds ratio.

[0039] The actual measurement of levels of a target analyte can be determined (for example, at the protein level) using any method(s) known in the art. A molecule or analyte such as a protein, polypeptide or peptide, or a group of two or more molecules or analytes such as two or more proteins, polypeptides or peptides, is "measured" in a sample when the presence or absence and/or quantity of said molecule or analyte or of said group of molecules or analytes is detected or determined in the sample, preferably substantially to the exclusion of other molecules and analytes. The terms "quantity," "amount," and "level" are synonymous and generally well-understood in the art. The terms as used herein may particularly refer to an absolute quantification of a molecule or an analyte in a sample, or to a relative quantification of a molecule or analyte in a sample, i.e., relative to another value such as relative to a reference value as taught herein, or to a range of values indicating a base-line expression of the molecule or analyte in a sample obtained from a healthy subject or, as appropriate, a sample obtained from a subject known to have EBV^+ GC and/or a particular type or stage of EBV^+ GC. These values or ranges can be obtained from a single patient or from a group of patients.

[0040] A target analyte is differentially present between the two samples if the amount of the target analyte in one sample is statistically significantly different from the amount of the target analyte in the other sample. As used herein, the phrase "differentially expressed" refers to differences in the quantity and/or the frequency of a target analyte present in a sample taken from patients having, for example, a particular disease as compared to a control subject.

[0041] For example, without limitation, a target analyte can be a polypeptide that is present at an elevated level or at a decreased level in samples of patients having a particular condition as compared to samples of control subjects. A target analyte can be differentially present in terms of quantity, frequency or both. In some cases, a target analyte is differentially present between the two samples if it is present at least about 120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% greater than it is present in the other sample, or if it is detectable in one sample and not detectable in the other.

[0042] Alternatively (or additionally), a target analyte is differentially present between the two sets of samples if the frequency of detecting the target analyte in samples of patients suffering from a particular disease or condition is statistically significantly higher or lower than in the control samples. For example, a target analyte is differentially present between the two sets of samples if it is detected at least about 120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% more frequently or less frequently observed in one set of samples than the other set of samples.

Articles of Manufacture

[0043] In another aspect, provided herein is a kit for determining and/or detecting at least one biomarker associated with EBV^+ GC. In some cases, the kit comprises a reagent composition that comprises components for detecting in a biological sample the presence of one or more biomarkers of EBV^+ GC. In some cases, the biomarkers of EBV^+ GC are antibodies selected from of anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2. In some cases, the kit may further comprise instructions for detecting EBV^+ GC or identifying a subject has having increased risk of EBV^+ GC according to the methods provided herein. In some cases, the kit further comprises materials for obtaining and preserving a biological sample, for example, from an individual.

[0044] In another aspect, provided herein is a kit for diagnosing a EBV^+ GC in a subject. In some cases, the kit comprises a reagent composition that comprises components for detecting in a biological sample obtained from the subject the presence of one or more biomarkers diagnostic of EBV^+ GC. In some cases, the biomarkers diagnostic of gastric cancer are antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2. In some cases, the kit may further comprise instructions for detecting EBV+gastric cancer or identifying a subject has having increased risk of EBV^+ GC according to the methods provided herein. In some cases, the kit further comprises materials for obtaining, retaining, and/or preserving a biological sample, for example, obtained from an individual.

[0045] In some cases, provided herein is a kit for the detection of EBV-specific antibodies in a subject comprising: a container for retaining a tissue sample; one or more

antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2; and a reagent for detection of the one or more antibodies bound to the corresponding antigen in the tissue sample. Preferably, the reagent is the reagent is capable of determining the level of an antibody against a gastric cancer-associated EBV protein by immunologic assay. In this manner, the kit is a diagnostic kit for detection of EBV^+ GC in the subject from whom the tissue sample is obtained. In other cases, the kit is a diagnostic kit for detection or quantification of gastric cancer-associated EBV antibodies in a biological sample. In some cases, the kits of this disclosure are used in tests such as, for example, immunoenzymatic tests, preferably ELISA, immunofluorescent, immunochemiluminescent, radioimmunological, immunochromatographic, immunodiffusion, and immunoprecipitation tests.

[0046] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

[0047] The terms "comprising", "comprises" and "comprised of as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements, or method steps. The phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," "having," "containing," "involving," and variations thereof, is meant to encompass the items listed thereafter and additional items. Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed. Ordinal terms are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term), to distinguish the claim elements.

[0048] The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

[0049] The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[0050] As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of" "only one of" or "exactly one of" "Consisting essentially of" when used in the claims, shall have its ordinary meaning as used in the field of patent law.

[0051] As used herein, the terms "approximately" or "about" in reference to a number are generally taken to include numbers that fall within a range of 5% in either direction (greater than or less than) the number unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value). Where ranges are stated, the endpoints are included within the range unless otherwise stated or otherwise evident from the context.

[0052] It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

[0053] The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention. The invention will be more fully understood upon consideration of the following non-limiting Examples. The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner.

EXAMPLES

Example 1—Epstein Barr Virus Immunoproteomic Profiles in EBV-Associated Gastric Cancer

[0054] This example describes characterization of gastric cancer (GC)-specific antibody responses to Epstein Barr Virus (EBV) to assess the virus' contribution to gastric carcinogenesis and to develop a non-invasive method for detecting EBV^+ GC. As described herein, despite the ubiquity of EBV infection in adults, ten novel anti-EBV IgG antibodies elevated in EBV^+ GC were discovered and blindly validated in an independent sample set. Characterization of these ten antibodies demonstrates that a humoral response to EBV can distinguish EBV involvement in gastric cancer, and that anti-EBV antibodies can be used in clinical diagnosis of EBV^+ GC, for epidemiologic studies, and for development of treatment guidelines for EBV^+ GC. The GC-specific antibody response to this common infection, which may provide a noninvasive method to detect EBV^+ GC and elucidate its contribution to carcinogenesis.

Materials and Methods

Abbreviations

[0055] AUC—area under the curve

[0056] CI—confidence intervals

[0057] EBER—Epstein-Barr encoded small RNA

[0058] EBV—Epstein-Barr virus

[0059] EDTA—ethylenediaminetetraacetic acid

[0060] ELISA—enzyme-linked immunosorbent assay

[0061] GC—gastric cancer

[0062] HL—Hodgkin lymphoma

[0063] IgA—immunoglobulin A

[0064] IgG—immunoglobulin G

[0065] MNI—median normalized intensity

[0066] NAPPA—Nuclear Acid Programmable Protein Array

[0067] NPC—nasopharyngeal carcinoma

[0068] OD—optical density at 450 nm

[0069] OR—odds ratio

[0070] ORF—open reading frame

[0071] ROC—receiver operating characteristics

[0072] Subjects: EBV^+ GC and EBV^- 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. 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% cardiac, 95% non-cardiac). 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% noncardiac; Table 2). 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).

TABLE 1

Identification of EBV-positive GC discriminatory IgG antibodies.

			Discovery Sample NAPPA		
Antibody	EBV protein or activity	Stage of EBV life cycle	Se (%) at MNI ≥2.0	Sp (%) at MNI ≥2.0	Se (%) at 95% Sp
anti-BALF2	single-stranded DNA binding protein	Early lytic	89	32	21
anti-LF2	protein that binds Rta	Unknown	43	94	36
anti-BORF2	ribonucleotide reductase, large subunit	Early lytic	68	59	36
anti-BaRF1	ribonucleotide reductase, small subunit	Early lytic	21	100	21[e]
anti-BRLF1	encodes lytic genes transactivator Rta	Immediate-early lytic	32	97	32[e]
anti-BLLF3	dUTPase	Early lytic	36	88	21
anti-BXLF1	thymidine kinase	Early lytic	32	91	32
anti-BDLF2	glycoprotein that binds BMRF2, an important factor for EBV attachment to epithelial cells	Late lytic	29	97	29[e]
anti-BOLF1	tegument protein binding phosphoprotein	Unknown	32	79	25

	Discovery Sample ELISA		Validation Sample ELISA				
Antibody	Se (%) at 95% Sp	Verified[a]	Se (%) at discovery cutoff[b]	Sp (%) at discovery cutoff[b]	p-value[c]	Validated[d]	AUC
anti-BALF2	43	yes	58	97	<0.001	yes	0.85
anti-LF2	43	yes	46	100	<0.001	yes	0.81
anti-BORF2	39	yes	46	99	<0.001	yes	0.84
anti-BaRF1	39	yes	46	97	<0.001	yes	0.79
anti-BRLF1	25	yes	21	97	0.006	yes	0.58
anti-BLLF3	39	yes	21	95	0.018	yes	0.71

TABLE 1-continued

Identification of EBV-positive GC discriminatory IgG antibodies.

anti-BXLF1	25	yes	17	99	0.006	yes	0.72
anti-BDLF2	32	yes	13	94	0.324	no	
anti-BOLF1	7	no	(not tested)				

Abbreviations:
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
[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

TABLE 2

Selected demographic and histologic characteristics of EBV-positive and EBV-negative GC in discovery and validation sample sets.

		Discovery sample set		Validation sample set	
		EBV-positive GC (28)	EBV-negative GC (34)	EBV-positive GC (24)	EBV-negative GC (65)
Age, median (range)		64 (35 to 86)	63.5 (47 to 78)	56 (25 to 73)	59 (28 to 77)
Male sex, n (%)		24 (86)	31 (91)	19 (79)	43 (66)
Lauren classification	Intestinal, n (%)	15 (54)	23 (68)	7 (29)	13 (20)
	Diffuse, n (%)	8 (29)	5 (15)	15 (63)	31 (48)
	Mixed, n (%)	4 (14)	5 (15)	0 (0)	0 (0)
	Undifferentiated, n (%)	1 (4)	1 (3)	2 (8)	13 (20)

[0073] Selection of EBV genes and EBV NAPPA array fabrication: The NAPPA was fabricated with the same procedure as previously reported. 89 EBV open reading frames (ORFs) from 85 total unique proteins for Type-1 EBV (B95-8) were included as part of a multi-microbe array, along with 1631 ORFs from *H. pylori* and several other microbes. 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 (Table 3). All clones were obtained from DNASU (available at dnasu.org/DNASU/Home.do on the World Wide Web; Tempe, Ariz., USA) in a NAPPA compatible pANT7-cGST expression vector.

TABLE 3

Replication cycle stage for expression of 89 EBV open reading frames.

Protein	Clone ID in DNASU	Stage of EBV life cycle
BHRF1	HhCD00595127	Early lytic
BaRF1	HhCD00595137	Early lytic
BMRF2	HhCD00595138	Early lytic
BSLF1	HhCD00595139	Early lytic
BLLF3	HhCD00595141	Early lytic
BLLF2	HhCD00595145	Early lytic
BZLF2	HhCD00595148	Early lytic
BRRF1	HhCD00595149	Early lytic
BBLF4	HhCD00595153	Early lytic

TABLE 3-continued

Replication cycle stage for expression of 89 EBV open reading frames.

Protein	Clone ID in DNASU	Stage of EBV life cycle
BBLF2/BBLF3	HhCD00595156	Early lytic
BGLF5	HhCD00595158	Early lytic
BGLF4	HhCD00595159	Early lytic
BDLF4	HhCD00595165	Early lytic
BcRF1	HhCD00595171	Early lytic
BXLF1	HhCD00595174	Early lytic
BVLF1	HhCD00595177	Early lytic
BALF2	HhCD00595185	Early lytic
BALF1	HhCD00595186	Early lytic
BARF1	HhCD00595187	Early lytic
BSLF2/BMLF1	HhCD00595190	Early lytic
BALF5	HhCD00595193	Early lytic
BHLF1	HhCD00595200	Early lytic
BORF2	HhCD00595201	Early lytic
BMRF1	HhCD00595202	Early lytic
BFLF2	HhCD00595128	Early lytic
BFLF1	HhCD00595129	Early lytic
BFRF1	HhCD00595131	Early lytic
BFRF2	HhCD00595132	Early lytic
BVRF1	HhCD00595176	Early lytic
BNLF2b	HhCD00595194	Early lytic
BNLF2a	HhCD00595206	Early lytic
BZLF1	HhCD00595191	Immediate-early lytic
BRLF1	HhCD00595192	Immediate-early lytic
BNRF1	HhCD00595124	Late lytic
BCRF1	HhCD00595125	Late lytic
BSRF1	HhCD00595140	Late lytic

TABLE 3-continued

Replication cycle stage for expression of 89 EBV open reading frames.

Protein	Clone ID in DNASU	Stage of EBV life cycle
BLRF1	HhCD00595142	Late lytic
BLRF2	HhCD00595143	Late lytic
BLLF1	HhCD00595144	Late lytic
BBRF3	HhCD00595157	Late lytic
BGLF2	HhCD00595163	Late lytic
BGLF1	HhCD00595164	Late lytic
BDLF3	HhCD00595167	Late lytic
BDLF2	HhCD00595168	Late lytic
BTRF1	HhCD00595172	Late lytic
BXLF2	HhCD00595173	Late lytic
BXRF1	HhCD00595175	Late lytic
BILF2	HhCD00595180	Late lytic
BALF4	HhCD00595184	Late lytic
BKRF2	HhCD00595195	Late lytic
BXLF2	HhCD00595203	Late lytic
BILF2	HhCD00595204	Late lytic
BFRF3	HhCD00595133	Late lytic
BPLF1	HhCD00595134	Late lytic
BRRF2	HhCD00595150	Late lytic
BKRF4	HhCD00595152	Late lytic
BBRF1	HhCD00595154	Late lytic
BBRF2	HhCD00595155	Late lytic
BDLF1	HhCD00595169	Late lytic
BcLF1	HhCD00595170	Late lytic
BVRF2	HhCD00595178	Late lytic
BdRF1	HhCD00595179	Late lytic
BPLF1	HhCD00595189	Late lytic
BBLF1	HhCD00595197	Late lytic
BORF1	HhCD00595198	Late lytic
LMP-2A	HhCD00595122	Latent
LMP-2B	HhCD00595123	Latent
EBNA-LP	HhCD00595126	Latent
EBNA-3B	HhCD00595146	Latent
EBNA-3C	HhCD00595147	Latent
EBNA-1	HhCD00595151	Latent
LMP-1	HhCD00595188	Latent
BARF0	HhCD00595205	Latent
EBNA2	HhCD00595207	Latent
EBNA3A	HhCD00595208	Latent
RPMS1	HhCD00595209	Latent
A73	HhCD00595210	Latent
BFRF1A	HhCD00595130	Unknown
BGLF3.5	HhCD00595160	Unknown
BGLF3	HhCD00595161	Unknown
BDLF3.5	HhCD00595166	Unknown
LF2	HhCD00595181	Unknown
LF1	HhCD00595182	Unknown
BILF1	HhCD00595183	Unknown
BKRF3	HhCD00595196	Unknown
BWRF1.1	HhCD00595199	Unknown
BPLF1	HhCD00595135	Unknown
BOLF1	HhCD00595136	Unknown
BGRF1/BDRF1	HhCD00595162	Unknown

[0074] EBV+GC associated antibody discovery on EBV NAPPA array: 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 (FIG. **7**A). 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., Mannedorf, 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 (FIG. **7**B).

[0075] 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 more than 10% seropositivity in either EBV-positive or EBV-negative GC were assessed for discrimination between these groups (FIG. **2**). 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.

[0076] RAPID ELISA verification and validation: Candidate biomarkers were verified in the discovery sample set by Rapid Antigenic Protein In Situ Display (RAPID) ELISA following a previously reported protocol. In brief, 96-well ELISA plates (Corning, N.Y., 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, Mass., 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 sample set. 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.

[0077] Statistical analyses: The difference in quantitative antibody response on NAPPA and differences between numbers of seropositive antibodies in EBV-positive and EBV-negative GC 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 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% Cl). 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, Tex., USA), GraphPad Prism 8.0.2 (Graph-

Pad Software, Inc., CA, USA) and R version 3.6 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Results

[0078] Antibody selection by NAPPA: Immunoprofiling of discovery samples by NAPPA identified a total of 41 antibodies seropositive in at least 10% of either EBV^+ GC or EBV^- GC patients, including 7 with both IgG and IgA, 33 with IgG only, and 1 with IgA only antibody (FIG. **2**). Twenty-six of the 40 IgG antibodies and 2 of the 8 IgA antibodies were common to both EBV^+ GC and EBV^- 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 5 IgA antibodies were only present in EBV-positive GC, whereas one IgA antibody but no IgG antibody was found only in EBV^- GC.

[0079] Median signal intensity for individual antibodies ranged up to 4.3-fold higher in EBV-positive as compared to EBV^- GC (FIG. **3**). Notably, there was no difference in signal intensity between EBV-positive and EBV^- 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.

[0080] 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^- GC samples had a median of 14 (MW=284.5, p=0.006). EBV^+ GC had significantly more IgG antibodies than EBV^- GC to immediate-early lytic, early lytic, and unknown phase proteins (FIG. **4**). Both sample groups had medians of 1.0 seropositive IgA antibody (p=0.737).

[0081] 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 antibodies met these criteria for EBV^- GC.

[0082] Verification and validation by RAPID ELISA: Using the same discovery sample set (Table 2), 8 of the 9 differential 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 8 antibodies were blindly validated to differ at $p<0.05$ between EBV^+ GC and EBV^- 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 7 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).

[0083] ELISA reactivity was markedly stronger for EBV^+ GC than EBV^- GC (FIG. **5**A). Using the cutoffs for 95% specificity of EBV^- GC in the discovery samples, the 7 validated markers all had ORs exceeding 5.0 for distinguishing EBV status in the validation samples, ranging up to 111 for anti-LF2 (FIG. **5**B).

[0084] All pair-wise correlations among the 9 anti-EBV antibodies were statistically significant with p-values <0.01 in the validation samples (FIG. **6**). For EBV^+ GC and EBV^- GC groups combined, correlation coefficients ranged from 0.35 (anti-BLLF3 vs. anti-BXLF1) to 0.79 (anti-BXLF1 vs. anti-LF2).

[0085] 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 3-marker combination of anti-BALF2, anti-BORF2, and anti-LF2.

Discussion

[0086] In summary, this example describes the first comprehensive proteome-level study to identify anti-EBV antibodies for EBV^+ GC, identifying 7 highly discriminatory IgG biomarkers. Our findings indicate that the EBV-positive 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. The discriminatory biomarkers were identified by comparing individuals having gastric cancer: comparing EBV^+ GC to EBV^- GC. As described herein, these biomarkers are useful to distinguish between these types of gastric cancer, but also be used to distinguish EBV^+ GC and healthy (non-GC) persons.

[0087] Of note, antibodies to BRLF1, BALF2 and BXLF1 were also increased in patients with the epithelial cell tumor NPC, but not in those with lymphoma. 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 in epithelial cells, while the other immediate-early EBV lytic protein is needed in B cells. BALF2 is the major single-stranded DNA binding protein and is required for viral DNA replication. BXLF1 encodes the viral thymidine kinase that catalyzes the phosphorylation of deoxythymidine to deoxythymidine monophosphate, which is important for viral DNA replication. 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. BORF2 can induce p53 expression to regulate G1/S transition arrest in the cell cycle. 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. LF2 is a type I interferon antagonist that prevents establishment of an antiviral response by blocking cellular IRF7-mediated innate immunity. It also inhibits viral replication by modulating BRLF1 (Rta) activity. 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.

[0088] EBV has a life cycle alternating between latency and lytic replication. Latency is manifested by persistence in host cells maintained with cell division, while lytic replication results in cell death and virus dissemination. With respect to timing of expression in the EBV replication cycle, 1 of our EBV-positive GC-specific target antigens is present

in immediate-early lytic phase (BRLF1) and 5 in early lytic phase (BALF2, BXLF1, BLLF3, BaRF1, BORF2). The stage for LF2 expression is unknown. While EBV+ 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. Our discovery that antibodies to immediate-early and early lytic proteins are elevated in EBV+ GC vs. EBV− 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.

[0089] EBV is also implicated in the etiologies of NPC, Burkitt lymphoma, HL, and non-Hodgkin lymphoma. 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. Furthermore, unlike NPC's where IgA antibodies are frequently expressed, EBV+ GCs largely were IgG responses.

[0090] Given the limited diagnostic options for EBV+ GC, apart from humoral 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 EBV+ GC.

[0091] The serology study described herein has several strengths. Covering 85 full length EBV proteins, it is believed to be the most comprehensive evaluation of EBV+ GC immunoproteomics. This approach provided for evaluation of more viral proteins and the interplay among them, extending previous targeted studies. Second, the 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, this study used post-diagnosis samples that may be reflective of the disease status, limiting interpretation regarding etiologic significance. To investigate causal pathways, prospective studies are 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.

[0092] Despite the near universal infection of adults with EBV, seven novel IgG antibodies were identified to discriminate EBV+ GC from EBV− GC. Unlike nasopharyngeal carcinoma, EBV-specific IgA response does not seem to play an important role in GC. A noninvasive blood test for EBV+ GC based on the IgG antibodies could have 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 development, maintenance, or progression of EBV-positive malignancies and represent potential new targets for precision therapeutics.

We claim:

1. A method for identifying a subject having increased risk of developing EBV-positive gastric cancer (EBV+ GC), the method comprising:

(a) reacting a biological sample obtained from a subject with a reagent composition that comprises components for detecting in the biological sample the presence of one or more antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2; and

(b) detecting the presence of the antibodies in the sample, wherein increased seroreactivity relative to a control for one or more of the antibodies is indicative of at least a two-fold increased risk of EBV+ GC.

2. The method of claim **1**, wherein the detected antibodies comprise anti-BALF2, anti-BORF2, and anti-BRRF1.

3. The method of claim **1**, wherein the biological sample is one or more of a whole blood sample, a serum sample, and a plasma sample.

4. The method of claim **1**, wherein the method detects EBV+ GC gastric cancer prior to symptom onset.

5. The method of claim **1**, wherein the determining step is carried out using an ELISA assay or a Western Blot assay.

6. The method of claim **1**, further comprising administering a vaccine-based gastric cancer treatment to the subject if identified as having an increased risk of EBV+ GC gastric cancer.

7. A method to detect EBV+ GC in a subject at risk of having EBV+ GC, the method comprising:

(a) contacting a biological sample obtained from the subject with a set of reagents, wherein the set of reagents specifically binds to at least three biomarkers in the biological sample, wherein the biomarkers are selected from the group consisting of BORF2, LF2, BDLF2, BXLF1, BRLF1, BaRF1, BGLF5, BOLF1, BRRF1, BALF2, BLLF3, and BSLF2;

(b) measuring the level of the at least three biomarkers in the biological sample; and

(c) detecting that the level of the at least three biomarkers is increased in the biological sample relative to a control sample from a subject without EBV+ GC, thereby detecting the presence of EBV+ GC in the subject.

8. The method according to claim **7**, wherein the at least three biomarkers comprise anti-BALF2, anti-BORF2, and anti-BRRF1.

9. The method of claim **7**, further comprising (d) administering an EBV+ gastric cancer therapy to the subject, wherein the EBV+ GC therapy is selected from the group consisting of chemotherapy, hormonal therapy, radiotherapy, immunotherapy, and surgical removal of stomach tissue.

10. The method of claim **7**, wherein the biological sample is one or more of a whole blood sample, a serum sample, and a plasma sample.

11. The method of claim **7**, wherein the determining step is carried out using an immunoassay.

12. A method of determining an Epstein Barr Virus (EBV) gastric cancer antibody signature comprising antibodies, contained in a biological sample from an individual, that specifically bind to immobilized EBV antigens, the method comprising:

(a) contacting the sample to a panel of immobilized EBV antigens under conditions that promote formation of antigen-antibody complexes; and

(b) identifying complexes formed by immobilized EBV antigens and antibody in the sample, to determine an EBV antibody signature.

13. The method of claim **12**, wherein the antibody signature is expressed as a level of antibody specifically binding to each immobilized antigen.

14. The method of claim **12**, further comprising comparing an antibody signature from one individual to the antibody signature from another individual.

15. The method of claim **12**, wherein one individual has a disease process, and one individual is a healthy individual and the method allows comparison of the antibody signature in the healthy individual and the individual with a disease.

16. The method of claim **15**, wherein the disease process comprises EBV^+ GC.

17. The method of claim **12**, wherein the immobilized EBV antigens comprise one or more of BORF2, LF2, BDLF2, BXLF1, BRLF1, BaRF1, BGLF5, BOLF1, BRRF1, BALF2, BLLF3, and BSLF2.

18. A kit for performing the method of claim **1**, the kit comprising a reagent composition that comprises components for detecting in a biological sample the presence of one or more antibodies selected from anti-BORF2, anti-LF2, anti-BDLF2, anti-BXLF1, anti-BRLF1, anti-BaRF1, anti-BGLF5, anti-BOLF 1, anti-BRRF1, anti-BALF2, anti-BLLF3, and anti-BSLF2.

19. The kit of claim **18**, further comprising instructions for diagnosing an EBV^+ GC in a subject.

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