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High Levels of Epstein-Barr Virus DNA in Latently Infected Gastric Adenocarcinoma

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Abstract

Gastric adenocarcinoma is the second leading cause of cancer death worldwide. Epstein-Barr virus (EBV) is present in the malignant cells of approximately 10% of cases. It is unclear whether EBV is being missed in some gastric adenocarcinomas due to insensitive test methods or partial EBV genome loss. In the current study, we screened 113 gastric adenocarcinomas from low and high incidence regions (United States and Central America) for the presence of EBV using a battery quantitative real-time PCR (Q-PCR) assays targeting disparate segments of the EBV genome (BamH1W, EBNA1, LMP1, LMP2, BZLF1, EBER1) and histochemical stains targeting EBVencoded RNA (EBER), the latent proteins LMP1 and LMP2, and the lytic proteins BMRF1 and BZLF1. EBV DNA was detected by Q-PCR in 48/75 United States cancers (64%) and in 38/38 Central American cancers (100%), which was a significant difference. EBER was localized to malignant epithelial cells in 8/48 (17%) United States and 3/38 (8%) Central American cancers. Viral loads were considerably higher for EBER-positive versus EBER-negative cancers (mean 162,986 versus 62 EBV DNA copies per 100,000 cells). A viral load of 2,000 copies per 100,000 cells is recommended as the threshold distinguishing *EBER*-positive from *EBER*-negative tumors. One infected cancer selectively failed to amplify the LMP2 gene because of a point mutation, while another cancer had an atypical pattern of Q-PCR positivity suggesting deletion of large segments of the EBV genome. Three different viral latency profiles were observed in the cancers

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based on constant expression of EBER and focal or variable expression of LMP1 or LMP2, without lytic protein expression. We conclude that EBV DNA levels generally reflect *EBER* status, and a panel of at least two Q-PCR assays is recommended for sensitive identification of infected cancers.

Keywords

Epstein-Barr virus; gastric adenocarcinoma; latency; replication; viral load

Gastric cancer represents the fourth most common type of cancer and the second leading cause of cancer death worldwide.(1) The incidence of gastric cancer varies up to ten fold by geographic region, suggesting that genetic or environmental factors influence carcinogenesis and that different carcinogenesis models may be operative.(2) Patients often present with advanced and incurable disease. Even with resection and chemotherapy, high rates of recurrence result in poor overall survival, especially in developing countries. For unclear reasons, the incidence of gastric cancer in the proximal one-third of the stomach, where *Helicobacter pylori* (*H. pylori*) infection is less frequent, has been increasing worldwide.(2, 3) Interestingly, Epstein-Barr virus (EBV) is more frequently identified in these proximal cancers.(4-8)

Patients who are at high risk of treatment failure are candidates for novel or aggressive treatment regimens. One promising therapeutic target is EBV, a ubiquitous gamma herpesvirus that has been implicated in the pathogenesis of a variety of benign and malignant diseases.(9, 10) EBV DNA is present in the malignant epithelial cells of about 10% of gastric adenocarcinomas across geographic regions. The gastric adenocarcinomas most likely to be EBV-related are those of the "diffuse" histologic subtype with abundant infiltrating lymphocytes, poor to moderate differentiation, and those involving the proximal stomach.(4, 5, 7, 11-13) EBV association is also noted in cancers of the gastric stump following surgery, especially with Billroth II reconstruction after gastrectomy for benign gastroduodenal disease.(7, 14-17)

The gold standard assay for EBV targets EBV-encoded RNA (*EBER*) by *in situ* hybridization in paraffin-embedded tissue sections.(10, 18) This assay detects the most abundant latent viral transcripts and also localizes the viral infection to malignant cells by microscopy. However, the sensitivity of *EBER* staining has been called into question by investigators who have shown, by molecular or immunohistochemical assays, that EBV is present in some *EBER*-negative tumors.(19-25) Therefore, it is unclear whether EBV is being missed in some gastric adenocarcinomas due to insensitive testing methods or partial EBV genome loss.

Reliable diagnosis of EBV-related malignancy requires not only detection of the viral genome or its gene products, but also localization of the virus to the malignant cell fraction. Viral LMP2 and EBNA1 are target analytes since they are often expressed in EBV-related gastric cancers and they can be localized to tumor cells by immunohistochemistry,(10, 26, 27) although EBNA1 histochemical stains are less reliable due to crossreactivity with a human protein.(28) LMP1 is rarely expressed, and the restricted pattern of viral gene

To detect EBV at the DNA level, semiquantitative PCR has been used to screen gastric adenocarcinoma tissues for presence of the EBV genome and to demonstrate that high viral load correlates with EBER localization to malignant cells.(34) Modern real-time PCR methods now permit even more precise measurement of EBV viral load in DNA extracted from tissue specimens.(10)

Using a battery of tests for EBV DNA, RNA and protein, we examined 113 gastric adenocarcinomas from high and low incidence regions. This included 75 from the United States and 38 from Western Honduras. Western Honduras has been characterized as a region of high gastric cancer incidence, with standardized annual incidence rates of approximately 39 cases per 100,000 for males and 21 per 100,000 for females.(35) In addition, endemic H. pylori infection has been confirmed in the region (85%). By comparison, the United States has a standardized annual incidence of 11 per 100,000 males and 6 per 100,000 females, with a lifetime risk of nearly 1%. To detect EBV in cancer tissues, we used a battery of sensitive and specific quantitative real-time PCR (Q-PCR) assays targeting six disparate regions of the EBV genome (*BamH1W, EBNA1, LMP1, LMP2, EZLF1, EBER1*). The virus was localized using *EBER in situ* hybridization and immunohistochemical assays targeting selected viral proteins expressed during latent infection (LMP1, LMP2A) or during active viral replication (BZLF1, BMRF1).

Materials and Methods

Gastric Adenocarcinoma Samples

Formalin fixed, paraffin-embedded blocks representing 113 gastric adenocarcinomas were obtained from UNC Hospitals tissue archives in Chapel Hill, NC (n=55), Massachusetts General Hospital in Boston, MA (n=20) and Western Regional Hospital, Santa Rosa de Copan, Honduras, Central America (n=38). The gastric adenocarcinomas obtained from Massachusetts General Hospital were selectively enriched for gastric stump site, with 16/20 of these being stump cancers. The remaining cancers from all three sites were serial cases.

Clinical information included age, anatomic site of the cancer within the stomach, and, for the Central American cases, *H. pylori* serologic status. *H. pylori* serology was not performed on the gastric cancer patients from the United States, rather histologic evidence was sought for infection by bacteria consistent with *H. pylori*, but evidence was found in only one US case. Note that the sensitivity of microscopic detection of *H.pylori* is low given that surface mucosa was sometimes not even present in the cancer specimen used for this study. Furthermore, there is loss of *H. pylori* colonization as cancer develops.(1) For each cancer, histologic subtype was classified as intestinal *versus* diffuse using a modern version of Lauren's criteria.(36) Paraffin sections were placed on coated glass slides for histochemical stains, or placed in a microfuge tube for manual DNA extraction as previously described in Ryan *et al.*(37)

Quantitative real-time PCR

A battery of Q-PCR assays targeting six disparate regions of the EBV genome was used to measure EBV DNA. We previously validated five of these Q-PCR assays targeting *BamH1W*, *EBNA1*, *LMP1*, *LMP2*, and *BZLF1* regions of the EBV genome(37), while the sixth assay targeting *EBER1* was developed by Ling *et al.*(38) A Q-PCR assay targeting the human *APOB* gene was used, as previously described, to control for efficacy of DNA extraction and to normalize for the number of cells amplified per reaction.(37)

PCR was performed and products were detected on ABI Prism 7900 and 7500 Real-Time PCR instruments with Sequence Detection System software (Applied Biosystems, Foster City, CA). Thermocycling conditions were: 50° C for 2 minutes, 95° C for 10 minutes, and then 95° C for 15 seconds and 60° C for 1 minute for 40 cycles. Each 25μ l reaction contained: 1X TaqMan Universal Master Mix and TaqMan probe (10pmol). Forward and reverse primers (15pmol each) were used for all assays except those targeting *LMP1* and *BZLF1* in which 30pmol was used. DNA template volume was 1 μ l, and failed *APOB* reactions were repeated at higher or lower template volumes. A standard curve was generated using serial 10-fold dilutions of Namalwa Burkitt lymphoma cell line DNA (at two copies of EBV genome per cell) varying from 50,000 copies to 0.5 copies of EBV DNA per reaction. This curve was acceptable if sensitivity was at least 50 copies of EBV DNA per PCR, a difference of 3.3 +/- 0.3 cycles was demonstrated between each of the 10-fold dilutions, and if the correlation coefficient was at least 0.99. To check for amplicon contamination, every run contained at least two "no template" controls in which nuclease-free H2O was substituted for template.

All experimental samples were run in duplicate and a mean viral load was calculated based on the ratio of the copies of EBV to *APOB* in a given volume of extracted DNA, with *APOB* quantity representing the number of cells in the reaction. The resulting ratio was adjusted to provide the number of copies of EBV DNA per 100,000 cells. Samples with no measurable EBV DNA were reported as having a viral load of zero.

EBV-encoded RNA (EBER) in situ hybridization

EBER in situ hybridization was performed on paraffin sections of cases having detectable EBV DNA by at least one Q-PCR assay (n=86). This was accomplished using either a manual method with fluorescein-labeled oligonulceotide EBER and oligo(d)T control probes and the Super-Sensitive Poly-HRP ISH Non-Biotin Detection Kit (Biogenex, San Ramon, CA) with methyl green counterstain, or by an automated method using fluorescein-labeled EBER and oligo(d)T control probes on the Ventana Benchmark *in situ* hybridization system (Ventana Medical Systems, Tuscon AZ). Validation work on 56 samples showed no discrepancies between the two staining procedures. The oligo(d)T probe served as a control for RNA preservation in histological sections. A tumor was considered *EBER*-negative if *EBER* staining was undetected or was only expressed in benign-appearing lymphoid cells, and *EBER*-positive if the signal was localized to malignant epithelial cells.

Immunohistochemistry for viral LMP1, LMP2, BMRF1, and BZLF1

Immunohistochemical stains for viral protein were performed on the 86 gastric cancer samples having detectable EBV DNA by Q-PCR. Stains for EBV LMP1 and LMP2 proteins were done as previously described(37) using citrate retrieval and the CS1-4 cocktail of mouse monoclonal antibodies against LMP1 (1:100, Dako, Capinteria, CA) and the E411 rat monoclonal antibody against LMP2A (1mg/ml, Asencion, Munich, Germany). Paraffin sections of EBV-related Hodgkin lymphoma served as positive controls.

Immunohistochemical analysis of the EBV replicative proteins BMRF1 and BZLF1 was performed using anti-BMRF1 clone G3-E31 (1:200 dilution, Research Diagnostics, Inc., Flanders, NJ) and anti-BZLF1 clone BZ.1 (1:25 dilution, Dako, Carpinteria, CA) on paraffin sections pretreated with citrate for antigen retrieval. Sections were incubated with primary antibody for 30 minutes at 37°C using the manufacturer's blocking and detection protocols in the Super-Sensitive Non Biotin HRP Detection Kit (Biogenex). Bound antibody was detected by diaminobenzidine chromogen (Biogenex) and tissues were counter-stained with hematoxylin (Dako). Oral hairy leukoplakia paraffin sections served as a positive control.

Qualitative LMP2 PCR and DNA Sequencing

To further investigate a case in which the EBV *LMP2* segment selectively failed to amplify by Q-PCR, a primer set was designed to amplify a 130bp region of the *LMP2* gene encompassing the 69bp segment that had been targeted by the standard *LMP2* Q-PCR assay. The following PCR primers were used: LMP2Ext forward 5'-

CTGTTTTGCAGCTGAGTCC-3' and LMP2Ext reverse 5'-

CAATGTTAAAAGGGCTGCACC-3'. The 50µl reaction consisted of: 1X PCR Buffer, 2mM MgCl₂ 2.5 units Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA), 0.2mM dNTPs (Applied Biosystems), 50pmol of each LMP2Ext primer, and nuclease-free water. Reaction conditions were: 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; and then 72°C for 10 minutes. The product was confirmed by electrophoresis in a 2% agarose gel containing 0.5mg/ml ethidium bromide. The product was sequenced in both directions by first removing unincorporated primers and dNTPs, and then incubating with shrimp alkaline phosphatase and exonuclease I (Amersham, Piscataway, NJ) at 37°C for 30 minutes, then 80°C for 15 minutes. The 12.5µl sequencing reaction consisted of: 1.1µl of template, 1µl LMP2Ext forward primer (5pmol/ ml), 5µl BD Terminator Dye (Applied Biosystems), and water. The reaction was performed on the ABI 9700 thermocycler under the following conditions: 96°C for 5 seconds; 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes; 60°C for 10 minutes followed by a hold at 4°C. The product was purified using the Qiagen DyeEx 2.0 Spin Kit, denatured at 95°C for 3 minutes, and separated by size in POP6 polymer by capillary electrophoresis on an ABI 3100 Gene Analyzer instrument. The sequence was compared to consensus EBV reference sequence in GenBank (NC 007605) and to wild type viral DNA from an EBER-positive AIDS lymphoma and another gastric carcinoma that were tested in parallel.

Results

Histopathologic findings

Gastric adenocarcinomas from the United States (n=75) and Central America (n=38) were classified by histopathology into intestinal pattern (n=50), diffuse pattern (n=55), or mixed pattern of tumor growth (n=8). The anatomic site of origin was classified as distal (n=93) for tumors occurring in the antrum or body, proximal (n=14) for tumors occurring in the cardia, or whole stomach (n=6). *H. pylori* infection was detected in 33/38 (87%) Central American gastric cancer patients by standard serological testing.

EBV DNA Measurement by Quantitative Real-Time PCR

Gastric adenocarcinoma tissues were screened for EBV genome using six separate Q-PCR assays targeting six different regions of the viral genome (*BamH1W*, *EBNA1*, *LMP1*, *LMP2*, *BZLF1*, and *EBER1*). Out of the 75 United States gastric cancers, 48 (64%) had detectable EBV DNA by at least one Q-PCR assay, and 11 (15%) were positive by all six Q-PCR assays (Table 1), while 27 (36%) were negative by all six assays. The Central American cases more frequently contained EBV DNA: 38/38 (100%) by at least one Q-PCR assay, and 11 (29%) by all six Q-PCR assays (Table 2).

Virologic correlates in gastric adenocarcinomas

EBER in situ hybridization is considered the gold standard assay for localizing latent EBV infection in lesional cells. *EBER in situ* hybridization revealed EBV within the malignant epithelial cells in 8/48 (17%) of the cancers from the United States and in 3/38 (8%) of the Central American cancers that had detectable EBV DNA by at least one Q-PCR. The *EBER*-negative cancer tissues often harbored scattered EBER-positive lymphocytes, including 17/40 (42%) United States and 12/35 (34%) Central American cancers. No *EBER* expression was seen in benign epithelial cells or stromal elements. In the malignant cells, *EBER* was either uniformly positive or uniformly negative, supporting that EBV infection may have occurred before malignant transformation and was transmitted to all daughter cells in the neoplastic clone.

Interestingly, the viral loads for gastric cancers with detectable EBV DNA could be split into two general categories: 1) viral load greater than 10,000 copies per 100,000 cells, or 2) viral load less than 2,000 copies per 100,000 cells. With two exceptions (discussed below), those viral loads consistently greater than 10,000 EBV DNA copies/100,000 cells (8 from the United States and 3 Central American) were all EBV-associated tumors as defined by *EBER* localization to the malignant epithelial cells, whereas those with low viral loads lacked *EBER* in the malignant cells. The viral loads in the *EBER*-positive gastric cancers were nearly 3,000 fold higher than in the *EBER*-negative cancers (mean = 162,986 versus 62 EBV DNA copies/100,000 cells, respectively; p=0.003). A cutoff value of 2,000 EBV copies per 100,000 cells is a reasonable and conservative level beyond which EBV was always localized to the malignant cells. A cost-effective screening strategy would be to reserve *EBER* staining for cases having viral loads over 2000 EBV copies per 100,000 cells. Among the *EBER*-negative malignancies, the 29 cases having visible *EBER*-positive lymphocytes by *in situ* hybridization had significantly higher EBV loads than those lacking visible *EBER* in lymphocytes (mean = 110 versus 30 EBV DNA copies/100,000 cells, respectively, by the *BamH1W* assay; p=0.02).

Association between EBV status and clinicopathologic findings

Surprisingly, EBV was more commonly detectable by Q-PCR in Central American than in United States gastric cancer tissues (p=0.0001). But EBV was not more commonly localized to the malignant epithelial cells in Central American compared with United States cancers (p=0.61). Statistical power was insufficient to examine subcategories, but trends showed that *EBER*-positivity was similarly frequent in the proximal tumors (1/10, 10%) compared to distal tumors (7/62, 11%), and it was slightly more common in stumps (2/12, 17%). In support of previous reports, *EBER*-positivity was significantly more common in diffuse compared to intestinal histology (p=0.01). However, when Q-PCR was used to identify the virus, detectable EBV DNA was not preferentially found in cancers with diffuse rather than intestinal histology (p=0.83).

All the Central American gastric cancers were located in non-cardia regions. Among US gastric cancers, *EBER*-positive cases were not preferentially localized in the cardia region of the stomach including cardia comprising a stump after prior surgery. Although our numbers were small: 3/8 *EBER*-positive cancers occurred in the antrum, 2/8 in the body, 1/8 were widespread, and 2/8 were stump cancers. A similar distribution of anatomical sites was observed for the 17 United States gastric cancers that expressed *EBER* only in lymphocytes, where 8/17 occurred in the body, 4/17 in the antrum, 3/17 in the cardia, 1/17 were widespread, and 1/17 was a stump cancer.

A negative correlation between *EBER*-positive cancer and *H. pylori* serologic status was observed in the Central American cases (p<0.0001). *H. pylori* status for the United States cases could not be evaluated because serological testing was not performed, and histologic visualization of *H. pylori* is not reliable in the setting of gastric cancer.

EBV latently infects gastric adenocarcinomas

Immunohistochemistry was performed on all gastric cancers having detectable EBV DNA by any of the Q-PCR assays to localize the viral infection and to further characterize it as latent or lytic based on the spectrum of expressed viral proteins. No lytic EBV infection was observed as signified by the absence of BMRF1 or BZLF1 replicative protein expression in the malignant cells or in reactive stromal tissue. The pattern of latent viral gene expression varied among the gastric cancers. Viral LMP2A staining was observed in 4/11 *EBER*positive cancers and all four were among the eight United States cases. When present, LMP2A was restricted to the cytoplasm of malignant epithelial cells and was expressed diffusely in 1 case and focally in up to 10% of malignant cells in 3 cases (Figure 1A). Viral LMP1 expression was seen in only 1/11 *EBER*-positive gastric cancers, Central American case #51. In that case, membranous/cytoplasmic LMP1 was diffusely expressed and restricted to the malignant epithelial cells (Figure 1B), and the cells did not coexpress LMP2. No staining for LMP1 or LMP2 was observed in *EBER*-negative gastric cancers, nor was either protein expressed in infiltrating lymphocytes or benign epithelial cells. Taken together, our results show three different viral latency profiles for EBV-associated gastric cancer: The majority of the *EBER*-positive cancers (4/8 United States and 2/3 Central American) were EBER+/LMP1-/LMP2A-. Less frequently, the latency profiles EBER+/LMP1-/LMP2A+ (4/8 United States) and EBER+/LMP1+/LMP2A-(1/3 Central American) were observed.

Viral genomic variation in an EBV-associated Central American Gastric Cancer

EBER-positive Central American gastric cancer #49 had selective dropout of amplifiable EBV DNA for the LMP2 locus whereas the viral loads were greater than 112,000 EBV DNA copies per 100,000 cells for the other five viral load assays (Table 2). Immunohistochemical analysis of this cancer revealed no LMP2A protein expression, however this is not necessarily a result of a viral genomic defect since most cancers in this series did not express LMP2A. Although the primers and probes were designed to target a highly conserved segment of the LMP2 gene, the selective non-amplification suggests either mutation or deletion interfering with primer/probe binding. To resolve this dilemma, amplification and sequencing was performed on the relevant *LMP2* gene segment. A point mutation was found near the 5' end of the reverse primer, only three bases in from where the polymerase initiates strand extension (Figure 2). This mutation involved a conversion from a cytosine to a thymine at position number 733 in the *LMP2* gene sequence compared to the wild type sequence found in the positive control and in GenBank Accession No. NC_007605. Our findings suggest that the *LMP2* 733C>T mutation hinders annealing of the reverse primer, resulting in reduced amplicon production.

Viral genomic variation in an EBER-negative US Gastric Cancer

The other exceptional case was an *EBER*-negative US gastric cancer case #24 with a single high *LMP1* Q-PCR result and no other evidence of the virus beyond rare *EBER*-positive lymphocytes by *in situ* hybridization. The high level of *LMP1* DNA is consistent with the levels of seen in EBV-associated gastric cancer rather than the levels seen in background infection of bystander lymphocytes. Repeat testing on four occasions verified the unusual results.

Discussion

This study is the first to demonstrate that quantitative real-time DNA amplification technology is equivalent to *EBER in situ* hybridization for identifying cases of gastric cancer that are EBV-related. We further showed that targeting viral DNA or *EBER* RNA is superior to immunohistochemical detection of selected viral proteins since these proteins are not consistently expressed at visible levels. Furthermore, expression patterns of viral proteins differed among cases, but always revealed latent infection. Use of multiple Q-PCR assays revealed that polymorphisms (mutation or deletion) of the viral genome may be more frequent than previously recognized.

When a battery of six different Q-PCR assays was applied to determine if one or a combination of these assays could be used to screen for EBV-related cancer, most of the Q-PCR assays were equally informative. A threshold could be set that clearly distinguished

EBER-positive from *EBER*-negative cancers regardless of whether *EBER*-positive infiltrating lymphocytes were seen. With the exception of two unusual cases discussed below, no *EBER*-negative cancer had a viral load over 1,629, and no *EBER*-positive cancer had a level below 10,558 EBV copies per 100,000 cells. A cutoff value of 2,000 copies per 100,000 cells is suggested as a threshold above which an *EBER* stain should be done to insure that the EBV signal is localized to the malignant cells, and below which *EBER* is likely to be restricted to benign lymphocytes, assuming that the tumor is adequately represented in the sample. Note that this cutoff is substantially higher than the threshold of 100 EBV copies per 100,000 cells that had been set in a prior study of non-gastric malignancies, including non-Hodgkin lymphomas.(37) This difference suggests that *EBER*-negative gastric cancer tissues tend to have higher EBV loads than do *EBER*-negative lymphoma tissues, implicating higher levels of EBV in the non-malignant cells of the stomach.

Prior work localized EBV to benign lymphocytes in gastritis lesions and other preneoplastic gastric lesions.(14, 29, 39-43) In the current study, scattered *EBER*-positive lymphocytes were found in some *EBER*-negative cancer tissues where they tended to correlate with measurable viral loads. No other benign cell types were found to contain either latent or lytic viral infection by histochemical stains. Although we did not study preneoplastic tissue or lesions, it will be important to determine if patients who eventually develop cancer have higher levels of EBV beforehand. In this regard, it is worth considering if the high incidence of gastric cancer in Central America is related to the relatively high EBV loads seen in every gastric cancer from that region regardless of *EBER* status. Further studies are needed to explore whether host genetic, immunologic, infectious, or nutritional factors caused the levels of EBV to be higher in Central American compared to United States gastric cancer tissue.

The laboratory assays described in the current study were clearly useful for identifying infected cancers, and further work is needed to explore their utility for measuring low level EBV in preneoplastic tissue or in blood. Among the six assays that were used, the BamH1W assay had the lowest limit of detection, while the LMP1 and LMP2 assays were more likely to miss low-level infection as judged by the frequency of amplification in our *EBER*-negative cancer tissues. For applications in which analytic sensitivity is critical, the BamH1W Q-PCR is recommended.

Our results build on prior work by others demonstrating that EBV is found in distinct clinicopathologic subtypes of gastric cancer, suggesting that the virus is not just an innocent bystander but rather it tracks with fundamental biologic and immunologic characteristics that are likely to influence tumor development and maintenance. Although the incidence of gastric cancer is much higher in Central America than in the United States, our work shows that the proportion of EBV-related cases (as defined by *EBER* expression in malignant cells) is not radically different between the two countries (8% versus 17%, respectively; p=0.23) and is comparable to rates described by others worldwide.(4, 5, 7, 11, 29, 32, 44, 45) The geographic variation is not as dramatic as with other EBV-related diseases such as Burkitt lymphoma in which EBV-positivity rates vary from over 95% in tropical Africa to 25% in

the United States, and nasopharyngeal carcinoma where rates vary from over 95% in Southern China to about 75% in the United States.(18)

A known contributing oncogenic factor is *H. pylori* infection. In fact, both EBV and *H. pylori* are considered class 1 oncogenic pathogens by the World Health Organization (WHO) and are associated with overlapping subsets of gastric carcinoma.(6, 46, 47) *H. pylori* is endemic among adults in Honduras, while the United States carrier rate is about 30% among adults. Unless treated with medications to eradicate the bacterium, the majority of individuals maintain chronic infection. In our Central American cohort on whom serologic testing was performed, a significant negative association was found between *EBER*-positive cancer and *H. pylori* infection. Further studies are warranted to determine if different mechanisms of carcinogenesis are operative.

Because mutation or partial genomic deletion could interfere with detection of viral DNA or gene products, a battery of six Q-PCR assays was used to assay multiple segments of the viral genome. In any given tumor specimen, the viral loads were fairly consistent across the six Q-PCR assays and were clearly informative with regard to *EBER in situ* hybridization status, suggesting that one or more of these Q-PCR assays could be used as a screening tool for EBV-related cancer in paraffin-embedded tissue. It is important to note that one of the gastric cancers in this series would have yielded a false negative interpretation of EBV status if only the *LMP2* Q-PCR assay had been used. In a prior study, false negative Q-PCR assays targeting either *LMP2*, *LMP1*, or *BZLF1* were seen in AIDS lymphomas.(37) These findings demonstrate the importance of targeting more than one segment of the viral genome to ensure sensitive detection of the virus.

US gastric cancer case #24 was exceptional in that a high LMP1 Q-PCR value was found despite undetectable virus by the other five Q-PCRs. The high level of *LMP1* DNA is consistent with the levels seen in EBV-associated gastric cancers, however the gold standard *EBER in situ* assay showed no *EBER* localization to malignant cells, and LMP1 immunohistochemistry was likewise negative. While it is feasible that this LMP1 Q-PCR result is false positive (perhaps crossreacting with a rare pathogen), one must consider the possibility that the result legitimately reflects a segment of EBV DNA. Interestingly, the *LMP1* gene is considered to be an oncogene in that it has many qualities that promote tumorigenesis. First, deletion of the LMP1 gene renders EBV incapable of immortalizing B cells.(48) Second, LMP1 functions as a TNF receptor that constitutively signals through multiple growth-promoting and anti-apoptotic pathways including EGFR, NFKB, PI3K and AKT.(49, 50) Its effect on the inhibitors of differentiation (ID1 and ID3) could help explain why infected carcinomas tend to appear so undifferentiated.(51) Transgenic mice expressing LMP1 behind a keratin promoter develop epithelial hyperplasia while LMP1 expressed behind an immunoglobulin promoter induces B cell lymphoma.(50) In this patient, it is feasible that a defective recombinant viral genome containing LMP1 could have been produced naturally by atypical rearrangement and partial deletion of the EBV genome. Integration of the defective LMP1-containing segment into host chromosomal DNA is a possible mechanism of persistence. The intact EBV genome may also persist in this patient as evidenced by rare *EBER*-expressing lymphocytes. If future work confirms that remnants of the EBV genome are indeed present within the malignant cells in cases such as this one, it

would lend support to the hit-and-run hypothesis by which EBV might have contributed early during tumorigenesis but then was largely eliminated from the epithelial cells prior to clonal expansion.

The current study demonstrates that latent, not lytic, EBV infection characterizes gastric adenocarcinomas. This confirms previous reports showing that virally encoded *EBER* and LMP2 are often expressed, but LMP1 is very rarely expressed.(29, 30, 32, 52-56) *EBER*, a non-polyadenylated transcript, is thought to promote growth of gastric epithelial cells at least in part through induction of insulin-like growth factor (IGF1).(57) Only one case in the current series expressed LMP1 by histochemical analysis, while 4/11 infected cases expressed LMP2A. It is important to characterize viral gene expression not only because viral gene products serve as targets to assist in laboratory diagnosis, but also because they may serve as targets for therapy. The immune system seems to tolerate latent viral infection in this and other infected malignancies.(58) Some progress has been made in managing EBV-related cancers by infusing cytotoxic T cells which have been expanded *in vitro* through stimulation by viral proteins.(59) Another promising strategy is to use differentiating agents that induce lytic viral replication, which incites a strong antiviral immune reaction.(60-62)

Prior research showed lytic EBV infection in some gastric adenocarcinomas using either histochemistry or rtPCR.(29, 32) In the current study, lytic viral protein expression was not detected by either BZLF1 or BMRF1 immunohistochemistry, suggesting that cells undergoing replicative infection are rare. These findings are relevant to patient care since one would predict that well-tolerated oral antiviral agents like gancyclovir, which target replicative infection, are unlikely to eliminate malignant cells. Nevertheless, even rare cells expressing lytic viral proteins may contribute to angiogenesis and tumor growth.(63) In fact, Kenney and colleagues succeeded in killing malignant cells using gancyclovir combined with standard chemotherapeutic or radiation therapies, presumably because gancyclovir is activated by phosphorylation in cells that the standard therapies induce to switch from latency to lytic viral replication.(64-66) Not only are the lytically infected cells killed, but adjacent cells die as phosphorylated gancyclovir is transferred to them and exerts its cytotoxic activity.(64) Once EBV-directed therapies are established to be useful in patient management, it will be all the more important to identify virally-infected cancers that would be predicted to respond to the therapy. Meantime, the utility of knowing that a particular patient's cancer is EBV-infected may be limited to prognosis(7, 67) and to measurement of circulating EBV DNA as a marker of tumor burden.(68) The laboratory assays described herein should prove useful for identifying affected patients.

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Abbreviations

(EBV)	Epstein-Barr virus
(US)	United States
(Q-PCR)	Quantitative polymerase chain reaction
(EBER)	Epstein Barr virus-encoded RNA
(H. pylori)	Helicobactor pylori

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Figure 1. Latent viral protein expression in gastric adenocarcinomas

A) Hematoxylin and eosin stain reveals gastric adenocarcinoma in United States Case #6. B) Immunohistochemistry shows LMP2A expression localized to the malignant cells of United States Case #6. C) Immunohistochemistry reveals LMP1 is expressed in the malignant cells of Central American Case #51. (A, B 400×; C 100×)

5' - ctgttttgcagctgagtcccctccttgg agctgtaactgtggtttccatga cg <u>ctgctgctactggctttcgtcctctgg</u> ct **ctčttcgccagggggc** ctaggtac tcttggtgcagcccttttaacattg -3'

Figure 2. EBV *LMP2* **DNA sequencing revealed a mutation interfering with primer binding** DNA sequencing was performed on a 130bp region of EBV *LMP2* encompassing the 69bp region targeted by the *LMP2* Q-PCR assay. The forward and reverse primer binding sites for the *LMP2* Q-PCR assay are in bold font, while the TaqMan probe site is underlined. The star represents the position of a point mutation (C>T base substitution at position 733) in *LMP2* exon 4 of Central American Case #49, which is predicted to result in substitution of serine by phenylalanine; this substitution of a large nonpolar for a small polar amino acid may have functional significance in addition to its apparent interference with laboratory testing.

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| EBERI | 85 945 | 453 506 | 255 939 | 336 394 | 307 206 | 24 526

 | 32 132 | 27 429

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 | 262 | 11

 | 0 | 50 | 0 | 0
 | 64 | 22 | 0 | 0 | 0 |
| BZLFI | 58 238 | 318 450 | 178 280 | 230 134 | 287 564 | 26 240

 | 98 141 | 38 912

 | 34
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 | 0 | 0 | 0 | 0
 | 0 | 18 | 0 | 0 | 1 |
| LMP2 | 12 441 | 217 712 | 18 750 | 104 257 | 149 268 | 20 183

 | 65 211 | 31 509

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| IdWI | 10 558 | 338 007 | 38 640 | 318 865 | 402 341 | 213 938

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| EBNAI | 57 969 | 569 004 | 117 818 | 370 534 | 390 856 | 219 321

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Lab Invest. Author manuscript; available in PMC 2009 July 01.

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Table 1

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Clinicopathologic Findings in EBV Q-PCR Positive United States Gastric Cancers

	<i>EBER in situ</i> hybridization	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	23 Negative 17 Lymphocytes + 8 Tumor +
	EBERI	0	0	4	63	9	0	6	275	0	0	0	0	0	28	0	0	0	0	0	0	0	0	0	24/48 Posituve
	BZLFI	15	36	0	0	0	0	0	0	0	0	0	5	0	56	0	0	21	0	129	9	0	11	2	25/48 Positive
es / 100 000 cells	LMP2	0	0	0	0	9	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	17/48 Positive
EBV DNA copi	LMPI	0	178	0	0	0	0	0	0	0	0	0	27	0	28	0	0	0	0	0	0	89	0	0	17/48 Positive
	EBNAI	15	0	0	0	12	0	0	0	0	33	5	27	3	17	0	65	0	0	0	15	0	0	0	31/48 Positive
	BamHIW	6	36	2	50	9	14	11	92	1	22	4	5	0	11	232	0	0	93	0	8	0	0	0	38/48 Positive
	APOB (# of cells)	3 289	562	30 257	798	16 198	709	9 268	218	14 907	9 105	7 466	3 723	11 976	1 784	431	614	943	108	774	6 597	5 594	2 782	5 152	48/48 Positive
	Hp Status*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Unk	Neg	Unk	Neg	Unk	Unk	Unk	Neg	Unk	Unk	1/36 Positive
	Site	Stump	Distal	Distal	Proximal	Distal	Proximal	Distal	Distal	Distal	Distal	Distal	Distal	Distal	Stump	Stump	Stump	Stump	Stump	Stump	Stump	Stump	Proximal	Proximal	27 Distal 7 Proximal 12 Stump 2 Other
	Histological Classification	Intestinal	Mixed	Diffuse	Diffuse	Mixed/Signet	Diffuse/Signet	Diffuse	Intestinal	Intestinal	Diffuse/Signet	Intestinal	Diffuse/Signet	Diffuse	Intestinal	Diffuse	Diffuse/Signet	Intestinal	Intestinal	Diffuse/Signet	Intestinal	Intestinal	Mixed/Signet	Intestinal	25 Diffuse 18 Intestinal 5 Mixed 14 Signet
	Case #	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	Totals (N=48)

Signet = Signet ring cells; Stump = Cancer arose in gastric remnant after prior surgery; Neg = negative; Unk = unknown.

 H_p status = Helicobacter pylori as determined by histopathology.

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| b) BamHIW EBNAI LMP1 LMP2 BZLF1 EBER1 112 875 186 573 413 866 2 215 594 141 963 444 96 295 554 282 447 18 140 175 280 249 742 64 047 145 306 30 121 12 660 37 162 68 457 222 489 1.333 533 489 222 29 50 101 0 20 0 2135 347 225 8 180 276 135 347 225 8 180 276 135 347 225 8 180 276 135 347 225 8 180 276 135 347 225 8 180 276 135 344 494 25 716 370 101 221 368 745 370 177 101 221 365 | Bantliw EBNAI LMP1 LMP2 BZLF1 EBER1 112 875 186 573 413 866 2 215 594 141 963 112 875 186 573 413 866 2 215 594 141 963 44 496 295 554 282 447 18 140 175 280 249 742 64 047 145 306 30 121 12 660 37 162 68 457 50 145 1333 533 489 222 222 489 1.333 533 489 222 210 145 489 1.333 533 489 222 135 347 225 8 180 276 76 135 347 225 8 180 276 776 142 469 306 22 95 177 76 142 449 236 247 716 370 76 370 221 95 716 370 | Imblit best best best best best best best bes | IWEBNAILMP1LMP2BZLF1EBER155 186573 413866 2 215594 141963 6 295554 282447 18140 175280 249742 7 145306 30121 12660 37162 68457 7 489 1.333 533 489 222 8 1.333 533 489 222 7 489 1.333 533 489 222 8 101 0 20 0 0 947 225 8 180 276 944 494 25 716 370 944 494 25 716 370 944 472 135 303 236 944 472 135 303 236 944 472 135 303 236 944 472 135 303 236 944 626 282 63 219 944 972 135 303 236 944 972 135 303 236 944 972 135 303 236 944 972 135 933 219 944 972 135 136 944 972 135 110 944 972 135 188 944 97 135 136 944 90 93 93 944 90 93 93 944 <th>EBNAI LMP1 LMP2 BZLF1 EBERI 186 573 413 866 2 215 594 141 963 295 554 282 447 18 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 489 1.333 533 489 222 50 101 0 20 0 51 101 0 200 37 162 68 457 489 1.333 533 489 222 68 57 50 101 0 200 37 162 68 457 57 489 1.333 533 489 222 57 57 347 225 8 180 276 7 7 469 306 222 95 177 370 7 444 494 25 716 370 74 110 221 944 472 135 333<th>EBNAILMP1LMP2BZLF1EBERI186 573143 8662215 594141 963186 573413 86622249 742295 554282 44718 140175 280249 742145 30630 12112 66037 16268 457145 30530 12112 66037 16268 457145 30630 12112 66037 16268 4574891.3335334892223472258180222347225818027634722581802764493062295177444494257163704444942571637022136874741102213687474110221368747411022136874741102213687474110221368747411022136874741102213613702369444721353032222826321994447213596944472135979444721359694447213597944947414944947416944<</th><th>EBNAI LMP1 LMP2 BZLF1 EBERI 186 573 413 866 2 215 594 141 963 295 554 282 447 18 140 175 280 249 742 295 554 282 447 18 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 50 101 0 20 0 0 50 101 0 20 0 0 0 51 1333 533 489 222 68 457 5 50 101 0 20 0 0 0 547 223 8 180 276 5 449 494 25 716 370 5 444 494 25 137 236 5 944 472 135 303 236 5 944 472 135 303 236 5</th><th>EBNAILMP1LMP2BZLF1EBER1186 573413 8662215 594141 963186 573413 86637 16268 457249 742295 554282 44718 140175 280249 742295 55430 12112 66037 16268 457145 30630 12112 66037 16268 457501010200050101020005010102070764693062281802763472258180276776469306229517717746930623747411022136874741107369444721353032367369444721353032361886122863747411022800536113694447213530323618862628263747411012405707913612431307410016612431307410013412431302011341343532940260763532940201134<th>EBNAILMP1LMP2BZLFIEBERI186 573141 3662215 594141 963186 573413 8662215 594141 963295 554282 44718 140175 280249 742295 55430 12112 66037 16268 457295 55430 12112 66037 16268 4574891.33353348922250101020050101020050101020050101027684501010203704693062295177469306257163704444444721353032213687474110944472135303236944472135303236944472135303236944472135303236944472135303236944905702994495932191889459493303236946909301009479102191349489394939494994949494944949594945<t< th=""><th>EBNAI LMP1 LMP2 BZLF1 EBERI 186573 413 866 2 215 594 141 963 295 554 282 447 181 40 175 280 249 742 295 554 282 447 18 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 489 1.333 533 489 222 50 101 0 20 0 347 225 8 180 222 489 1.333 533 489 222 51 110 0 20 0 444 494 25 716 370 444 494 25 716 370 221 368 74 716 110 221 368 74 110 716 222 96 5370 236 716 223 0 536 1177 110</th><th>EBNAI LMP1 LMP2 BZLF1 EBERI 186 573 413 866 2 215 594 141 963 186 573 413 866 2 215 594 141 963 295 554 282 447 181 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 50 101 0 20 249 742 50 101 0 20 249 742 50 101 0 20 68 457 510 30121 12 660 37 162 68 457 533 533 489 222 68 50 101 0 20 0 449 494 255 716 370 221 368 74 74 110 222 363 219 370 236 444 472 135 303 236 222 283 303 236 110</th></t<><th>EBNAILMP1LMP2BZLFIEBERI186 573$413 866$2$215 594$$141 963$$247$186 573$413 866$2$215 594$$141 963$$297 42$295 554$282 447$$181 40$$175 280$$2497 42$$2497 42$145 306$30121$$12660$$371 62$$68
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<th>EBNAI LMP1 LMP2 BZLF1 EBERI 186 573 413 866 2 215 594 141 963 295 554 282 447 18 140 175 280 249 742 295 554 282 447 18 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 50 101 0 20 0 0 50 101 0 20 0 0 0 51 1333 533 489 222 68 457 5 50 101 0 20 0 0 0 547 223 8 180 276 5 449 494 25 716 370 5 444 494 25 137 236 5 944 472 135 303 236 5 944 472 135 303 236 5</th> <th>EBNAILMP1LMP2BZLF1EBER1186 573413 8662215 594141 963186 573413 86637 16268 457249 742295 554282 44718 140175 280249 742295 55430 12112 66037 16268 457145 30630 12112 66037 16268 457501010200050101020005010102070764693062281802763472258180276776469306229517717746930623747411022136874741107369444721353032367369444721353032361886122863747411022800536113694447213530323618862628263747411012405707913612431307410016612431307410013412431302011341343532940260763532940201134<th>EBNAILMP1LMP2BZLFIEBERI186 573141 3662215 594141 963186 573413 8662215 594141 963295 554282 44718 140175 280249 742295 55430 12112 66037 16268 457295 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 | EBNAI LMP1 LMP2 BZLF1 EBERI 186 573 413 866 2 215 594 141 963 295 554 282 447 18 140 175 280 249 742 295 554 282 447 18 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 50 101 0 20 0 0 50 101 0 20 0 0 0 51 1333 533 489 222 68 457 5 50 101 0 20 0 0 0 547 223 8 180 276 5 449 494 25 716 370 5 444 494 25 137 236 5 944 472 135 303 236 5 944 472 135 303 236 5 | EBNAILMP1LMP2BZLF1EBER1186 573413 8662215 594141 963186 573413 86637 16268 457249 742295 554282 44718 140175 280249 742295 55430 12112 66037 16268 457145 30630 12112 66037 16268 457501010200050101020005010102070764693062281802763472258180276776469306229517717746930623747411022136874741107369444721353032367369444721353032361886122863747411022800536113694447213530323618862628263747411012405707913612431307410016612431307410013412431302011341343532940260763532940201134 <th>EBNAILMP1LMP2BZLFIEBERI186 573141 3662215 594141 963186 573413 8662215 594141 963295 554282 44718 140175 280249 742295 55430 12112 66037 16268 457295 55430 12112 66037 16268 4574891.33353348922250101020050101020050101020050101027684501010203704693062295177469306257163704444444721353032213687474110944472135303236944472135303236944472135303236944472135303236944472135303236944905702994495932191889459493303236946909301009479102191349489394939494994949494944949594945<t< th=""><th>EBNAI LMP1 LMP2 BZLF1 EBERI 186573 413 866 2 215 594 141 963 295 554 282 447 181 40 175 280 249 742 295 554 282 447 18 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 489 1.333 533 489 222 50 101 0 20 0 347 225 8 180 222 489 1.333 533 489 222 51 110 0 20 0 444 494 25 716 370 444 494 25 716 370 221 368 74 716 110 221 368 74 110 716 222 96 5370 236 716 223 0 536 1177 110</th><th>EBNAI LMP1 LMP2 BZLF1 EBERI 186 573 413 866 2 215 594 141 963 186 573 413 866 2 215 594 141 963 295 554 282 447 181 140 175 280 249 742 145 306 30 121 12 660 37 162 68 457 50 101 0 20 249 742 50 101 0 20 249 742 50 101 0 20 68 457 510 30121 12 660 37 162 68 457 533 533 489 222 68 50 101 0 20 0 449 494 255 716 370 221 368 74 74 110 222 363 219 370 236 444 472 135 303 236 222 283 303 236 110</th></t<><th>EBNAILMP1LMP2BZLFIEBERI186 573$413 866$2$215 594$$141 963$$247$186 573$413 866$2$215 594$$141 963$$297 42$295 554$282 447$$181 40$$175 280$$2497 42$$2497 42$145 306$30121$$12660$$371 62$$68 457$$247$$489$$1.333$$533$$489$$222$$6457$$247$$50$$101$$0$$20$$0$$0$$0$$347$$225$$8$$180$$2276$$8$$347$$225$$8$$180$$276$$0$$449$$494$$25$$716$$370$$276$$444$$494$$25$$716$$370$$276$$444$$494$$25$$716$$370$$236$$444$$494$$25$$716$$370$$236$$444$$494$$25$$716$$370$$236$$444$$472$$135$$303$$236$$177$$424$$313$$0$$53$$01$$100$$166$$124$$0$$0$$0$$201$$134$$124$$213$$00$$00$$136$$01$$124$$213$$00$$00$$201$$134$$124$$00$$00$$00$$100$$166$$124$$00$$201$$134$$00$$124$$00$$00$$00$<th>EBNAILMP1LMP2BZLFIBSLFI186573413 8662215 594141 963186573413 8662215 594141 963295 554282 44718 140175 280249 742145 3063012112 66037 16268 45753353348922268 4575603012112 66037 16268 45757014302020501010202051305533489222533062295177444494257163704444942571637052628263219188626282632191885280002062628263219188626282632191886262286321918862622863219188626228632191887120536113472300260744100100166745333236134741330267433323474133074333236743332367433323674<td< th=""><th>EBNAILMP2LMP2BZLF1EBER1$186573$$413866$$2$$215594$$141963$$141963$$186573$$413866$$2$$215594$$141963$$2477$$295554$$282447$$18140$$175280$$249742$$8475$$295534$$280121$$12660$$37162$$68457$$68457$$489$$30121$$12660$$37162$$68457$$68457$$489$$1333$$533$$489$$222$$68457$$50$$1011$$0$$276$$68457$$7845$$489$$1333$$533$$489$$222$$68457$$469$$3061$$207$$68457$$2265$$533$$444$$494$$25$$74$$1100$$766$$444$$494$$25$$744$$1100$$766$$444$$494$$25$$716$$370$$236$$944$$4472$$135$$236$$370$$236$$924$$222$$633$$219$$188$$236$$612$$222$$633$$219$$188$$226$$626$$282$$633$$219$$188$$226$$924$$213$$00$$533$$61$$00$$124$$313$$0$$216$$134$$106$$124$$213$$0$$216$$124$$106$$124$$213$$0$$216$$00$$106$$124$$213$</th><th>EBNAI LMP1 LMP2 BZLFI EBERI 186573 413 866 2 215 594 141 963 295 554 282 447 18 140 175 280 249 742 145 306 30121 12 660 37 162 68 457 489 1.333 533 489 222 68 489 1.333 533 489 222 68 74 489 1.333 533 489 222 68 74 50 101 0 20 20 0 222 347 225 8 180 276 94 444 494 25 716 370 97 444 494 25 716 370 97 944 47 135 303 236 177 944 47 13 74 110 97 124 0 5 303 236 14</th></td<></th></th></th>
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66 IntestinalDistal $S+$ 66 IntestinalDistal $S+$ 66 IntestinalDistal $S+$ 100 IntestinalDistalNeg 85 IntestinalDistalNeg 100 | Mixed/SignetDistal $S+$ 29 IntestinalDistal $S+$ 31 Diffuse/SignetDistal $P+$ 13 IntestinalDistal $P+$ 13 IntestinalDistal Neg 10 Diffuse/SignetDistal $S+$ 66 Diffuse/SignetDistal $S+$ 64 Diffuse/SignetDistal $S+$ 64 Diffuse/SignetDistal $S+$ 66 Diffuse/SignetDistal $S+$ 71 IntestinalDistal $S+$ 100 IntestinalDistal $S+P+$ 100 | Mixed/SignetDistal $S+$ 29 IntestinalDistal $S+$ 31 Diffuse/SignetDistal $P+$ 13 IntestinalDistal $P+$ 13 IntestinalDistal $S+$ 66 Diffuse/SignetDistal $S+$ 66 Diffuse/SignetDistal $S+$ 66 Diffuse/SignetDistal $S+$ 66 IntestinalDistal $S+$ 66 IntestinalDistal $S+$ 71 IntestinalDistalNeg 85 IntestinalDistal $S+P+$ 71 Diffuse/SignetDistal $S+/P+$ 71 Diffuse/SignetDistal $S+/P+$ 71 Diffuse/SignetDistal $S+/P+$ 71 | Mixed/SignetDistal $S+$ 29 IntestinalDistal $S+$ 31 Diffuse/SignetDistal $P+$ 13 IntestinalDistal $P+$ 13 IntestinalDistal Neg 10 Diffuse/SignetDistal $S+$ 44 Diffuse/SignetDistal $S+$ 66 Diffuse/SignetDistal $S+$ 66 Diffuse/SignetDistal $S+$ 66 IntestinalDistal $S+$ 66 IntestinalDistal $S+$ 71 Diffuse/SignetDistal $S+$ 100 IntestinalDistal $S+$ 100 IntestinalDistal $S+$ 100 Diffuse/SignetDistal $S+$ 100 IntestinalDistal $S+$ 100 IntestinalDistal $S+$ 100 IntestinalDistal $S+$ 100 Diffuse/SignetDistal $S+$ 100 IntestinalDistal $S+$ 100 IntestinalDistal $S+$ 100 IntestinalDistal $S+$ 177 Diffuse/SignetDistal $S+$ 177
 | Mixed/SignetDistal $S+$ 29 IntestinalDistal $S+$ 31 Diffuse/SignetDistal $P+$ 13 IntestinalDistal $P+$ 10 IntestinalDistal $S+$ 44 Diffuse/SignetDistal $S+$ 71 Diffuse/SignetDistal $S+P+$ 71 Diffuse/SignetDistal $S+$ 71 Diffuse/SignetDistal $S+$ 71 DiffuseDistalDistal $S+$ DiffuseDistal $S+$ 71 | Mixed/SignetDistal S_{i+} 2 IntestinalDistal S_{i+} 31 Diffuse/SignetDistal P_{i+} 13 IntestinalDistal Neg 10 IntestinalDistal S_{i+} $6($ Diffuse/SignetDistal S_{i+} $6($ Diffuse/SignetDistal S_{i+} $6($ Diffuse/SignetDistal S_{i+} $6($ Diffuse/SignetDistal S_{i+} 71 IntestinalDistal Neg 85 IntestinalDistal S_{i+} 71 Diffuse/SignetDistal S_{i+} 71 Diffuse/SignetDistal S_{i+} 71 Diffuse/SignetDistal S_{i+} 71 Diffuse/SignetDistal S_{i+} 71 DiffuseDistal S_{i+} 71 DiffuseDistal S_{i+} 72 DiffuseDistal S_{i+} 21 |
| | Intestinal Distal S+ 3 194 | Intestinal Distal S+ 3 194 Diffuse/Signet Distal P+ 1 315 | IntestinalDistalS+3 194Diffuse/SignetDistalP+1 315IntestinalDistalNeg1 052
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 | IntestinalDistal $S+$ $3 194$ iffuse/SignetDistal $P+$ $1 315$ IntestinalDistal Neg $1 052$ iffuse/SignetDistal $S+$ 602 iffuse/SignetDistal $S+$ $4 476$ DiffuseDistal $S+$ $3 806$ DiffuseDistal $S+$ $3 806$ | IntestinalDistal $S+$ $3 194$ iffuse/SignetDistal $P+$ $1 315$ IntestinalDistalNeg $1 052$ iffuse/SignetDistal $S+$ 602 iffuse/SignetDistal $S+$ 4476 DiffuseDistal $S+$ $3 806$ DiffuseDistalDistal $S+$ $3 806$ IntestinalDistalDistal $S+$ $3 806$ IntestinalDistalDistal $S+$ $3 806$

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Table 2

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Clinicopathologic Findings in Central American Gastric Cancers

	I EBER in situ hybridization	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	Tumor -	titive 23 Negative 12 Lymphocytes + 3 Tumor +
	EBERI	116	0	0	0	47	9	12	104	68	L	8	10	49	28/38 Posi
	BZLF1	93	9	233	L	54	8	0	69	203	0	0	20	18	33/38 Positive
s / 100 000 cells	LMP2	0	0	0	0	0	0	0	0	0	0	0	0	0	12/38 Positive
EBV DNA copie	IMPI	0	0	0	0	94	12	0	0	0	0	0	0	0	17/38 Positive
	EBNAI	0	16	320	0	173	10	371	0	136	0	0	199	183	27/38 Positive
	BamHIW	0	11	08	1	22	9	12	104	68	L	8	10	49	36/38 Positive
	APOB (# of cells)	1 726	8 757	847	10 060	6 356	11 728	809	288	738	4 454	1 255	2 009	3 281	38/38 Positive
	Hp Status [*]	\mathbf{S}^+	\mathbf{S}^+	\mathbf{S}^+	Neg	\mathbf{S}^+	\mathbf{S}^+	S+/P+	\mathbf{S}^+	\mathbf{S}^+	\mathbf{S}_+	Neg	S^+	S^+	30/38 Positive
	Site	Distal	Proximal	Distal	Distal	Distal	Distal	Distal	Distal	Distal	Distal	Distal	Distal	Distal	35 Distal 3 Proximal
	Histological Classification	Intestinal	Intestinal	Intestinal	Intestinal	Diffuse	Intestinal	Intestinal	Intestinal	Intestinal	Diffuse/Signet	Diffuse/Signet	Diffuse/Signet	Diffuse	19 Diffuse 18 Intestinal 1 Mixed 12 Signet
	Case #	74	75	76	77	78	79	80	81	82	83	84	85	86	Totals (N=38)

Signet = Signet ring cells; Stump = Cancer arose in gastric stump after prior surgery; Neg = negative; Unk = unknown.

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* Hp status = Helicobacter pylori infection as determined by serology positive (S+) or histopathology positive (P+).

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