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Case–control study of Epstein–Barr virus and *Helicobacter pylori* serology in Latin American patients with gastric disease

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Background: Chronic tissue damage induced by *Helicobacter pylori* (*HP*)-driven inflammation is considered the main risk of gastric carcinoma (GC). Epstein–Barr virus (EBV) infection has also been associated with GC. In this study, we aim to address the role of EBV in inflammatory GC precursor lesions and its added risk to *HP* infection.

Methods: Antibodies against EBV, *HP* and the bacterial virulence factor CagA were measured in sera from 525 Mexican and Paraguayan patients with gastric disease. Gastric samples were characterised according to the updated Sydney classification and associations were estimated between antibody responses and severity of both tissue damage and inflammation.

Results: We found significant associations (odd ratios and trends) between EBV and *HP* copositivity and premalignant lesions and intestinal-type GC. The EBV and *HP* coinfection was also significantly associated with increased infiltration of immune cells. No association was found between EBV and the less inflammation-driven diffuse-type GC.

Conclusions: Our study suggests that EBV co-participates with *HP* to induce severe inflammation, increasing the risk of progression to intestinal-type GC.

Gastric carcinoma (GC) is the fifth most common type of cancer and the third cause of death by cancer worldwide, affecting particularly Asian and Latin American countries (Ferlay *et al*, 2013). According to the Lauren classification, there are two main histological types of gastric adenocarcinoma, intestinal and diffuse, that presumably evolve through different mechanisms. Intestinal-type GC progresses through inflammatory lesions characterised by increased tissue damage, starting with a nonatrophic gastritis (NAG) that becomes an atrophic gastritis (AG), intestinal metaplasia (IM), dysplasia and eventually GC (the so-called Correa sequence) (Correa *et al*, 1975). Although diffuse GC also initiates through NAG, it does not progress through intermediate atrophic/inflammatory lesions, and it has been suggested that it may be importantly driven by causes other than inflammation, including germline mutations. In agreement, familial GC cases resulting from inherited E-cadherin mutations usually develop diffuse type of GC (Guilford *et al*, 1998); still, the carcinogenic process remains unknown for most cases.

Gastric carcinoma is a cancer of a recognised infectious aetiology, with *Helicobacter pylori* (*HP*) considered the main risk

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factor and, more recently, also linked to infection by Epstein–Barr virus (EBV) (Murphy *et al*, 2009; Camargo *et al*, 2011). Both pathogens are usually acquired early in life, with ~50% of the adult world population infected by *HP* and 90% by EBV (Thorley-Lawson and Gross, 2004; Fuccio *et al*, 2010). Carcinogenic pathogens are classified as direct or indirect acting according to their transforming mechanisms (Morales-Sanchez and Fuentes-Panana, 2014). The direct-acting agents are usually found in monoclonal form in tumour cells and keep the transformed phenotype through aberrant expression of pathogen and/or cellular oncogenes and tumour suppressor genes. Indirect agents promote the transformation process without housing in the tumour cell, either triggering local chronic inflammation and oxidative stress that persistently damage the infected tissue or triggering immunosuppression affecting tumour immunosurveillance mechanisms. Although *HP* is considered the prototype cancer-inducing agent through chronic inflammation/tissue damage mechanisms, a direct-transforming bacterial oncogene, *cagA* (*cytotoxin-associated gene A*), has been recently documented (Ohnishi *et al*, 2008; Miura *et al*, 2009). On the other hand, EBV is considered a direct transforming pathogen through expression of its own death/proliferation regulatory genes (Thornburg *et al*, 2006; Frappier, 2012; Shair *et al*, 2012; Saha and Robertson, 2013).

The EBV infection has been associated with several types of B-cell lymphomas and upper digestive tract carcinomas. The EBV infection usually persists in B cells, with most infected individuals carrying the virus asymptomatically in a latent stage in these cells. It is not clear how EBV infects the gastric mucosa and whether infection induces an inflammatory reaction, as observed with *HP*. We have recently presented evidence of an association between EBV reactivation antibodies and severe inflammatory responses in the gastric mucosa of pediatric patients (Cardenas-Mondragon *et al*, 2013). In the present study, a role of EBV in premalignant and malignant lesions was addressed in adult patients from two Latin American countries with similar GC risk and *HP* prevalence (Flores-Luna *et al*, 2012). For this, antibodies against the viral capsid antigen (VCA), an EBV reactivation antigen, *HP* and the CagA virulence factor were analysed for association with the type of gastric lesion and the degree of inflammation. We found evidence suggesting a critical EBV activity promoting inflammation of the gastric epithelium that, together with *HP*, increases the risk of developing premalignant lesions and intestinal-type GC.

MATERIALS AND METHODS

Ethics statement. The Scientific and Ethics Committees from each of the participating hospitals approved this study: Hospital de Especialidades and Hospital de Oncologia of the Centro Médico Nacional Siglo XXI (Instituto Mexicano del Seguro Social; IMSS), Hospital General de México 'Eduardo Liceaga' and Hospital de Cancerología of the Secretaría de Salud (SS) in Mexico City, and the Universidad Nacional de Asunción in Paraguay. All patients were informed about the nature of the study and those willing to participate signed a written informed consent before specimen collection.

Study design. This is a case–control study of patients with gastric disease in which antibodies against an EBV reactivation antigen, *HP* and the CagA virulence factor were analysed for association with the type of gastric lesion and the degree of inflammation. For all analyses performed premalignant (AG, IM and dysplasia) and malignant lesions (cases) were compared with NAG (controls), the earliest inflammatory lesion in the progression to intestinal and diffuse GC.

Study population. The study included 525 adult patients (≥ 30 years old) with any spectrum of gastric lesion from Mexico and Paraguay, two Latin American countries with reported similar rates of *HP* infection, prevalence of CagA-positive strains and GC incidence (Flores-Luna *et al*, 2012). Patients were recruited between October 1999 and July 2002 after attending the Gastroenterology or Oncology Units because of gastric symptoms or a probable GC. Patients were recruited from the following hospitals: Hospital de Especialidades and Hospital de Oncología of the Centro Médico Nacional Siglo XXI (Instituto Mexicano del Seguro Social (IMSS)), Hospital General de México 'Eduardo Liceaga' and Hospital de Cancerología of the Secretaría de Salud (SS) in Mexico City, and the Universidad Nacional de Asunción in Paraguay. A total of 813 patients were recruited, of whom 236 were eliminated from Mexico and 52 from Paraguay because of lacking complete information about either the histopathological or serological analysis. Thus, 309 (Mexico) and 216 (Paraguay) patients were included in the study.

Healthy blood donors. Sera from 129 healthy blood donors (median age = 41.6 ± 7.7; 0.98 male to female ratio) were collected between September 2010 and April 2012 from the blood bank of the Centro Médico Nacional Siglo XXI (IMSS in Mexico City). Clinical information was registered in questionnaires at the time of inclusion with no reports of gastric symptomatology. The median of antibody titres for this group was: 72.7 (anti-EBV IgG), 1.2 (anti-*HP* IgG) and 1.0 (anti-CagA IgG).

Data collected. Sociodemographic data and clinical information were registered in questionnaires at the time of inclusion. The information collected included age, gender, clinical symptoms and clinical diagnosis based on endoscopy, histology and clinical presentation. Patients with antibiotic, bismuth compounds, proton pump inhibitors and/or nonsteroidal anti-inflammatory drugs or antiacid treatments 3 weeks before sample collection as well as those who had received cancer treatment were excluded from the study.

Histopathological examination. Three biopsies from the antrum and three from the body of the stomach were used for the histopathological diagnosis. All biopsies were fixed in formalin, embedded in paraffin and a section stained with haematoxylin and eosin (HE). The HE-stained sections were used to measure and classify the inflammatory reaction according to the updated Sydney system (Dixon *et al*, 1996). The pathologist involved in this study received direct training from a group of experts led by Dr Pelayo Correa. This group of experts validated all the samples collected after standardisation of the criteria using consensus protocol reading (Kasamatsu *et al*, 2010). Samples were classified according to the level of tissue damage and type of lesion as NAG, AG, IM, dysplasia, intestinal- and diffuse-type GC. Samples were also classified according to the level of immune cell infiltration of mononuclear (MN) cells (inflammation) and polymorphonuclear (PMN) cells (activity of the lesion) (Dixon *et al*, 1996). After reading all samples, the final diagnosis for both types of lesion and level of infiltration was that of the most severe reading observed in the six biopsies analysed for each patient.

Collection of blood and analysis of antibodies. A sample of venous blood (4 ml) was drawn from all patients. Stored serum samples were used to analyse IgG, IgM and IgA antibodies against EBV VCA, as well as IgG antibodies against *HP* whole-cell extracts and CagA. Anti-EBV VCA antibodies were determined using ELISA commercial kits (HUMAN, Wiesbaden, Germany) for IgG anti-VCA (catalogue 51204) and for IgM anti-VCA (catalogue 51104), as well as IgA anti-VCA (catalogue 1414; Diagnostic Automation, Inc., Calabasas, CA, USA) following the manufacturer's instructions and as previously described (Cardenas-Mondragon *et al*, 2013). Anti-EBV IgG-positive samples were defined after comparison with a cutoff value (COV). This COV was calculated with the following formula: COV = (mean

absorbance of negative controls + ((0.1) (mean absorbance of positive controls)). Sample values are expressed in HU ml^{-1} = mean absorbance of sample/[(mean absorbance values of positive control) (100)]. The sample is considered positive when the mean absorbance value (after two or four readings) was above the COV calculated + 15%. The COV is independently calculated for each ELISA plate and a mean of 20.1 was obtained between plates. The IgG antibodies against *HP* and CagA were determined using ELISA tests previously used and validated in a Mexican population (Camorlinga-Ponce *et al*, 1998; Cardenas-Mondragon *et al*, 2013). Patients were considered positive for *HP* antibodies when ELISA units were $\geq$1.0, and for CagA when ELISA units were $\geq$1.5, according to the validated cutoffs (Camorlinga-Ponce *et al*, 1998).

Statistical analysis. The data set was analysed using different statistical tools assessing whether the data followed a normal or nonnormal distribution. We then compared Mexico and Paraguay using the media and the s.d. for all descriptive analyses: differences in age were estimated by the Student's *t*-test, and for categorical variables (sex, type of gastric lesions; EBV, *HP* and CagA serology) frequencies were obtained, and differences were estimated by the proportion test. Because no significant differences were found, both populations were added and analysed together. The proportion test was also used to analyse differences in the frequency of seropositive patients between gastric lesions: premalignant and malignant lesions against NAG, or intestinal-type against diffuse-type GC. For all comparisons between more than two categories, the Mantel–Haenszel χ^2 with linear tendency was used. In all these analyses the least advanced lesion was used as control: NAG for premalignant and malignant lesion, and none/mild PMN and MN infiltration as control for severe immune cell infiltration. To assess the risk provided by EBV, *HP* or CagA to develop premalignant and malignant lesions or severe immune cell infiltration, the odd rates (ORs) were estimated. The group of EBV and *HP* double-positive patients was compared with the group infected with only *HP* or EBV. A similar analysis was performed with HPCagA + /EBV + against HPCagA − /EBV + and HPCagA + /EBV − . Premalignant and malignant lesions were compared with NAG and severe immune infiltration against none or mild. Because sex and age are confounders, ORs were adjusted by them using logistic regression with 95% confidence intervals (CIs). Sex- and age-adjusted ORs were also used to estimate whether increased anti-EBV antibody titres were associated with premalignant and malignant lesions. For this analysis the EBV antibody titre was categorised by tertiles based in their distribution in NAG followed by a comparison of the highest to the lowest tertiles. Tests for trend were conducted by modelling tertile median serological values to asses increased risk when progressing from NAG to premalignant to malignant lesions; from non/mild to moderate to severe immune cell infiltration; and from low to moderate to high anti-EBV antibody titres. Data were analysed using the statistical Stata 12.0 software program (Stata Corporation, College Station, TX, USA) and Epi Info 7 TM (Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA)).

RESULTS

Study population. The study included 525 adult patients who sought medical attention for gastric diseases in Mexico and Paraguay. The demographic characteristic of the patients and the seroprevalence of anti-EBV, anti-*HP* and anti-CagA antibodies are summarised in Table 1. A total of 225 (42.9%) samples were classified as NAG with typical epithelial cell morphology and no glandular atrophy, and 300 samples presented atrophy and were grouped according to the presence of malignant changes: 186 (35.4%) premalignant lesions (AG = 27, IM = 152 and dysplasia = 7) and 114 (21.7%) GCs. Of these 114, 50 GCs were intestinal type and 64 were diffuse type.

Table 1. General description of the study population

Variable	Mexico	Paraguay	Total
Number studied (%)	309	216	525
Age (mean ± s.d.)[a]	54.7 ± 13.9	54 ± 15.1	54.4 ± 14.4
Sex, male/female ratio	126/183 = 0.69	118/98 = 1.2	244/281 = 0.87
Gastric lesion, *n* (%)			
Nonatrophic gastritis[b]	124 (40.1)	101 (46.8)	225 (42.9)
Premalignant lesions[b]	123 (39.8)	63 (29.2)	186 (35.4)
Gastric cancer (GC)[b]	62 (20.1)	52 (24.1)	114 (21.7)
Intestinal type (%[c])[b]	30 (48.4)	20 (38.5)	50 (43.9)
Diffuse type (%[c])[b]	32 (51.6)	32 (61.5)	64 (56.1)
EBV positive, *n* (%)[b]	291 (94.2)	206 (95.4)	497 (94.7)
HP positive, *n* (%)[b]	270 (87.4)	189 (87.5)	459 (87.4)
CagA positive, *n* (%)[b]	219 (70.9)	163 (75.5)	382 (72.8)

Abbreviations: CagA = cytotoxin-associated gene A; EBV = Epstein–Barr virus; *HP* = *Helicobacter pylori*.
[a]Student's *t*-test.
[b]Proportion test.
[c]Referred to GC (100%).

Reactivation of EBV has been documented to occur in the mucosa of the upper GI tract and antibodies against lytic-cycle antigens mirror the level of viral reactivation and correlate with the risk to develop nasopharyngeal carcinoma (NPC; Ji *et al*, 2007). To address whether EBV reactivation also correlates with GC progression, IgG and IgA antibodies against VCA were measured in all patients. The IgM antibodies were also initially measured, but because only a few samples were positive, and IgM-positive patients preferentially presented NAG, we did not continue that analysis. In all, 497 (94.7%) patients were positive for EBV antibodies, with a similar prevalence in both countries (Table 1). Most patients were positive for IgG antibodies, with only 120 (22.9%) also positive for IgA. The median of the anti-VCA IgG titre was 80.4 HU ml^{-1} (range 18–181.1 units), whereas IgA tests were not quantitative. Seroprevalence to *HP* and CagA was 87.4% and 72.8%, respectively. The *HP* infection frequencies in both countries were also highly similar. The coefficient of variation (CV) of all serological measurements was very similar for both countries, with CV average values of 45.1 for EBV, 85 for *HP* and 77.8 for CagA.

EBV serology positively associates with premalignant lesions and intestinal type GC. The frequency of EBV positivity (IgG + with either IgA + or IgA −) was estimated for each disease group and premalignant lesions (AG, IM and dysplasia) and malignant lesions were compared with NAG, the earliest stage in GC progression (Table 2). A proportion test showed significant values for premalignant lesions ($P = 0.009$) and intestinal-type GC ($P = 0.039$). A significant OR was also found with premalignant lesion (OR = 3.5), whereas intestinal GC showed an infinite (∞) value because all cases were EBV positive (Table 2). Moreover, the frequency of positives from NAG to premalignant lesion and to intestinal-type GC showed a significant trend ($P = 0.003$). On the other hand, no association was found between EBV seropositivity and the diffuse type of GC. The EBV IgA-positive patients did not show any significant association (data not shown). Similar results were obtained comparing with sera from a group of 129 healthy blood donors (data not shown), although the analysis with this group was not pursued because of lack of tissue to evaluate gastric disease. Regarding *HP*, we observed *HP* or CagA associated with premalignant lesions, the frequency of positives to both of these risk factors decreased in intestinal type of GC but it was maintained in the diffuse type. This trend in *HP* serology

Table 2. EBV and *H. pylori* association with advanced stages of gastric disease

	Gastric lesion				
			Gastric cancer		
IgG titres	NAG[a]	Premalignant lesions	Intestinal	Diffuse	Total GC
Total, M + P	*n* = 225	*n* = 186	*n* = 50	*n* = 64	*n* = 114
EBV					
Positive, *n* (%)	207 (92)	**182 (97.9)**	50 (100)	58 (90.6)	108 (94.7)
P[b]		**0.009**	**0.039**	0.489	0.375
OR[c] (95% CI)		**3.5 (1.1–10.8)**	∞	0.7 (0.2–2.1)	1.2 (0.4–3.4)
P for trend[d]			**0.003**	0.577	0.163
HP					
Positive, *n* (%)	193 (85.8)	166 (89.3)	40 (80)	60 (93.8)	100 (87.7)
P[b]		0.319	0.352	0.099	0.652
OR[c] (95% CI)		1.7 (0.9–3.2)	0.9 (0.3–2.5)	**3.2 (1.01–10.9)**	1.6 (0.7–3.5)
P for trend[d]			0.756	0.068	0.493
CagA					
Positive, *n* (%)	157 (69.8)	147 (79)	28 (56)	50 (78.1)	78 (68.4)
P[b]		0.067	0.150	0.256	0.827
OR[c] (95% CI)		**1.8 (1.1–3.0)**	0.9 (0.4–2.1)	1.8 (0.9–3.9)	1.2 (0.7–2.0)
P for trend[d]			0.647	0.052	0.824

Abbreviations: CagA = cytotoxin-associated gene A; CI = confidence interval; EBV = Epstein–Barr virus; GC = gastric cancer; *HP* = *Helicobacter pylori*; IgG = immunoglobulin G; M + P = total patients, Mexico + Paraguay; NAG = nonatrophic gastritis; OR = odds ratio. Numbers in bold denote statistical significance ($P<0.05$).
[a]Used as control group.
[b]Proportion test for positive samples.
[c]OR adjusted for age and sex.
[d]The χ^2 for trend of positives (%) found from nonatrophic gastritis (NAG) to premalignant lesion to cancer.

Table 3. The *HP*/EBV coinfection and risk to develop advanced gastric disease

	Gastric lesion								
				Gastric cancer					
	Nonatrophic gastritis[a]	Premalignant lesion		Intestinal		Diffuse		Total GC	
Groups	*N*	*N*	OR[b] (95% CI)	*N*	OR[b] (95% CI)	*N*	OR[b] (95% CI)	*N*	OR[b] (95% CI)
HP + /EBV + *vs*	175	164	**8.4 (1.8–38.9)**	40		55	0.9 (0.3–2.9)	95	1.5 (0.5–4.7)
HP + /EBV −	18	2	*P* = **0.007**	0	∞	5	*P* = 0.912	5	*P* = 0.502
HP + /EBV + *vs*	175	164	**2.0 (1.01–3.9)**	40	1.0 (0.4–2.8)	55	**4.5 (1.2–17.4)**	95	2.0 (0.8–4.6)
HP − /EBV +	32	18	*P* = **0.047**	10	*P* = 0.928	3	*P* = **0.027**	13	*P* = 0.114
HP CagA + /EBV +	143	145	**6.8 (1.4–33)**	28		45	0.7 (0.2.-2.3)	73	1.1 (0.3–3.6)
HP CagA + /EBV −	14	2	*P* = **0.017**	0	∞	5	*P* = 0.567	5	*P* = 0.885
HP CagA + /EBV +	143	145	1.7 (0.9–3.2)	28	0.9 (0.3–2.6)	45	1.1 (0.4–2.6)	73	1.0 (0.5–2.2)
HP CagA − /EBV +	32	19	*P* = 0.132	12	*P* = 0.899	10	*P* = 0.869	22	*P* = 0.966

Abbreviations: CagA = cytotoxin-associated gene A; CI = confidence interval; EBV = Epstein–Barr virus; GC = gastric cancer; *HP* = *Helicobacter pylori*; OR = odds ratio. Numbers in bold denote statistical significance ($P>0.05$).
[a]Used as control group.
[b]OR adjusted for age and sex.

during GC progression has been previously documented by us (Camorlinga-Ponce *et al*, 2008) and others (Kokkola *et al*, 2003).

Because infection by *HP* is considered the main risk factor to develop GC, we grouped patients according to their infection status as follows: 434 patients were positive for both pathogens (*HP* + / EBV +), 63 were positive only for EBV, 25 were positive for *HP* and 3 patients were negative for both *HP* and EBV. To better understand the added risk of both pathogens to develop gastric lesions, we have taken the individuals infected by both pathogens and compared them with the ones infected only by *HP* (to estimate the added risk of EBV infection) or compared them with the ones infected only by EBV (to estimate the added risk of *HP* infection) (Table 3). A significant OR = 8.4 was observed for premalignant lesions when *HP* + /EBV + was compared with *HP* + (which addresses the role of EBV, $P = 0.007$), whereas in the case of intestinal-type GC, all patients were EBV + , giving a *P*-value of ∞. No significant association was obtained with diffuse-type GC. When *HP* + /EBV + was compared with the EBV + group (which addresses the role of *HP*), the association between coinfection and premalignant lesions was also significant (OR = 2.0; $P = 0.047$). However, results with the types of GC differed; there was no association with the intestinal-type GC, but a significant association was observed with the diffuse-type GC (OR = 4.5; $P = 0.027$). A similar analysis was performed comparing CagA + /EBV + with CagA + /EBV − patients, finding a significant value for premalignant lesions (OR = 6.8; $P = 0.017$; Table 3).

The frequency of EBV- and *HP*-positive patients in the different disease groups is shown in Supplementary Figure 1, in which it can be observed that EBV positivity increases as the inflammatory lesion progresses (from NAG to premalignant lesion and intestinal GC), whereas *HP* decreases in intestinal GC patients. In contrast, the frequency of *HP* remains high in diffuse GC. Supplementary Table 1 shows a proportion test comparing the frequency of EBV or *HP* seropositivity between intestinal and diffuse GCs, showing that whereas EBV was significantly associated with intestinal GC ($P=0.026$), *HP* and CagA were associated with diffuse GC ($P=0.036$ and $P=0.041$, respectively).

EBV serology positively associates with severe gastric inflammation. The association of EBV with intestinal-type GC and its precursor lesions suggests a possible role of EBV in collaboration with *HP* to induce a more severe inflammatory process, considered as the main driver for this oncogenic pathway. To test this, the level of inflammation in each lesion was graded according to the updated Sydney system (Dixon *et al*, 1996) and ORs (for both infiltration of MN or PMN cells) were estimated between EBV+/HP+ and single infected patients, either *HP*+ or EBV+ (Table 4). Because only a few samples presented severe PMNs, moderate and severe groups were combined. The analysis showed that patients with a mixed EBV and *HP* infection presented significant ORs for severe infiltration of both MN (OR of 5.9 in NAG; and OR of 18.3 in premalignant lesions) and PMN cells (OR of 11.5 in NAG, and OR of 10.2 in premalignant lesions) than patients with single infection. Also, an analysis of the trend of progression from less to more severe inflammation showed significant values for both MN and PMN infiltration in *HP*+/EBV+ patients with either NAG or premalignant lesions ($P<0.005$; Table 4). Nonsignificant ORs of 3 and 7.5 for severe MN and PMN infiltration, respectively, were observed in intestinal-type GC. Similar positive associations were found when the double-positive EBV and CagA groups were compared with the group of CagA positive but EBV negative plus the EBV positive but CagA negative (Supplementary Table 2). Nonsignificant numbers were observed for diffuse-type GC (Supplementary Table 3).

Increased levels of antibodies against EBV were significantly associated with severity of disease. We then analysed whether increased antibody titres against EBV-VCA were also associated with premalignant lesion and intestinal-type gastric disease (Table 5). This analysis showed that increased antibody titres had a significantly increased association with premalignant lesions (*P*-value for trend = 0.023) and with intestinal GC (*P*-value for trend = 0.038). In contrast, no such significant trend was observed for diffuse GC.

Table 4. Risk of increased inflammation in double-infected *vs* single-infected patients

	HP+ EBV+	*HP*+ EBV− and *HP*− EBV+		*P* for trend[a]
	N	***N***	**OR[b] (95% CI)**	
MN infiltration				
Nonatrophic gastritis				
Mild[c]	32	24	1.0	
Moderate	105	21	**3.7 (1.8–7.6)**	
Severe	38	5	**5.9 (2–17.3)**	
				0.0001
Premalignant lesion				
Mild[c]	8	4	1.0	
Moderate	115	15	3.3 (0.9–12.6)	
Severe	41	1	**18.3 (1.6–211.1)**	
				0.004
Intestinal-type GC				
Mild[c]	3	2	1.0	
Moderate	29	5	3.3 (0.4–26.3)	
Severe	6	3	3 (0.1–12.8)	
				0.915
PMN infiltration				
Nonatrophic gastritis				
None[c]	15	23	1.0	
Mild	59	12	**7.7 (3–19.4)**	
Moderate/severe	101	15	**11.5 (4.7–27.9)**	
				0.0000
Premalignant lesion				
None[c]	10	8	1.0	
Mild	46	4	**8.9 (2.2–35.8)**	
Moderate/severe	108	8	**10.2 (3.1–33.9)**	
				0.0002
Intestinal-type GC				
None[c]	4	3	1.0	
Mild	15	4	1.6 (0.2–13.6)	
Moderate/severe	20	3	7.5 (0.8–72.3)	
				0.172

Abbreviations: CI = confidence interval; EBV = Epstein–Barr virus; GC = gastric cancer; *HP* = *Helicobacter pylori*; MN = mononuclear; OR = odds ratio; PMN = polymorphonuclear. Numbers in bold denote statistical significance ($P<0.05$).
[a]The χ^2 for trend of positives (%) found from mild to moderate to severe (for MN) or none to mild to moderate-severe (for PMN) infiltration.
[b]OR adjusted for age and sex.
[c]Used as comparison group.

DISCUSSION

In this study, we provide serological evidence suggesting that despite the decreased level of antibodies against *HP* in intestinal-type GC, there is a significant association of EBV and *HP* coinfection with this GC and its precursor lesions. We did not observe the same for the diffuse type of GC, although *HP* remained positively associated, suggesting that EBV reactivation does not importantly contribute to this pathway of gastric carcinogenesis. The intestinal and diffuse types of GC presumably evolve through different mechanisms. Intestinal-type GC progresses through inflammatory precursor lesions characterised by increased tissue damage (AG to IM to dysplasia). In agreement with the EBV association with this pathway, we also found a positive association between EBV and severe infiltration of PMN and MN immune cells, suggesting that EBV is an important trigger of inflammation. On the contrary, diffuse GC does not progress through intermediate atrophic/inflammatory lesions, and it has been suggested that it may be importantly driven by causes other than inflammation.

Increasing evidence supports a link between EBV and GC, with a recent meta-analysis concluding that ~9% of all GCs are positive to EBV (Murphy *et al*, 2009). However, Murphy *et al* (2009) only considered reports supporting a direct transforming role, in which EBV is infecting most tumour cells (e.g., searched by *in situ* hybridisation of viral transcripts). We have also found evidence of tumour cell infection by EBV in a similar percent of GCs (Martinez-Lopez *et al*, 2014). Early reports found EBV specifically associated with lymphoepiteliomas, a rare type of GC histologically similar to NPC (Shibata *et al*, 1991). More recent meta-analyses have found the virus in both intestinal- and diffuse-type GCs (Lee *et al*, 2009; Murphy *et al*, 2009; Li *et al*, 2010b), or preferentially associated with the diffuse type of GC (Camargo *et al*, 2011).

The association of *HP* with early inflammatory precancerous lesions is well documented. However, only a few studies have analysed the participation of EBV infection in these lesions. Some studies have found evidence that EBV infects epithelial cells of the atrophic gastric mucosa with a relatively low frequency (≤3% positive samples) (Hungermann *et al*, 2001; Zur Hausen *et al*,

Table 5. The EBV antibody titre association with gastric disease

	Gastric lesion								
				Gastric cancer					
	Nonatrophic gastritis[b]	Premalignant lesion		Intestinal		Diffuse		Total GC	
EBV IgG titres[a]	*N*	*N*	OR (95% CI)	*N*	OR (95% CI)	*N*	OR (95% CI)	*N*	OR (95% CI)
Total, M + P									
3.5–52.62	75	48	1.0	12	1.0	22	1.0	34	1.0
52.63–87.09	75	56	1.2 (0.7–1.9)	13	1.1 (0.5–2.5)	19	0.9 (0.4–1.7)	32	0.9 (0.5–1.7)
87.10–181.1	75	82	**1.7 (1.1–2.8)**	25	2.1 (0.97–4.5)	23	1.04 (0.5–2)	48	1.4 (0.8–2.4)
P for trend[c]			**0.023**		**0.038**		0.856		0.172

Abbreviations: CI = confidence interval; EBV = Epstein–Barr virus; GC = gastric cancer; IgG = immunoglobulin G; OR = odds ratio; M + P = total patients, Mexico + Paraguay. Numbers in bold denote statistical significance ($P<0.05$).

[a]Units HU ml^{-1}.

[b]Used as control group.

[c]The χ^2 for trend of positives (%) found from low to mild to high antibody titres.

2004), whereas other studies favour frequencies of $\geq 50\%$ (Ryan *et al*, 2012). Similarly, a few studies have addressed EBV serology in gastric disease with conflicting results (Levine *et al*, 1995; Shinkura *et al*, 2000; Koshiol *et al*, 2007; Schetter *et al*, 2008; Kim *et al*, 2009). Interestingly, we observed a positive association between EBV and severe infiltrate of MN and PMN cells in NAG (table 4), suggesting that the virus is inducing inflammation very early in the oncogenic pathway leading to intestinal type GC.

The EBV first and foremost documented mechanism for cell transformation is through direct infection and expression of viral oncogenes within the transforming cell. On the contrary, *HP* is considered the prototype cancer-inducing agent through chronic inflammation indirect mechanisms. However, a direct-transforming bacterial oncogene, *cagA*, has been recently documented (Ohnishi *et al*, 2008; Miura *et al*, 2009), and an *HP* association with diffuse type of GC has been extensively documented (Huang *et al*, 1998). Because in our study we measured antibodies against an EBV reactivation antigen, our data suggest a positive association between EBV reactivation and severe inflammation in the gastric mucosa. We have recently made the same observation in pediatric patients, in which *HP* sole infection was not associated with severe inflammation, and the presence of EBV appeared necessary for that effect (Cardenas-Mondragon *et al*, 2013). It has been suggested that EBV reactivation from infected B cells facilitates infection of epithelial cells (Sixbey and Yao, 1992; Imai *et al*, 1998; Faulkner *et al*, 1999; Shannon-Lowe and Rowe, 2011), and the titre of anti-EBV reactivation antibodies has been shown to correlate with an increased risk of progression to NPC (Fachiroh *et al*, 2006; Ji *et al*, 2007; Li *et al*, 2010a).

How EBV and *HP* interact at the tissue or cellular level is not understood. Two possible mechanisms are envisioned: one is simply through additive inflammatory responses causing increased damage to the tissue. In that scenario, interleukin-1β (IL-1β), tumour necrosis factor-α (TNFα) and interleukin-8 (IL-8) are significantly elevated and associated to increased infiltration of immune cells in GC (Noach *et al*, 1994; Yamaoka *et al*, 1996; El-Omar *et al*, 2003) and in NPC (Huang *et al*, 1999; Chang *et al*, 2011; Li *et al*, 2012). A second mechanism is through more intimate interactions between EBV and *HP* genes. For instance, viral reactivation from infected B cells *in vitro* is induced by activation of the PLCγ signalling pathway and CagA is a strong activator of PLCγ (Churin *et al*, 2003).

Taken together, all these data suggest that EBV may contribute to GC carcinogenesis by both direct and indirect mechanisms: direct in $\sim$10% of cases in which the virus transforms the epithelial cells through expression of viral/cellular oncogenes; and indirect in perhaps a larger percent of GCs, in which viral reactivation favours severe chronic inflammatory responses, leading to increased tissue damage. In the latter scenario, there would not be clonal expansions of EBV-infected cells as the virus may reside in a cell different than the tumour cell. Considering that *HP* also encodes the CagA oncoprotein, EBV and *HP* may transform the gastric epithelium through a combination of direct and indirect mechanisms, similar to hepatitis B and C virus (HBV and HCV)-induced hepatocellular carcinoma (Nikolaou *et al*, 2013). The importance of this observation is that individuals with a high risk of progression to GC, because of augmented viral reactivation and advanced gastric lesions, may benefit from pharmacological treatment targeting EBV lytic-cycle proteins.

Study limitations. This is a case–control study of patients with gastric disease in which premalignant and malignant lesions were compared with NAG, the earliest inflammatory lesion in the progression to intestinal and diffuse GCs. Molecular studies addressing how EBV and *HP* cooperate to increase inflammatory responses together with longitudinal studies in a cohort of patients addressing GC progression are necessary to obtain more conclusive evidence supporting a causative indirect transforming role for EBV in gastric carcinogenesis. In addition, we initially anticipated using *HP* and EBV double-negative patients as the control group, but the low number of these patients ($n=3$) precluded its use; hence, the *HP* + /EBV + group was compared with patients with single *HP* or EBV infection. However, this may be a more rigorous comparison to address the joint role of both pathogens in gastric disease. In our stratified statistical analysis we often ended with cells with low numbers ($n\leq 10$), including no EBV-negative patients with intestinal-type GC. Still, our data show significant associations of EBV + /*HP* + with intestinal GC and its inflammatory precursor lesions, and no associations with diffuse GC, in spite that some of these patients show severe inflammation. Finally, in this study statistical data were adjusted only for age and sex confounders, and other documented confounders, such as smoking, salt ingestion and so on, should be addressed in future studies.

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AUTHOR CONTRIBUTIONS

MGC-M and RC-T performed the experiments, JT and EMF-P designed the study, JT, MC-P and EK recruited all patients included in the study, LF-L, MGC-M and AG-D performed the statistical analysis, MGC-M, JT, LF-L and EMF-P wrote the manuscript.

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