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• *GASTRIC CANCER* •

Epstein-Barr virus-associated gastric carcinoma: Evidence of age-dependence among a Mexican population

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Abstract

AIM: To investigate features of Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) among a Mexican population.

METHODS: Cases of primary gastric adenocarcinoma were retrieved from the files of the Departments of Pathology at the Instituto Nacional de Cancerología and the Instituto Nacional de la Nutrición in Mexico City. The anatomic site of the gastric neoplasia was identified, and carcinomas were histologically classified as intestinal and diffuse types and subclassified as proposed by the Japanese Research Society for Gastric Cancer. EBV-encoded small non-polyadenylated RNA-1 (EBER-1) *in situ* hybridization was conducted to determine the presence of EBV in neoplastic cells.

RESULTS: We studied 330 consecutive, non-selected, primary gastric carcinomas. Among these, there were 173 male and 157 female patients (male/female ratio 1.1/1). EBER-1 was detected in 24 (7.3%) cases (male/female ratio: 1.2/1). The mean age for the entire group was 58.1 years (range: 20-88 years), whereas the mean age for patients harboring EBER-1-positive gastric carcinomas was 65.3 years (range: 50-84 years). Age and histological type showed statistically significant differences, when EBER-1-positive and -negative gastric carcinomas were compared. EBER-1 was detected in hyperplastic- and dysplastic-gastric mucosa surrounding two EBER-1-negative carcinomas, respectively.

CONCLUSION: Among Latin-American countries, Mexico has the lowest frequency of EBVaGC. Indeed, the Mexican population >50 years of age was selectively affected. Ethnic variations are responsible for the epidemiologic behavior of EBVaGC among the worldwide population.

Key words: Epstein-Barr virus; Stomach; Lymphoepithelioma-like carcinoma; Gastric carcinoma; EBV-A; EBER-1; LMP-1

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INTRODUCTION

Gastric cancer (GC) is the second leading cause among cancer deaths in the world[1] and is one of the most frequent malignant neoplasms in Mexico[2]. Although the etiology of gastric carcinoma is now accepted as multifactorial, infectious agents play a central role in the mechanism of neoplastic transformation. The bacterium *Helicobacter pylori* (*H pylori*) has been implicated in a high percentage of gastric adenocarcinomas[3], in intestinal- as well as diffuse-type adenocarcinomas, according to the Lauren histoepidemiologic classification[4]. Another infectious agent, Epstein-Barr virus (EBV) or gamma type 4 herpes virus, has also been proved to be associated with gastric carcinoma in approximately 10% of cases[5]. This association has been reported in intestinal- and diffuse-type adenocarcinomas, as well as in nearly 100% of cases labeled lymphoid stroma-rich, lymphoepithelioma-like (LEL) carcinomas. The etiological role of EBV in GC development has been suspected on the basis of the uniform expression of Epstein-Barr nuclear antigen (EBNA)-1 protein, and EBV-encoded small non-polyadenylated RNA (EBER)-1 in all GC cells, the episomal monoclonality of the EBV genome, the elevated serum antibodies against EBV-related antigens among EBV-GC patients, and the unique 'lace pattern' morphology in some

early-stage EBV-GCs.

EBV-associated gastric carcinoma (EBVaGC) accounts for 1.7-16% of gastric carcinomas throughout the world, excluding LEL carcinomas[6]. The lowest frequency has been recorded in the UK, whereas the highest was in the USA. The definitive explanation for this figure remains unclear, but is probably related with genetic variations among different populations, as well as cultural and environmental influences among different geographic regions. Among Latin Americans, Mexican individuals are less likely to develop GC in association with EBV infection; in a previous study, we reported a prevalence of 8.15%[7]. In these series, diffuse-type EBV-GCs were seen exclusively, and EBER-1 was demonstrated in 100% of LEL carcinomas. In the present study, we expanded the number of cases under scrutiny and provided evidence that the risk for EBVaGC was significantly increased among patients >50 years of age in Mexico.

MATERIALS AND METHODS

Patient population

We retrieved cases of gastric adenocarcinoma from the files of the Departments of Pathology at the Instituto Nacional de Cancerología (1983-2000) and the Instituto Nacional de la Nutrición (1980-1995) in Mexico City. The results of a partial analysis of 135 cases were published previously elsewhere[7]. Eligible cases were included whenever they possessed complete demographic and pathologic information, as well as paraffin blocks with appropriate and well-preserved neoplastic tissue for molecular analysis. The age and gender of patients, and anatomic site, histological type, and depth of invasion of gastric carcinomas were obtained from records at the corresponding Department of Pathology.

Pathologic features

The anatomic site of gastric neoplasia was identified as upper (proximal) third, middle third, or lower (distal) third[8]. On the basis of predominant histological pattern, carcinomas were classified as intestinal- or diffuse-type according to the Lauren criteria[4] and subclassified as proposed by the Japanese Research Society for Gastric Cancer as follows[9]: intestinal types tub1 (well-differentiated adenocarcinoma with distinct glandular pattern and columnar epithelium throughout, moderate or small amount of stroma); tub2 (moderately differentiated adenocarcinoma with small or incomplete tubular structures with cubical or flat epithelium, amount of stroma variable from case to case), and muc (mucinous carcinoma); diffuse types, including por1 (poorly differentiated adenocarcinoma with solid, sheet-like proliferation with an alveolar pattern and indistinct tubular differentiation), por2 (poorly differentiated adenocarcinoma with acinar and trabecular pattern, usually showing diffuse infiltration with abundant fibrous stroma), and sig (signet-ring cell carcinoma). A special category, LEL carcinoma, similar to por1 adenocarcinoma but with dense lymphoid infiltrate exceeding total mass of carcinoma cells, was included. The depth of invasion was specified as mucosa; submucosa; or muscularis propria, subserosa, or serosa.

In situ hybridization

Molecular analysis was conducted as previously described[10]. Briefly, we retrieved one representative formalin-fixed, paraffin-embedded tissue sample from each carcinoma containing the neighboring non-neoplastic gastric mucosa. Two slides with 5 μm sections were prepared from each paraffin block. A set of slides were conventionally stained with hematoxylin and eosin, whereas the remainder were enhanced for EBER-1 *in situ* hybridization. The remaining paraffin-block sections were deparaffinized, rehydrated, predigested with pronase, prehybridized, and hybridized overnight at 37 ℃ with a concentration of 0.5 ng of digoxigenin-labeled probe. After sections were washed with 0.5×saline sodium citrate, hybridization was detected by anti-digoxigenin, antibody-alkaline phosphatase conjugate. Sections from a patient with known EBV-positive gastric carcinoma were used for a positive control, and sense probe to EBER-1 was used for a negative control for each procedure.

EBV genotyping

Preparation of DNA Each formalin-fixed and paraffin-embedded specimen was cut into 10-μm-thick slices, and a DNA sample was prepared following the method reported previously[11]. Each deparaffinized sample was treated with proteinase K (200 μg/mL) at 37 ℃ overnight followed by phenol/chloroform extraction and ethanol precipitation. Finally, the extracted DNA sample was dissolved in 50 μL of TE buffer.

Genotype-specific primer sets and probes Four different regions, the EBNA-3C, *Bam*HI-F, *Bam*HI-I, and *Xho*I sites in LMP-1, were used to determine viral genotypes. Types A and B can be determined by using the EBNA-2, -3A, -3B, or -3C gene[12-14]. In the present study, we chose EBNA-3C for genotyping because we experienced a higher detection rate of the primer set than those of the EBNA-2 region found in previous studies[15,16]. Types A and B, identified by PCR amplification of EBNA-3C region, corresponded to a 153- and a 246-bp band, respectively, and were confirmed by Southern blot hybridization with type-specific internal probes [14]. Wild-type F and f variant were identified by the presence of a 186-bp fragment in amplification of the *Bam*HI F region; after *Bam*HI cleavage, a 186-bp fragment could be identified in the case of wild-type F, and a 127-bp fragment could be identified in the case of the f variant. Wild-type F and f variants were confirmed by Southern blot hybridization with the internal probe as described previously[15].

For the *Bam*HI-I region, a 205-bp fragment was amplified by using primer sets as described previously[17], and types C and D were distinguished after cleavage by *Bam*HI-restriction enzyme. Type C had a 205-bp fragment, and type D had cleaved fragments with 130 and 75 bp. Types C and D were also confirmed by Southern blot hybridization with a cloned *Bam*HI-I DNA fragment probe.

To detect the *Xho*I polymorphism in exon 1 of the LMP-1 gene, we amplified a 497-bp DNA fragment with a primer set as previously described[18]. When two fragments, 340- and 157-bp long, were observed after *Xho*I digestion of the PCR product, the case was considered to contain the *Xho*I cleavage site. The 497-bp fragment of the PCR product of the B95-8 cell line was used as a probe to confirm the *Xho*I

cleavage site of LMP-1 by Southern blot hybridization[19].

PCR and Southern blot hybridization The PCR template contained the appropriate primer pair (1 μmol/L each), deoxyribonucleotide triphosphates (200 μmol/L each), and *Taq* polymerase (Takara Shuzo, Kyoto, Japan) in a total of 100 μL of PCR buffer. PCR products or PCR products digested with *Bam*HI and *Xho*I were confirmed by electrophoresis in 2% agarose gel and by staining with 0.5 μg/mL of ethidium bromide. Then, electrophoretic pattern was photographed under ultraviolet light. Electrophoretic DNA was transferred onto a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, UK) by capillary blotting using 0.4 N NaOH solution. Membranes were prehybridized with hybridization buffer for 0.5-1 h at 42 ℃. After the probe was added, hybridization was carried out overnight at 42 ℃. Probes of types A and B, and *Bam*HI-F were labeled with Dig oligonucleotide 3'-end labeling kit and detected using a Dig luminescent detection kit (Boehringer Mannheim, Germany). For detecting the *Bam*HI-I fragment and *Xho*I polymorphism in LMP-1, hybridization was carried out using the ECL direct labeling and detection kit (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions.

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (95%CIs) were obtained from logistic regression analysis, making comparisons between EBER-1-positive and EBER-1-negative gastric carcinomas with regard to age, gender, decade, anatomic site, histologic type, and depth of invasion.

RESULTS

Patient characteristics

We studied 330 consecutive, non-selected cases of gastrectomies due to primary gastric carcinoma. Among the 330 cases, there were 173 male and 157 female patients. The mean age was 58.1 years (range: 20-88 years) for all the patients, 59.9 years (range: 22-88 years) for male patients, and 56.1 years (range 20-88 years) for female patients. EBER-1 was detected in 24 (7.3%) of the 330 cases, 13 in men (7.5%) and 11 in women (7.0%). The mean age for patients harboring EBER-1-positive gastric carcinomas was 65.3 years: male patients 66.2 years (range: 51-74 years) and female patients 64.4 years (range: 50-84 years). The male/female ratio was 1.1/1 for the entire group and 1.2/1 for those with EBER-1-positive carcinomas.

Pathologic findings

With regard to the anatomic site of the primary neoplasia, 44 (13.3%) carcinomas were localized in the upper-third, 128 (38.8%) were in the middle portion, and 156 (47.3%) were in the lower-third of the stomach. In two cases (one male and one female), the anatomic location could not be determined; the entire stomach showed neoplastic infiltration in the male patient, and information on the original location of primary neoplasia was not available in the female patient. Both cases were EBER-1-negative. The distribution of carcinomas according to anatomic site and histological type, and the anatomic site and histological type of EBER-1-positive carcinomas are shown in Tables 1 and 2, respectively. Fourteen cases corresponded to early carcinomas, and only 4 were confined to mucosa; 10 cases invaded the submucosal layer. The remaining 316 cases were advanced carcinomas affecting muscular, subserosal, and serosal layers, as well as adjacent organs. EBER-1 was positive in all LEL carcinomas, in 4 out of 141 intestinal-type adenocarcinomas and in 11 out of 180 diffuse-type adenocarcinomas. The EBER-1 *in situ* hybridization signal was uniformly distributed in the nuclei of all 24 positive cases (Figures 1-6). A characteristic

Table 1 Distribution of EBER-1-positive gastric carcinomas by anatomic site[1] and gender

	Total (EBER-1+/total) %		Males (EBER-1+/total) %		Females (EBER-1+/total) %	
Total	24/330	7.3	13/173	7.5	11/157	7.0
Upper	3/44	6.8	3/31	9.7	0/13	0
Middle	13/128	10.2	7/67	10.4	6/61	9.8
Lower	8/156	5.1	3/74	4.1	5/82	6.1

[1]In two cases, anatomic location could not be determined. All (one male and one female) were EBER-1-negative.

Table 2 Distribution of EBER-1-positive gastric carcinomas by histologic type and gender

	Total (EBER-1+/total) %		Males (EBER-1+/total) %		Females (EBER-1+/total) %	
I-type	4/141	2.8	3/87	3.5	1/54	1.9
Tub1	0/42	0	0/28	0	0/14	0
Tub2	4/80	5.0	3/47	6.4	1/33	3.0
Muc	0/19	0	0/12	0	0/7	0
D-type	20/189	10.6	10/86	11.6	10/103	9.7
Por1	8/64	12.5	3/31	9.7	5/33	15.2
Por2	2/45	4.4	2/19	10.5	0/26	0
Sig	1/71	1.4	1/32	3.1	0/39	0
LEL	9/9	100	4/4	100	5/5	100

I-type: Intestinal-type adenocarcinoma; *D-type*: Diffuse-type adenocarcinoma.

lace pattern was evident in the intramucosal component of three EBER-1-positive carcinomas, two por1 plus tub2 and one tub2 plus por1 adenocarcinomas. Twenty-two of twenty-four EBER-1-positive cases extended beyond the submucosa, whereas two carcinomas, one from a female and one from a male patient, did not exceed the submucosal layer.

There were two EBER-1-negative carcinomas accompanied by EBER-1-positive gastric lesions. The first case, a 52-year-old male patient (Figures 7 and 8), had EBER-1 expression in regenerative epithelium of gastric mucosa adjacent to an EBER-1-negative primary adenocarcinoma (por1). The second case was a 46-year-old female patient whose EBER-1-negative adenocarcinoma (por1) was in the immediate vicinity of dysplastic gastric glands with EBER-1 expression (Figures 9 and 10).

Among the demographic and pathologic variables analyzed, age and histologic type had statistically significant differences, when EBER-1-positive and EBER-1 negative gastric carcinomas were compared (Table 3). In addition, comparison among patients more or less than 60 years of age showed significant differences ($P = 0.008$).

EBV genotype

We examined the genotype of seven EBV strains detected from

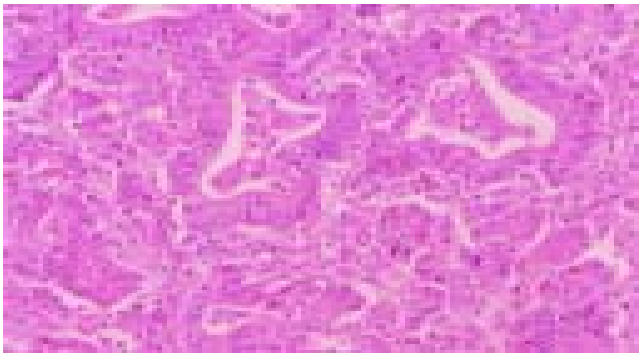

Figure 1 Moderately differentiated, intestinal-type (tub2) adenocarcinoma. Irregular neoplastic tubular structures are seen throughout the field (hematoxylin and eosin stain).

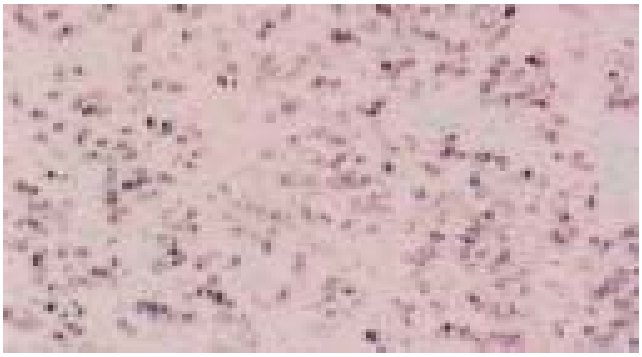

Figure 4 Same case as in Figure 3. A uniform nuclear signal of EBER-1 is seen in neoplastic cells (*in situ* hybridization).

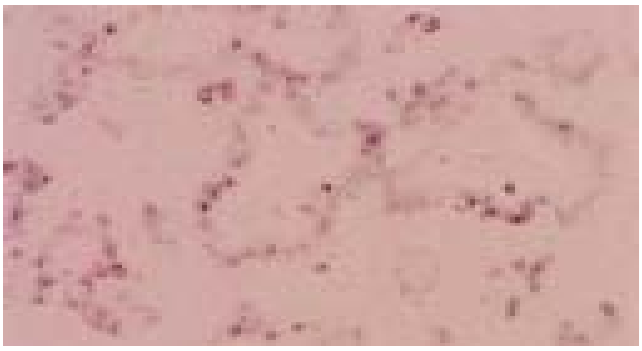

Figure 2 Same case as in Figure 1. EBER-1 nuclear positivity is limited to neoplastic cells lining the tubular structures (*in situ* hybridization).

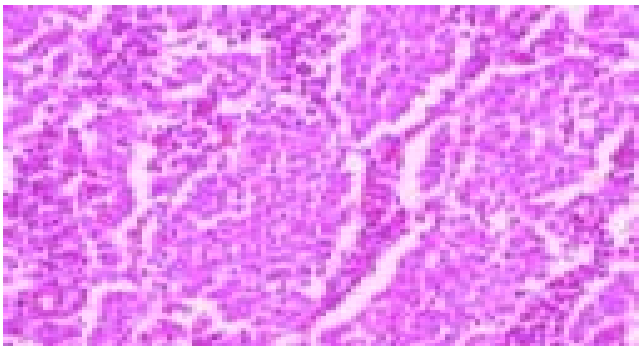

Figure 5 Poorly differentiated, LEL carcinoma. Clusters of neoplastic cells are separated by lymphoplasmacytic infiltrate.

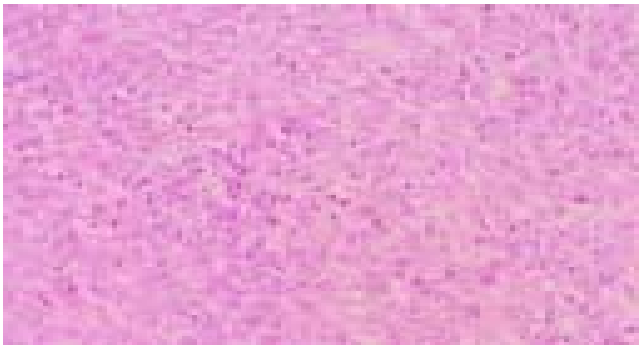

Figure 3 Diffuse-type (por1) adenocarcinoma. Sheets of neoplastic cells are distributed in an indistinct pattern (hematoxylin and eosin stain).

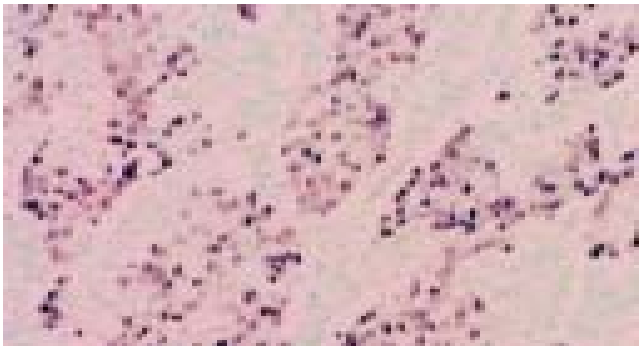

Figure 6 Same case as in Figure 5. An EBER-1-positive signal is detected in the nuclei of neoplastic cells (*in situ* hybridization).

EBER-1-positive cases; genotype could be determined in five of them. All were type A, wild-type F, and type D. In analysis of the *Xba*I cleavage site in LMP-1, we found that the cleavage site was lost in four cases and was maintained in one case.

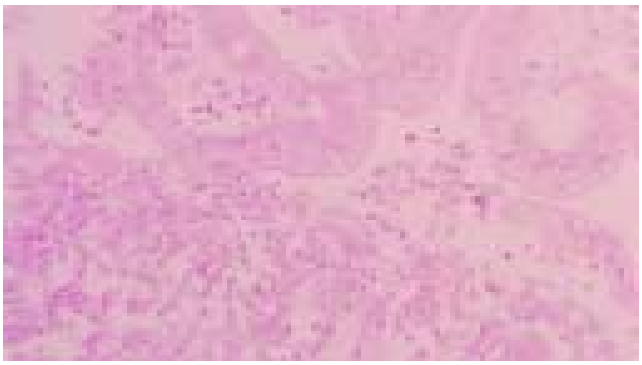

Figure 7 Lining gastric epithelium shows regenerative changes characterized by nuclear growth without atypia. There are few neoplastic cells in the underlying lamina propria (hematoxylin and eosin stain).

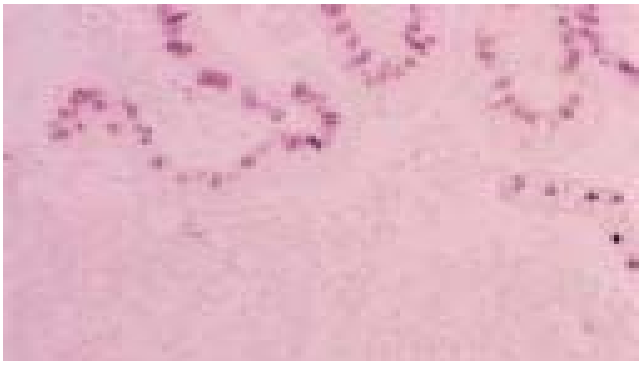

Figure 8 Same case as in Figure 7. The EBER-1-positive nuclear signal is restricted to regenerative epithelium. Note the EBER-1 nuclear negativity of neoplastic cells infiltrating the lamina propria (*in situ* hybridization).

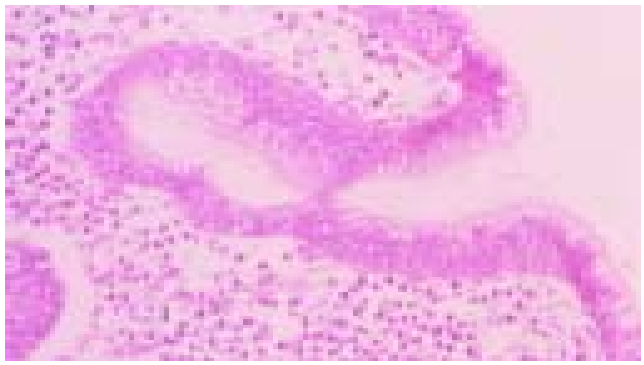

Figure 9 Lining gastric epithelium shows high-grade dysplasia, characterized by cell stratification and crowding, increased nuclear/cytoplasm ratio, nuclear atypia, and prominent eosinophilic nucleoli (hematoxylin and eosin stain).

Figure 10 Same case as in Figure 9. Dysplastic epithelium is intensely positive for the EBER-1 nuclear signal (*in situ* hybridization).

Table 3 Comparison of demographic and pathologic variables between EBER-1-positive and EBER-1-negative gastric carcinomas[1]

	EBER-1+/total	OR	95%CI	*P*
Gender				0.859
Female	11/157	1	Reference	
Male	13/173	0.9	0.4-2.2	
Age (yr)				0.013
20-49	0/87	<0.1		
50-69	14/170	0.6	0.2-1.3	
70-88	10/73	1	Reference	
Decade				0.787
1980-1989	11/130	1	Reference	
1990-2000	13/200	0.9	0.4-2.1	
Tumor site				0.229
Cardia	3/35	1.9	0.5-7.5	
Middle	13/137	2.2	0.9-5.5	
Antrum	8/156	1	Reference	
Lauren classification				0.005
Intestinal	4/141	1	Reference	
Diffuse	20/189	4.9	1.6-14.8	
Depth				0.273
Early	2/14	2.4	0.5-11.8	
Advanced	22/316	1	Reference	

[1]Odds ratios and 95% confidence intervals were obtained from logistic analysis. Age was adjusted in the analysis of variables other than age.

DISCUSSION

In this study, we found a 7.3% prevalence of EBVaGC in Mexico. In Latin America, this frequency is in contrast with that reported by Koriyama *et al*., (11.2%)[20] and Lopes *et al*., (11.3%)[21] in Brazil, Carrascal *et al*., in Colombia (13%)[22], and Corvalan *et al*., in Chile (16.8%)[23]. Excluding LEL carcinomas, the prevalence of EBVaGC in Mexico was 4.7%, whereas in Chile it was 15.8%. In a Brazilian study by Koriyama *et al*.[20], and a Colombian study by Carrascal *et al*.[22], there were no LEL carcinomas. Nonetheless, in the study by Lopes *et al*.[21], a high prevalence of LEL carcinomas (66.7%) among EBVaGC patients was found in a Brazilian population; thus, the prevalence of EBVaGC excluding LEL carcinomas is the lowest (3.8%). Conversely, the prevalence of LEL carcinoma was 7.6% in Brazil, 2.7% in Mexico, and 1.1% in Chile. The male/female ratio (1.2/1) was, as previously noted[7], the lowest among the series reported worldwide. Moreover, after excluding LEL carcinomas, Mexico remains among countries with the low prevalence of EBVaGC worldwide[6].

The frequency of EBVaGC among GC patients of Mexican ancestry in the USA ranged from 10.2%[24] to 12%[25], which is higher than the frequency (7.3%) reported by us. This peculiar migratory phenomenon has also been seen in other countries such as Japan and China. In Japan, the mean frequency of EBVaGC is 6.2%, but among patients of Japanese descent, those who are living in Hawaii, the frequency is 10.2%. In Taiwan, the frequency of EBVaGC among patients of Chinese descent is 11.2%, in comparison to 6.8% in China[26]. This figure probably indicates that besides ethnic and genetic backgrounds, environmental factors are involved in the development of EBVaGC.

A high frequency of EBVaGC at older ages is evident in our Mexican study. Not a single case of EBVaGC was observed among patients aged <50 years. This feature was previously highlighted by Gulley *et al*.[25], who examined American patients of Mexican descent in the USA and found EBVaGC cases only among those aged 56 years or older. Age dependence of EBVaGC frequency was statistically significant in their study ($P = 0.04$). The absence of EBVaGC in a set of patients of Mexican ancestry aged <56 years was also reported by Vo *et al*.[24], although the age difference they reported was not statistically significant. A similar age dependence was reported in China[26], where EBVaGC frequency was higher among those aged 60 years or older than those aged <60 years ($P = 0.03$); interestingly, the frequency of EBVaGC (7.8%) in their study is quite similar to that reported by us (7.3%).

In Brazil, Lopes *et al*.[21], also did not find any patient less than 52 years of age, although other Latin-American studies such as those of Koriyama *et al*.[20], and Corvalan *et al*.[23], did not show any age dependence, reporting EBVaGC cases in patients <50 years. Contrary to the age dependence observed in the present study, a large-scale Japanese study reported a high prevalence of EBVaGC in young men[27]. Furthermore, the same authors showed a significant decreasing trend in EBV prevalence with increasing age for males ($P = 0.04$). Carrascal *et al*.[22], also reported an age-dependent decrease of EBVaGC frequency among Colombian individuals with GC (P for trend = 0.022).

The fact that EBV-associated cancer cannot be detected in other digestive tract organs including the colon and esophagus indicates the importance of epithelial change(s) specific to the stomach[28]. EBV-latent infection products were reported to be expressed in predisposing conditions for gastric carcinoma[29,30]. Our observation showing that EBVaGC could not be found among patients <50 years of age supports the involvement of gastric-mucosal changes occurring late in human life in Mexico, as well as in Brazil and China, and relatively early in Japan and Colombia.

EBVaGC has been related to atrophic gastritis, and EBV DNA has been isolated from epithelial cells in gastric mucosa carrying chronic atrophic gastritis[29-31]. Indeed, intestinal metaplasia may enhance EBV entrance into epithelial cells via adherence of the virus to the secretory component of polymeric immunoglobulin A[32]. Our finding of two cases of EBV non-associated gastric carcinoma, one positive for EBER-1 in adjacent hyperplastic mucosa -a finding not previously described -and the other with an EBER-1-positive signal in dysplastic mucosa -a finding originally reported by Shibata and Weiss[33] -also suggests that the most plausible mechanisms for EBV entry into gastric epithelial cells are those related to previous mucosal damage and cooperation with some unknown promoter factors. In the present study, we did not observe any EBER-1 expression in normal gastric mucosa, even surrounding LEL-EBVaGC or infiltrating lymphocytes. Furthermore, we analyzed endoscopic gastric biopsies from 116 Mexican individuals >40 years of age carrying gastritis with mild atypia, and we did not find any EBER-1-positive case (unpublished data).

In addition to the age dependence of EBVaGC, the present study shows other characteristics of EBVaGC such as distal presentation among female patients and no male preponderance, altogether supporting that ethnicity and genetic backgrounds may address this particular outcome of EBV infection in the Mexican population. Among genetic backgrounds, an immunogenetic constitution may influence the outcome of EBV infection. Human leukocyte antigens (HLA) of the major histocompatibility complex have been implicated in susceptibility to develop EBV-related malignancies[34]. Very recently, we reported an association between the *HLA-DQB1**0501 allele and GC, predominantly in those labeled as diffuse-type carcinomas[35]; unfortunately, EBV status could not be assessed.

In Mexico, EBV antibody prevalence at 4-6 years of age is about 75%[36]. All EBV strains detected in EBVaGC and subjected to EBV genotyping were type A. Previous molecular studies on nasal T-lymphocyte/natural killer-cell lymphomas (nT/NKL) in Mexico[37] documented that EBV type A (EBV-1) is more frequent than EBV type B (EBV-2), as in nT/NKL and sino-nasal-B-cell lymphomas, and as in reactive tonsils from healthy individuals, thus suggesting that viral infection with EBV-1 strain is highly predominant among the Mexican population. In addition, the same authors[37] found a similar incidence of EBV LMP-1 deletions in Mexican individuals harboring nT/NKL as compared with normal subjects. Mori *et al*.[38], found no significant differences in DNA sequences of the LMP-1 region of EBV strains isolated from EBVaGC patients and throat washing samples of healthy individuals. So far no studies

have revealed differences in the genotype of EBV detected in EBVaGC *vs* that found in healthy individuals.

In conclusion, EBVaGC occurs less in Mexico than among other Latin-American populations, but it is as frequent in male as it is in female patients >50 years. In Mexican women, EBVaGC affects the middle and distal portions of the stomach but not the proximal portion. Finally, the participation of sequential steps in the mechanism of neoplastic transformation in EBVaGC, in a similar manner to the cascade of events described by Correa[39] in gastric carcinogenesis, cannot be ruled out.

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