Table II - Age and gender distribution by tumor location or histology in gastric adenocarcinoma

	Males				Females			
	N	Age Mean	SD*1	Range	N	Age Mean	SD*1	Range
Tumor location								
Cardia	2	61.0	–	50-72	1	37	–	37
Middle	8	51.9	19.4	23-74	7	53.6	12.5	36-68
Antrum	3	44	8.5	35-52	1	66	–	66
Unknown	24	51.3	13.9	23-80	6	38.5	11.1	30-60
Histology								
Intestinal type*2								
tub1*3	3	62.3	2.5	60-63	0	–	–	–
tub2*3	11	59.2	14.4	30-74	5	51.0	18.5	30-68
muc*3	1	60	–	60	0	–	–	–
Diffuse type*2								
por1*3	3	54.0	12.2	40-62	2	57.5	–	55-60
por2*3	15	45.3	14.2	23-80	7	43.1	12.0	34-66
sig*3	4	40.0	12.1	23-50	1	37	–	37
Total	37	51.4	14.7	23-80	15	47.3	14.0	30-68

**1 The standard deviation of age was not calculated when there were less than 3 cases with information on age.*
**2 Lauren's classification.*
**3 Japanese classification.*

tion of EBV-GC has a wide confidence interval (95%CI=0.05-10.2%) due to the small sample size.

On the basis of the relatively high frequency of EBV-GC in the American Continents and low frequency in Asian Continents, it has been speculated that EBV-GC frequency is high in countries where gastric cancer risk is low and vice versa. Although no national data on Pakistan are available, statistics from the Karachi Cancer Registry (33) suggests that the incidence (per 100,000) of gastric cancer is 3.9 and 3.0 for men and women, respectively. The present study has shown that Pakistan has a low EBV-GC frequency while its gastric cancer incidence is relatively low and does not support the hypothesis mentioned above.

The Ebv-GC is known to have a uniform expression of EBNA-1 and EBERs in all carcinoma cells in addition to the episomal monoclonality of the EBV genome (34), elevated serum anibodies against EBV-related antigens (35,36), and the unique "lace pattern" morphology in some early-stage-EBV-GCs (37). Those features strongly suggest important etiological roles of EBV in the development of EBV-GCs. Recently, however, zur Hausen et al. reported that EBV can only infect neoplastic gastric cells, and thus the infection is a late event in gastric carcinogenesis (5).

According to the Karachi cancer registry data (33), cancer of the oral cavity is the second most frequent cancer among both males and females if the cases with cancers of the tongue and mouth are added together. The incidence of esophageal cancer is also relatively high among males (6.5 per 100,000 person year) and females (6.9 per 100,000 person year). Interestingly, there is little difference in the incidence of oral and esophageal cancers between males and females, and this feature is different from those observed in other countries; in many countries, males have a higher inci-

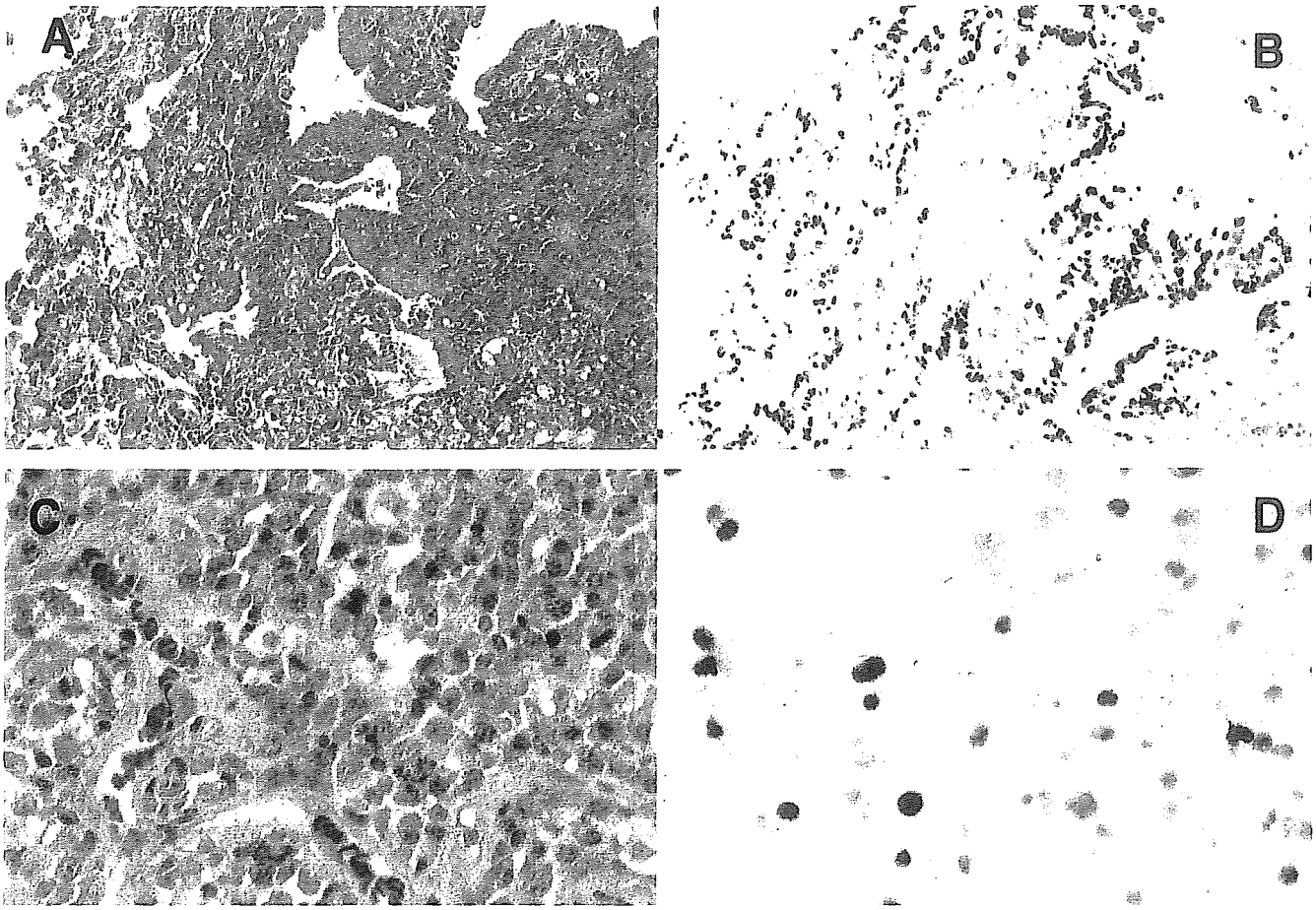

Fig. 1 - The EBER-positive and –negative gastric adenocarcinoma cases - **(A)** Hematoxylin and eosin staining for the EBER-positive case - **(B)** EBER-1 positive in tumors by ISH - **(C)** Hematoxylin and eosin staining for the EBER-negative case - **(D)** EBER-1 negative in tumors but positive in lymphocytes by ISH.

dence of esophageal cancer than females. These observations suggest that factors common to both men and women are predominant risk factors of oral and/or esophageal cancers in Pakistan.

Regarding the OSCC cases, there was no case with EBER expression in the present study. Several studies have examined EBER expression by ISH among OSCC cases (9, 11-16), and the frequency of EBER-positive cases ranges from 0 to 67%. Interestingly, three studies reporting EBER-positive OSCC cases investigated Japanese patients (12,14,15). However, four studies including one Japanese study reported no EBER-positive OSCC (9,11,13,16). These results indicate geographical variations in the frequency of the EBER-positive OSCC as suggested by Tsuhako et al. (12), who reported various frequencies of EBER-positive OSCC among Japanese populations from different regions.

Recently, Shimakage et al. (14), examined EBER-expression in 24 Japanese OSCC cases, and 16 (67%) of them were EBER-positive by PCR and ISH analyses. In addition to that, they found the expression of LMP1 and EBNA2, potential oncogenic proteins, and BZLF1 expression, which is a trans-activator that initiates the switch from the latent cycle to the lytic cycle of EBV in epithelial cells of the EBER-positive tumors but not in all cases. Their results suggested a potential role of EBV in the carcinogenesis of OSCC. However, further investigations are needed to confirm these results.

Mori et al. (17) reported the first case of EBER- and LMP1-positive ESCC. However, subsequent studies, including the present one, failed to detect EBER-positive ESCC by ISH (9,18,19,21,22) except one study reported from Taiwan. Wang et al. (21) detected the EBV genome in 11 out of 31 ESCC cases

by PCR and ISH methods. However, they could not detect LMP1 expression in these cases. Interestingly, they also detected EBER expression in neighboring normal epithelial cells of ESCC in 7 of the 11 EBER-positive cases. Mori et al. (17) also suggested an association between EBV and esophageal undifferentiated carcinoma with lymphoepithelioma-like histology. However, Kijima et al. reported that none of the 107 ESCC, including 11 cases with lymphoepithelioma-like histology, were EBER-positive (21).

In conclusion, the frequency of EBV-associated gastric carcinoma was relatively low in Pakistan. The present study could not confirm the involvement of EBV in the carcinogenesis of oral and esophageal SCC.

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Epstein-Barr Virus-Associated Gastric Carcinoma in Lima, Peru

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We examined 254 gastric carcinomas (GCs) diagnosed in four hospitals in Lima, Peru, and its suburban area during the period between 1994-2001. Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) was identified by the *in situ* hybridization (ISH) technique to detect EBV-encoded small RNA (EBER) in gastric tissue. EBVaGCs, where EBER ISH staining was observed in all carcinoma cells, accounted for 3.9% (10 /254) of gastric adenocarcinomas, the lowest frequency ever reported in Latin American countries. EBVaGC incidence rates in Peru, which we estimated on the basis of the present study and cancer incidence in Lima, were 0.8 per 100,000 among men and 0.5 per 100,000 among women. These estimates are much lower than those reported in our previous studies in Colombia (4.1 and 1.4 per 100,000 among men and women, respectively), a neighboring country, and in Japan (6.4 and 1.1 per 100,000 among men among women, respectively). Interestingly, EBVaGC in Peru showed no evident male predominance, as opposed to the findings reported in a majority of studies. Other clinicopathological features of EBVaGC in Peru were similar to those found in literature: EBVaGC showed no age dependence, a predominance in the non-antrum part of the stomach, and high frequencies in histological subtypes of moderately differentiated tubular adenocarcinoma and solid poorly differentiated adenocarcinoma. There was a case of well-differentiated adenocarcinoma showing a partial EBER-1-positive staining. In this carcinoma, the tumor in the body (middle third of the stomach) was EBER-1 positive but the tumor in the stomach antrum showed no noticeable EBER-1 ISH staining. We suspect this was a case of synchronous double carcinomas. Further studies are needed to identify the cause of the low frequency and lack of male predominance of EBVaGC in Peru.

Key Words: Epstein-Barr Virus, Peru, Gastric carcinoma

More than 90% of adults in the world are infected with Epstein-Barr virus (EBV), which is implicated in the etiology of some types of lymphomas derived from B cells and some epithelial malignancies, including nasopharyngeal carcinoma (1). In 1992, Shibata and Weiss (2) reported the presence of the EBV genome in 16% of gastric adenocarcinomas in a small North American series, using the *in situ* hybridization (ISH) technique to detect the EBV-encoded small RNA (EBER) genome in gastric tissue. They reported that EBER ISH staining was observed in all carcinoma cells of an EBV-associated gastric carcinoma (EBVaGC) and was not detected in adjacent normal epithelial cells and surrounding lymphocytes. Subsequent studies revealed the proportion of EBVaGCs to be different from country to country, ranging from 2% to 16% (3). In our studies conducted in Chile and Colombia, two Latin American countries having relatively high gastric cancer incidence rates, the proportions of EBVaGCs were 17% and 13%, respectively, suggesting relatively high frequencies in the South American continent (4,5). The major clinicopathological features of EBVaGC are a predominance in the non-antrum part of the stomach, high frequencies in histological subtypes of moderately differentiated tubular adenocarcinoma (tub2) and solid poorly differentiated adenocarcinoma (por1), and no evident age dependence (6,7). Regarding sex distribution, many studies showed an evident male predominance of EBVaGC. In the present study,

we examined the frequency of EBVaGCs and their clinicopathological features in another South American country, Peru.

Materials and Methods

Subjects. Paraffin-embedded formalin-fixed tissues from 254 consecutive patients with gastric adenocarcinoma diagnosed during the period between 1996 and 2001 were obtained from the following hospitals: Policlinico Peruano Japones in Lima (1996-2001); Hospital Regional de Chiclayo in Chiclayo (1994-1999), Hospital Regional Hermilio Valdizan in Huanuco (1996-2001), and Hospital Edgardo Rebagliati Martins in Lima (2000). Lymphoma and other types of gastric malignancies were excluded from the present study. There were no remnant carcinomas, which are gastric carcinomas arising in the remnant part of the stomach after partial gastrectomy.

Histological classification and macroscopic findings. The gastric carcinomas were classified as the intestinal or diffuse type according to the Lauren classification (8), and subclassified according to the classification scheme of the Japanese Research Society for Gastric Cancer (9). Briefly, histological patterns were classified as follows: well-differentiated tubular adenocarcinoma (tub1), moderately differentiated tubular adenocarcinoma (tub2), solid poorly differentiated adenocarcinoma (por1), non-solid poorly differentiated adenocarcinoma (por2), signet ring cell carcinoma (sig), and mucinous carcinoma (muc).

Tumor location, defined as the predominant location of a tumor, was divided into the cardia of the stomach (the upper third of the stomach), the middle part of the stomach, and the antrum of the stomach (the lower third of the stomach) according to the guidelines of the Japanese Research Society for Gastric Cancer (10).

In situ hybridization. We examined the EBER-1 expression in the paraffin-embedded tissue obtained from the main tumor by ISH assay as described previously (11). A case was considered EBER-1 positive on the basis of positive signals in carcinoma cells under microscopy. Paraffin sections from a known EBER-1-positive gastric cancer case were used as the positive control, and a sense probe for EBER-1 was used as the negative control in every assay.

Statistical analysis. We conducted univariate logistic regression analyses to examine the associations of EBVaGCs with gender, age, tumor location, and histological type. The test for trend in the analysis of age used the continuous variable of age. All of the P values presented in this study were two-sided.

Results

We examined 254 gastric cancer cases. EBER-1 expression in carcinoma cells was detected in ten cases (3.9%). There were no lymphoepithelioma-like carcinomas (LELCs). Table I shows the EBVaGC frequency by gender, age, tumor location, and histology. The proportion of EBVaGCs among the entire set of gastric cancer cases showed no evident dependence on gender or age. The EBVaGC proportion did not change over the years (data not shown), either. EBVaGCs were more frequently found in the middle part of the stomach than in the other sites, but the difference was not statistically significant (P=0.459).

Table II lists all the EBVaGC cases identified in the present study. There was a case of well-differentiated adenocarcinoma (tub1) showing a partial EBER-1 positive staining (Case 5 in Table II). In this case, the tumor in the body (middle third of the stomach) was EBER-1 positive but the tumor in the stomach antrum (the lower third of the stomach) showed no noticeable EBER-1 ISH staining (Fig.1). This case was regarded as EBVaGC in Table II.

Discussion

The present study showed that only 3.9% of gastric carcinomas in Peru were EBER-1 positive. This is the lowest frequency reported among the Latin American countries, and is among the lowest in the world (3). A study conducted in Chile reported the highest frequency of EBVaGCs in Latin America; EBVaGCs accounted for 16% of gastric carcinomas, an about four-fold-higher frequency than the Peruvian figure. Using stomach cancer incidence rates in Lima, Peru (19.1 per 100,000 person-years among males and 13.7 100,000 person-years among females), reported in Cancer Incidence in Five Continents VII (12), and male- and female-specific frequencies of EBVaGCs observed in the present study (4.2% among men and 3.9% among women), we estimated that the incidence rates of EBVaGC among male and female Peruvians were 0.8 and 0.5 per 100,000, respectively. These estimates are much lower than those reported in our

Table I - Clinicopathological features of EBVaGCs in Peru

	Total	EBER+	%	P value
Total	**254**	**10**	**3.9**	
Gender				*P* for sex difference = 0.917
Female	128	5	3.9	
Male	120	5	4.2	
Age				*P* for age trend = 0.763
< 50	30	1	3.3	
50-59	45	2	4.4	
60-69	69	4	5.8	
70-79	82	2	2.4	
80≤	28	1	3.6	
Tumor location				*P* for site difference= 0.464
Antrum	111	3	2.7	
Body	73	6	8.2	
Cardia, Fundus	21	1	4.8	
Whole	7	0	0	
Histology				*P* for subtype difference = 0.491
tub1	51	1	2.0	
tub2	79	3	3.8	
por1	74	4	5.4	
por2	22	2	9.1	
muc	27	0	0	
sig	1	0	0	
Lauren classification				*P* for subtype difference= 0.459
Intestinal	131	4	3.1	
Diffuse	123	6	4.9	

previous studies in Colombia and Japan. In Colombia, EBVaGC incidence rates among men and women were estimated to be 4.1 and 1.4 per 100,000, respectively (5); in Japan, the corresponding estimates among men and women were 6.4 and 1.1 per 100,000, respectively (5). At this moment, we cannot offer any good explanation for the apparently low incidence of EBVaGC in Peru.

EBVaGC usually shows a male predominance: the sex ratio of EBVaGC patients is several-fold higher than EBV-negative GC patients (6,7). Even gastric LELCs, which are almost always EBV associated (13), show a male predominance: male LELCs are almost always EBVaGC but female LELCs are not (5,14). Interestingly, as opposed to the majority of studies, the present study did not show a male predominance of EBVaGC. However, the relatively low sex ratio is not limited to our Peruvian study but was also noted in studies conducted in Chile (4) and Mexico (14), where the sex ratios of EBVaGC were 1.5 (1.4 after excluding LELCs) and 1.2 (1.0 after excluding LELCs), respectively. A low sex ratio was observed in an international study conducted by Qiu et al. (15) in China and Japan, too. The mechanisms underlying varying degrees of male predominance in EBVaGC are yet to be elucidated. The age distribution of EBVaGC is not well understood, either. In most of the studies, as was the case in the present study, the proportion of EBVaGCs showed no evident age dependence (6). A study in Colombia showed, however, a strong age-dependent decrease of EBVaGC (5). On the other hand, an international study conducted in China and Japan also showed a relatively higher frequency of EBVaGC among those

Table II - Characteristics of the EBVaGC patients studied

Case	Hosp*	Gender	Age	Year	Location	Histology#
1	Pol	F	50	1998	Antrum	por1
2	Pol	F	51	2000	Middle (Body)	por2, tub2
3	Pol	M	68	2000	Antrum	tub2, por
4	Pol	M	61	2000	Antrum	por1
5	Pol	F	71	1996	Middle (Body)	tub1
6	Chi	M	42	1999	Middle (Body)	tub2, por
7	Huan	F	68	2000	Middle (Fundus)	por1
8	Reb	M	66	2000	Middle (Body)+Antrum	por1
9	Reb	F	76	2000	Middle (Body)	tub2, por
10	Reb	M	84	2000	Middle (Body)	por2

** Pol: Policlinico Peruano Japones in Lima, Chi: Hospital Regional de Chiclayo in Chiclayo, Huan: Hospital Regional Hermilio Valdizan in Huanuco, Reb: Hospital Edgardo Rebagliati Martins in Lima.*
 # The histological classification shown in the list uses the classification scheme of the Japanese Research Society for Gastric Cancer except for "por", which is poorly differentiated adenocarcinoma.

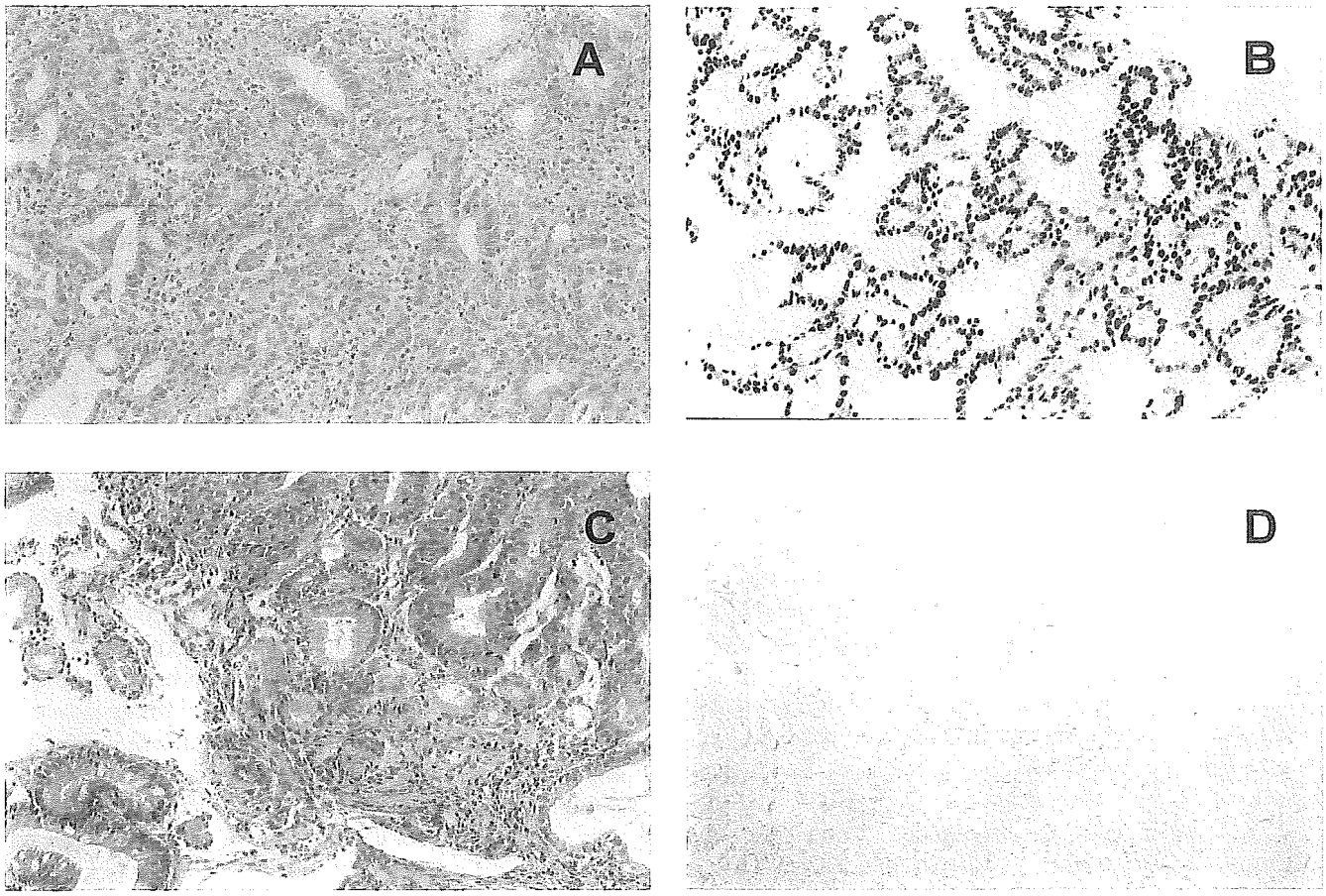

Fig. 1 - A case of suspected double carcinoma (Case 5 in Table II)
A. Hematoxylin and eosin staining (x10) **B.** EBER-1 in situ hybridization (x10, EBER-1 positive).
C. Hematoxylin and eosin staining (x10) **D.** EBER-1 in situ hybridization (x20, EBER-1 negative).

aged older than 60 than among those aged 60 or younger (15). Koriyama et al. (7), examining by far the largest number of EBVaGCs in Japan, showed the age distributions of intestinal- and diffuse-type EBVaGCs to be different from each other: the estimated incidence of intestinal-type EBVaGCs had its peak in patients in their seventies, while diffuse-type EBVaGCs appeared to have a much older peak incidence, if any. Their results suggest that the natural history of EBVaGC may differ in those two histologic subtypes. However, the discrepancies of age dependence among various countries do not appear to be explained by differences in the histologic distributions in those countries. In the present study, we could not test the tendency presented by Koriyama et al. due to the small number of EBVaGCs in our study. To understand the sex and age distribution of EBVaGC, further studies on the prevalence of EBV infection, age at infection with EBV, and EBV genotypes seem warranted. It may also be of interest to compare lifestyles and genetic backgrounds such as human leucocyte antigen (HLA) in EBVaGCs and EBV-negative GCs, since HLA distribution was reported to be different between EBVaGCs and EBV-negative GCs (16).

EBVaGC is known to show uniform EBER staining in all the carcinoma cells (2). That was also the case in the present study except for Case 5 (Table II), where EBER-1 staining was observed in the carcinoma in the middle part of the stomach but not in the carcinoma in the antrum. We suspect that this is the case of double synchronous carcinomas, one in the stomach antrum (EBER-1 negative) and the other in the body of the stomach (EBER-1 positive). To our knowledge, this is the first report of possible synchronous double adenocarcinomas that could not be identified unless EBER-1 ISH was conducted. Recently, however, zur Hausen et al. reported that EBV can only infect neoplastic gastric cells, and thus the infection is a late event in gastric carcinogenesis (17). We cannot deny the other possibility that the observed double adenocarcinomas have arised from the same tumor with only one area infected by EBV.

In summary, the present study showed that the proportion of EBVaGCs in Peru was 3.9%. The figure is the lowest among those reported in Latin American countries. An interesting feature of Peruvian EBVaGC was a lack of a male predominance. Other clinicopathological features of EBVaGC in Peru were similar to those found in literature: EBVaGC showed no age dependence, a predominance in the non-antrum part of the stomach, and high frequencies in histological subtypes of tub2 and por1. Further studies are needed to identify the cause of the low frequency and lack of male predominance of EBVaGC in Peru.

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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

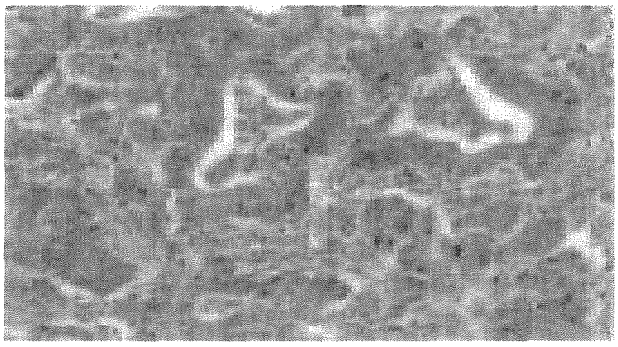

Figure 1 Moderately differentiated, intestinal-type (tub2) adenocarcinoma. Irregular neoplastic tubular structures are seen throughout the field (hematoxylin and eosin stain).

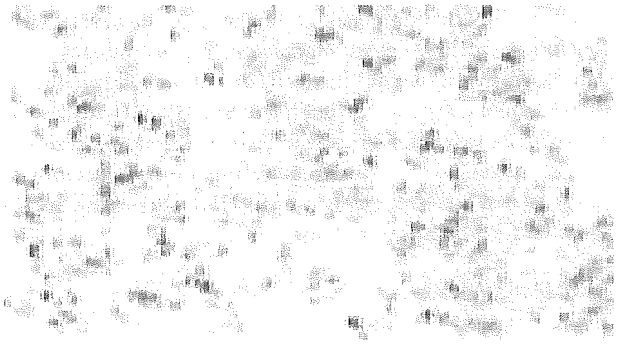

Figure 4 Same case as in Figure 3. A uniform nuclear signal of EBER-1 is seen in neoplastic cells (*in situ* hybridization).

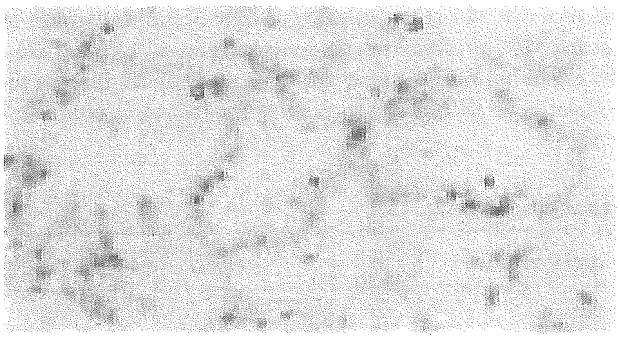

Figure 2 Same case as in Figure 1. EBER-1 nuclear positivity is limited to neoplastic cells lining the tubular structures (*in situ* hybridization).

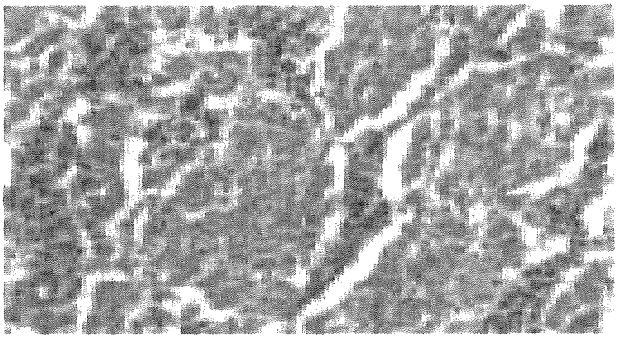

Figure 5 Poorly differentiated, LEL carcinoma. Clusters of neoplastic cells are separated by lymphoplasmacytic infiltrate.

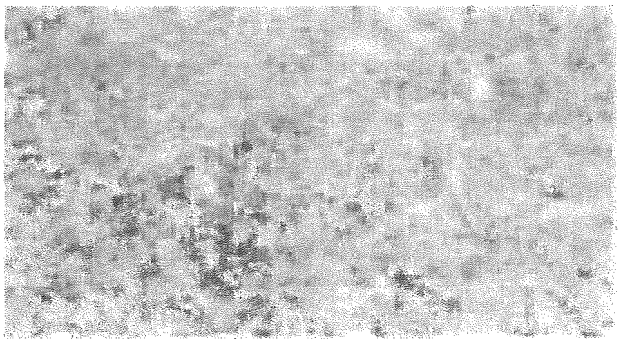

Figure 3 Diffuse-type (por1) adenocarcinoma. Sheets of neoplastic cells are distributed in an indistinct pattern (hematoxylin and eosin stain).

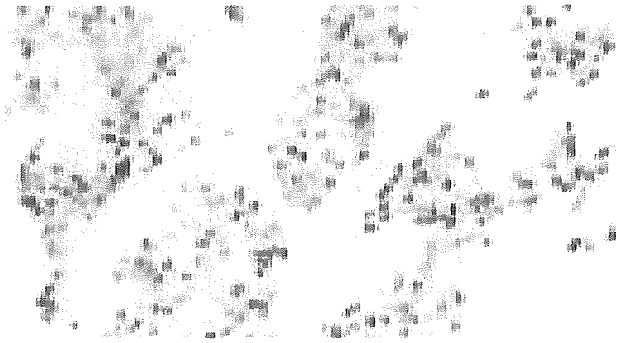

Figure 6 Same case as in Figure 5. An EBER-1-positive signal is detected in the nuclei of neoplastic cells (*in situ* hybridization).

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 *Xho*I cleavage site in LMP-1, we found that the cleavage site was lost in four cases and was maintained in one case.

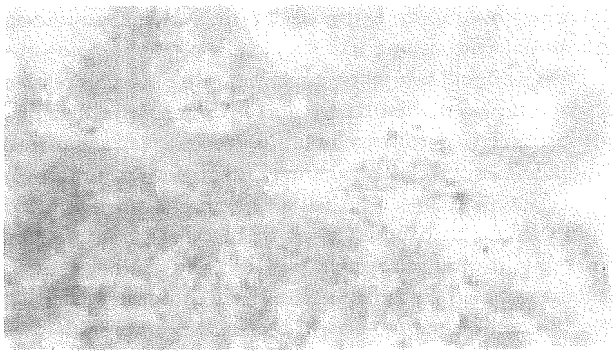

Figure 7 Lining gastric epithelium shows regenerative changes characterized by nuclear growth without atypia. There are few neoplastic cells in the underlying lamina propria (hematoxylin and eosin stain).

Figure 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).

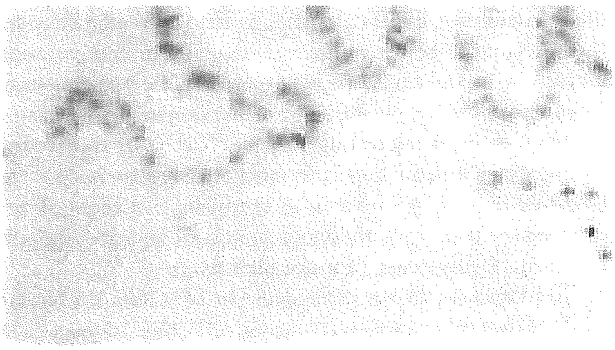

Figure 8 Same case as in Figure 7. The EBER-1-positive nuclear signal is restricted to regenerative epithelium. Note the EBER-1 nuclear negativity of neoplastic cells infiltrating the lamina propria (*in situ* hybridization).

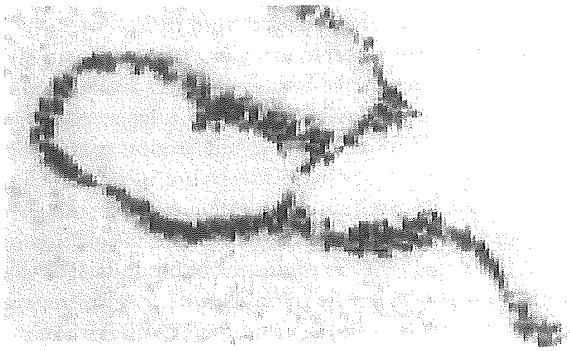

Figure 10 Same case as in Figure 9. Dysplastic epithelium is intensely positive for the EBER-1 nuclear signal (*in situ* hybridization).

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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ARTÍCULOS DE INVESTIGACIÓN

Características clínico-moleculares del cáncer gástrico cardial asociado al virus Epstein Barr

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Clinical and molecular features of cardial gastric cancer associated to Epstein Barr virus

***Background**: Mortality caused by cardial gastric cancer in Chile, is increasing. Previously we demonstrated an association between Epstein Barr virus and this specific location of gastric cancer.* ***Aim**: To perform a clinical and molecular characterization of cardial gastric cancer associated to Epstein Barr virus.* ***Material and methods**: Epstein Barr virus was identified in 93 cardial gastric tumors, by in situ hybridization. Clinical and pathological features, survival and expression of p53 and c-erbB2 were compared between tumors with or without the presence of the virus.* ***Results**: Twenty two (23.6%) tumors expressed Epstein Barr virus. No difference in sex or age of patients with tumors positive or negative for the virus was observed. Epstein Barr positive tumors had a tendency to have a higher frequency of Bormann III endoscopic appearance and a lower frequency of p53 accumulation (p=0.06). Five years survival was 67% and 42% of tumors positive and negative for the presence of the virus, respectively (p=0.57).* ***Conclusions**: Our results, although not significant, show a tendency towards unique characteristics of cardial gastric tumors associated to Epstein Barr virus (Rev Méd Chile 2005; 133: 753-60).*
*(**Key Words**: Epstein-Barr virus infections; Herpes virus 4, human; Stomach neoplasms)*

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El cáncer gástrico (CG) representa la primera causa de muerte por enfermedades neoplásicas en Chile y aunque su tasa de mortalidad se ha estabilizado[1], la frecuencia del CG de ubicación cardial se encuentra en aumento[2-4]. En efecto, autores chilenos[2,3] han demostrado que los tumores cardiales han incrementado entre 12 y 15% en los últimos 30 años, llegando a representar el 42,3% de las localizaciones. De modo similar, investigadores americanos[4] han reportado un incremento anual de 4,4% entre 1976 y 1987, sin observar modificaciones en la frecuencia del CG, en otras localizaciones. Se ha postulado que el CG cardial, sería una forma particular de CG, dado que se comunica una peor sobrevida a 5 años en esta localización en comparación con CG de otras ubicaciones[5]. Por otra parte, se ha descrito que la frecuencia de mutaciones de los oncogenes p53 y Her2/neu sería diferente entre CG cardial y antral[6,7] y Hansen et al[8] han señalado que *Helicobacter pylori* no tendría un rol tan relevante en la patogénesis del CG cardial como el observado en la ubicación antral. Incluso se ha observado un probable rol protector de *Helicobacter pylori* en el desarrollo del CG cardial[9]. Tomadas en conjunto, estas observaciones sugieren que el CG cardial sería una forma emergente y particular de CG.

El virus de Epstein-Barr (VEB), tradicionalmente asociado a neoplasias linfoides[10], también ha sido descrito en tumores epiteliales como carcinoma nasofaríngeo y linfoepiteliomas de distintos órganos, incluyendo el estómago[11,12]. En esta ubicación, los linfoepiteliomas no representan más de 5% de los tumores gástricos[13], sin embargo, en los últimos años se ha demostrado una emergente asociación entre VEB y CG[14]. En efecto, diversas publicaciones señalan una asociación de 6,9% a 18% entre VEB y CG[15-17] con características clínico-patológicas únicas, como predominio en hombres, localización alta (cardias y tercio medio del estómago) y una frecuencia similar de los subtipos "intestinal" y "difuso"[18]. Recientemente hemos analizado las características clínico-patológicas del CG asociado a VEB en Chile, encontrando una asociación de 16,8% (31/185), una de las más altas del mundo, y un perfil clínico-patológico único[19]. Este perfil se caracteriza por una distribución similar entre ambos sexos, una fuerte asociación con ubicación cardial y un predominio del patrón histológico "difuso"[19]. Dado que estas características han sido descritas en México[20] y en descendientes mexicanos en Estados Unidos[21], hemos sugerido la presencia de un perfil único del CG asociado a VEB en Latinoamérica[19].

Ya que el CG cardial representa una entidad propia y emergente en Chile y que una de las principales características del CG asociado a VEB es la fuerte localización cardial, el objetivo de nuestro estudio fue analizar características clínico-patológicas, moleculares y de sobrevida del CG cardial en sus formas asociada y no asociada al VEB.

Materiales y métodos

Pacientes. El estudio se realizó sobre la base de 305 piezas quirúrgicas de pacientes operados por CG en el Servicio de Cirugía del Hospital San Borja Arriarán y archivados en el Instituto Chileno-Japonés de Enfermedades Digestivas del mismo hospital entre 1993 y 1999. De este material y utilizando la definición de Locke et al[22] que definen como mucosa gástrica cardial a la región entre la unión gastroesofágica y los primeros 5 cm de estómago, se identificaron 93 casos, que son la base del presente estudio. Las características clínicas de estos casos se obtuvieron de la revisión de fichas médicas y las características patológicas de informes anátomo-patológicos correspondientes. Se consignó edad, sexo, tamaño tumoral (<5 y >5 cm), infiltración de pared gástrica y compromiso ganglionar (sin consignar número de ganglios comprometidos) de acuerdo a la Unión Internacional contra el Cáncer[23] y tipo histológico de acuerdo a la clasificación de Lauren[24]. En los tumores que infiltraban muscular propia o serosa, se consignó además la forma macroscópica según la clasificación de Bormann[25].

Hibridación in situ para la identificación del virus de Epstein-Barr. La presencia de VEB se realizó determinando la expresión del ARN pequeño no poliadenilado intranuclear-1 (EBER-1),