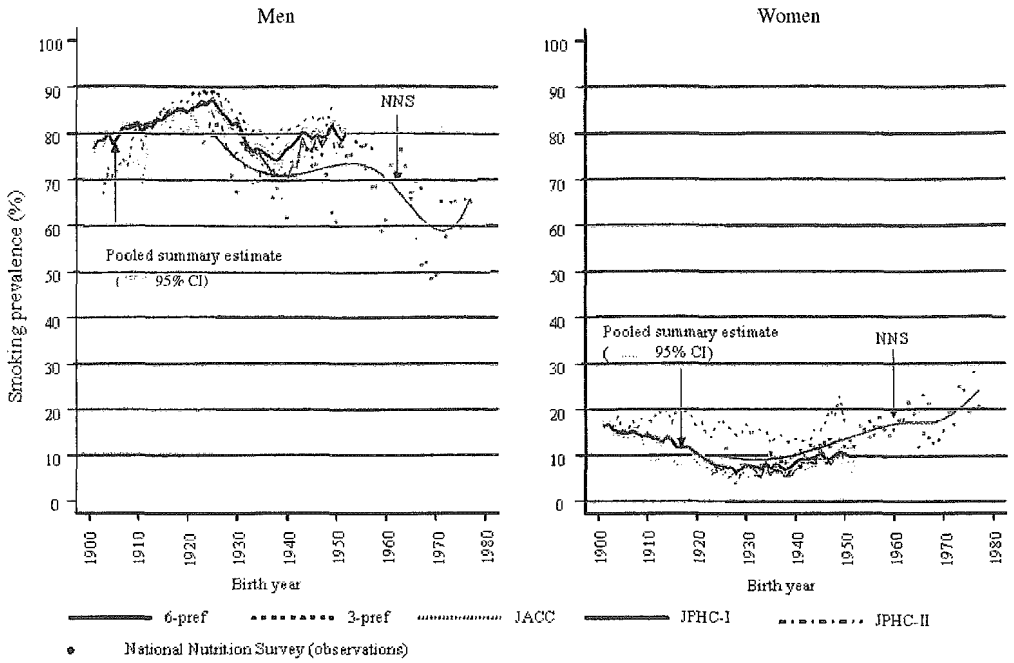

Fig. 7. Ever smoking prevalence by birth cohort among Japanese men and women, according to study-specific pooled summary estimate and 95% confidence intervals (1900–1952 birth cohorts) and the National Nutrition Survey (NNS, 1925–1977 birth cohorts). JACC study, The Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, sponsored by the Japanese Ministry of Education, Science, Sports and Culture; HOHC study, The Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Disease.

population. After adjusting for the comparatively high mortality of smokers, smoking trends across all birth cohorts did not differ materially.

Historical incidents did not have uniform effects across birth cohorts as a result of the different ages and different smoking career stages of the cohorts. The most important incident affecting smoking habits in the first half of the 20th century might be World War II (WWII, 1939–1945). In general, during and just after WWII, Japan experienced an extreme shortage of cigarettes; however, rationing provided small numbers of cigarettes to most men aged 20 or more. This wide distribution may have established smoking habits in a large number of adolescent and young adult males born in the late 1910s and 1920s. From the end of WWII to the beginning of Japan's post-WWII economic growth, cigarettes continued to be in short supply but were not distributed by rationing. Therefore, men born during 1930s had less opportunity to begin smoking in their adolescence, and their cohorts showed a corresponding dip in ever smoking prevalence. In recent years, even though the younger generation has adopted a nonsmoking lifestyle, smoking prevalence among Japanese men remains high. Therefore, the cessation of smoking should be strongly promoted among Japanese men.

Our results also demonstrate a more complex pattern of smoking habits among Japanese women. Before WWII, Japanese society did not accept smoking by young women. Therefore, Japanese women began smoking later in life. This was especially true for older generations. During the 1960s, a rapid increase in cigarette marketing was seen in Japan, along with an expansion of women's social position and participation in the labor force. Therefore, younger generations have been more tolerant towards women who smoke, and, accordingly, smoking habits have grown rapidly among these younger generations. In conclusion, for women, Japanese health policy should emphasize the prevention of smoking initiation, regardless of age, with a priority placed on targeting younger birth cohorts.

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Association of a distinctive strain of Epstein-Barr virus with gastric cancer

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Epstein-Barr virus (EBV) has been linked to gastric carcinoma (GC) with worldwide geographical variations attributable to types and variants of EBV. Here, we compare EBV strains between EBVaGC and healthy donors in Latin America, a high frequency area for EBVaGC. Tumor samples from 73 EBVaGC cases and throat washings from 329 healthy adults were examined for types 1 and 2 EBV and polymorphism at BamHI-F and BamHI-W1/I1 boundary regions and XhoI restriction site in LMP1 gene. Type 1 and prototype F of BamHI- F polymorphism accounted 59 (81%) and 69 (95%) of EBVaGC cases and 257 (78%) and 267 (81%) of healthy donors, respectively. Types I and "i" of BamHI W1/I1 polymorphism accounted 2 (3%) and 62 (85%) of EBVaGC and 85 (26%) and 170 (52%) of healthy donors, respectively ($p<0.001$). XhoI+ and − polymorphism accounted 60 (82%) and 4 (5%) of EBVaGC and 142 (43%) and 92 (28%) of healthy donors, respectively ($p<0.001$). Cosegregation analysis demonstrated that most of the 62 type "i" EBVaGC cases harbor XhoI+ strain (81%). However, among 143 type "i" healthy adults, both XhoI polymorphism were present in relatively similar frequencies (XhoI+ 58% and XhoI− 42%) (OR 9.0; 95% CI 1.2–69). Our findings are against to the proposed hypothesis that EBV strains are geographically but not disease-restricted. We conclude that most of the EBVaGC cases harbor a distinctive EBV strain (type "i"/XhoI +), but in healthy donors, this strain was as common as other strains. This finding is contrary to the proposed hypothesis that EBV strains are geographically but not disease-restricted and identified a healthy population group that share the same strain that predominate in EBVaGC cases.

Key words: gastric cancer; Epstein-Barr virus; genotypes

Epstein-Barr virus (EBV), an ubiquitous human herpesvirus, causes infectious mononucleosis after primary infection in young adults, and is closely associated with lymphoid neoplasms, such as B-cell lymphomas among immunosuppressed patients, Burkitt lymphoma, and Hodgkin lymphoma Hodgkin's disease.[1,2] EBV has also been associated with epithelial malignancies like nasopharyngeal carcinoma (NPC), lymphoepitheliomas of several organs,[3] and during the last decade, it has been linked to gastric carcinoma (GC).[4] This latter association has been evidenced by the presence of uniform expression of EBV-encoded small RNA type-1 (EBER-1) in all GC tumor cells,[5] the detection of monoclonal EBV episomes in GC cells[6] and the elevation of serum antibodies against viral capsid antigen in EBV-associated GC (EBVaGC) patients but not in EBV-negative GC patients or healthy controls.[6–8] Worldwide, EBVaGC represents about 10% of GC;[4] however, the frequency of this association varies from country to country and an inverse correlation between GC mortality and frequency of EBVaGC has been found;[9] *e.g.*, countries with a low GC mortality rate, such as the U.S.A. and Germany,[10] showed the highest frequencies of EBVaGC (16–18%)[11,12] and in countries with a high GC mortality rate, like Japan and China,[10] the proportion of EBVaGC accounts only for 6–7%.[13,14] In Latin America, we reported a frequency as high as 17% of EBVaGC, with a significant association to cardia location and diffuse histology.[15,16]

The 2 major types of EBV, type 1 and 2, differ in the sequence of EBNA-2, 3A, 3B, 3C and LP genes and in their capacity to transform B-lymphocytes into a state of continuous proliferation.[17] Type 1 EBV is the predominant strain in Western and Asian countries while type 2 EBV is frequently found in Africa.[18,19] In addition, 3 major variants of EBV have been identified based on restriction fragment length polymorphism (RFLP) of BamHI and XhoI restriction endonuclease map of the prototype B95.8 genome.[20–23] At BamHI-F region, the prototype F has a worldwide distribution but "f" variant, featured by the presence of an extra BamHI site, is found only in Southern China where it is associated with NPC.[24] The polymorphism at BamHI W1/I1 boundary region identifies 2 types, type I and "i". Type I lacks the BamHI site and predominates among healthy people and EBV-associated diseases in Japan and China.[20,25,26] Type "i", which keeps BamHI restriction site prevails in healthy donors and EBV-associated disease in Western countries.[21] Finally, the lack of XhoI restriction site at exon 1 of the LMP1 gene defines the XhoI − genotype, which is common in Asia[27] while the XhoI + variant is frequently observed in Western countries.[22]

Taken together, these observations suggest that geographical distribution of EBVaGC might be explained by different EBV genotypes or variants around the world. In this study, we compare types and variants of EBV among EBVaGC cases and healthy adults in Latin America.

Material and methods

Specimens

We examined formalin-fixed and paraffin-embedded tumor samples of 73 EBVaGC cases (44 from Chile and 29 from Colombia) and throat washing samples collected from 329 healthy adults (140 from Chile and 189 from Colombia) by gargling with 15 ml of phosphate-buffered saline. In addition, 5 EBVaGC cases in which both tumor samples and throat washing gargles were also available were examined. The EBV expression status of the EBVaGC cases was examined previously[15,16] using *in situ* hybridization with oligonucleotide probes specific for the EBER-1 gene as described in detail elsewhere.[28] The Institutional Review Board of the San Borja-Arriaran Hospital, Santiago, Chile and the Institutional Review Board of the Faculty of Health, Universidad del

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TABLE I – LIST OF PRIMERS AND PROBES USED IN THE PRESENT STUDY

	Sequence	Type by probe or size after RE[1] digestion	Reference
EBNA-3C primers			
Sense	5′-AGAAGGGGAGCGTGTGTTGT-3′		
Antisense	5′-GGCTCGTTTTTGACGTCGGC-3′		Sample *et al.* (1990)
Probes			
Type A	5′-GAAGATTCATCGTCAGTGTC-3′	153 bp[2]	
Type B	5′-CCGTGATTTCTACCGGGAGT-3′	246 bp	
Bam-HI-F primers			
Sense	5′-TCCCACCTGTTACCACATTC-3′	Prototype F = 198 bp	Lung *et al.* (1994)
Antisense	5′-GGCAATGGGACGTCTTGTAA-3′	Variant "f" = 127+71 bp	
BamHI-W1/I1 primers			
Sense	5′-ACCTGCTACTCTTCGGAAAC-3′	Type I = 205 bp	Lung *et al.* (1994)
Antisense	5′-TCTGTCACAACCTCACTGTC-3′	Type "i" = 130+75 bp	
XhoI site in LMP1 gene primers			
Sense	5′-AACAGTAGCGCCAAGAGGAG-3′	XhoI− = 113 bp	Sandvej *et al.* (1997)
Antisense	5′-ATGGAACACGACCTTGAGAGG-3′	XhoI+ = 67+46 bp	

[1]Restriction enzyme.–[2]Base pairs.

Valle, Cali, Colombia approved this study and all healthy individuals as well as EBVaGC gave informed consent.

Preparation of DNA

Cellular material from throat washing was collected by centrifugation at 22,000*g* for 40 min and the resulting pellet was resuspended in 100 μl of extraction buffer (1 M Tris, pH 8.0, 50 mM EDTA, 0.5% Tween 20) with 100 μg/ml Proteinase K. After overnight incubation at 37°C and boiling at 100°C for 10 min for Proteinase K inactivation, samples were subjected to phenol–chloroform extraction and ethanol precipitation. Finally, DNA was dissolved in 40 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE) buffer and kept at −30°C until amplification. Formalin-fixed, paraffin-embedded archival material was cut in 5 μm slices, treated with xylene and ethanol and centrifuged at 22,000*g* for 20 min, and the resulting pellet was resuspended in 100 μl of extraction buffer as described earlier.

Primers and probes

Primers and probes used in this study are shown in Table I. For distinguishing type 1 and 2 EBV strains, we used primers and probes described by Sample et al.[29] These probes recognized divergent sequences in the U2 region encoding EBNA-3 gene[30] and produced 153-and 246-bp, respectively. The BamHI-F region was amplified with primers described by Lung et al[20,31] that yield a 198-bp fragment. To distinguish the prototype F from the "f" variant, the 198-bp fragment was digested by BamHI restriction enzyme to yield a 198-bp fragment in the case of the F prototype and 127-bp and 71-bp fragments in the case of "f" variant. A 205-bp fragment of the BamHI-W1/I1 boundary region was amplified using the primer pair described by Lung et al.[20,31] Type I, 205-bp fragment, and type "i", 130- and 75-bp fragments, were determined by BamHI restriction enzyme digestion. Analysis of XhoI restriction site polymorphism in exon 1 of LMP1 gene was performed with a set of primers to produce a 113-bp amplified fragment.[32] Digestion with XhoI restriction enzyme resulted in 67-and 46-bp fragments for the XhoI + type and the undigested 113-bp PCR product indicates the XhoI − type. The cell line B95-8 served as positive control for type 1, prototype F, type I and XhoI + virus. The cell lines AG786 and Akata served as positive controls for type 2 and XhoI − virus, respectively. The cloned BamHI-"f" and BamHI-"i" DNA fragments served as positive controls for the "f" variant and type "i", respectively. The MOLT-4 cell line infected with human herpesvirus 6 served as negative control.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed with 2 μl of DNA in a 25 μl reaction mixture containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 uM dNTP, 0.5 μM of each primer and 1.25 U Taq Polymerase (Invitrogen Corp., CA). The amplification profile for EBNA-3 gene, the BamHI-F region and the BamHI-W1/I1 boundary region amplification were 1 cycle at 95°C for 5 min, followed by 40 cycles of 92°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The program ended with 5 min at 72°C. For XhoI restriction site amplification, the profile was 1 cycle at 95°C for 5 min, followed by touchdown PCR [6 touchdown cycles at 94°C for 30 sec, 66°C for 30 sec with a decrease of 1°C each cycle, and 72°C for 30 sec, followed by 39 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec], with a final extension at 72°C for 5 min.

Southern blot analysis

Specificity of the PCR reactions for type 1 and 2 EBV strains was confirmed by southern blot hybridization with specific probes. After 2% agarose gel electrophoresis, the electrophoretic pattern was transferred to a Hybond N+ nylon membrane (Amershan, Aylesbury, UK) by capillary blotting in 0.4 N NaOH solutions. Membranes were prehybridized with hybridization buffer and each probe was labeled with peroxidase using ECL direct labeling kit (Amersham, Aylesbury, UK). After adding the probe, hybridization was carried out overnight at 42°C. The hybridization signal was detected using ECL detection kit (Amershan, Aylesbury, UK) according to manufacturer's instructions.

Restriction fragment length polymorphism analysis

To perform an analysis of RFLP at BamHI-F region, BamHI-W1/I1 boundary region and XhoI restriction site, amplified PCR products were purified by phenol/chloroform extraction followed by ethanol precipitation with glycogen carrier. DNA pellets were resuspended in 50 μl of distilled water and 5 μl aliquots were digested of either BamHI, for BamHI-F and BamHI-W1/I1 boundary region RFLP, or XhoI, for the XhoI restriction site polymorphism. Restriction enzyme digestion was performed in 20 μl volumes with 10 U. of restriction enzyme for overnight according to manufacturer's instructions (Invitrogen Corp., CA). Resultant products were electrophoresed through 8% polyacrylamide gel in TBE (45 mmol/l Tris-Borate) and visualized by silver-staining method.[33–35]

Cloning and sequencing DNA

Amplified XhoI restriction site products from 5 EBVaGC cases were purified from low melting point agarose gels and cloned into the pGMET vector, using the Wizard PCR prep kit and pGMET cloning methods (Promega Corp, WI). Before precipitation for sequencing, plasmids were checked for correct insert size by PCR.

TABLE II – EPSTEIN-BARR VIRUS GENOTYPING IN EPSTEIN-BARR-VIRUS-ASSOCIATED GASTRIC CARCINOMA AND THROAT WASHING OF HEALTHY ADULTS

	EBVaGC[1] (*N* = 73)		TW[2] (*N* = 329)		*p* value[3]	Age-adjusted *p* value[4]
	N	%	*N*	%		
Gender						
Female	14	19	173	53	<0.001	–
Male	55	75	156	47		
Unknown	4	5	0	0		
Mean age (SD)	58.1	(14)	29.5	(10)	<0.001	–
Range	19–83		18–66			
EBNA-3C						
Type 1	59	81	257	78	0.045	0.222
Type 2	11	15	22	7		
NA[5]	3	4	50	15		
BamHI-F						
Prototype F	69	95	267	81	0.037	0.053
"f" variant	0	0	17	5		
NA[5]	4	5	45	14		
BamHI-W1/I1						
Type I	2	3	85	26	<0.001	<0.001
Type "i"	62	85	170	52		
NA[5]	9	12	74	22		
XhoI site						
–	4	5	92	28	<0.001	<0.001
+	60	82	142	43		
NA[5]	9	12	95	29		

[1]Epstein-Barr-virus-associated gastric carcinoma.–[2]Throat washing of healthy adults.–[3]*p* values were obtained by χ^2 test.–[4]*p* values were obtained by exact method.–[5]No amplified fragment.

A small sample of each clone was boiled in 10 µl water and 1 µl aliquot was amplified using T3 and T7 primers (Promega Corp, WI) under standard conditions using 30 cycles of PCR with annealing temperature of 55°C. Of the products, 5 µl was checked on agarose gel, and 5 µl aliquots of the remainder were used for sequencing. Clones were bidirectionally sequenced through cycle sequencing, using the Big Dye Terminator kit (Perkin Elmer, CT) on the automated ABI Prism 310 sequencer (Applied Biosystems, CA). Clones were sequenced at least 2 times to ensure sequence fidelity.

Statistical analysis

We conducted a case-control comparison for EBV strains between EBVaGC cases and healthy donors by using χ^2 test or two-sided Fisher's exact test. The results were considered to be statistically significant at a P of less than 0.05. Logistic regression analysis was conducted to compare the association between BamHI W1/I1 boundary and XhoI restriction site polymorphism. Exact P values and estimation of common odds ratios were obtained using stratified contingency tables by age (<30, 30–49 and 50<=). Statistical analyses were conducted using the EPICURE package of statistical programs for analysis of epidemiological data (Hirosoft, WA) or StatXact 4 (CYTEL Software Corporation, MA).

Results

We examined 73 EBVaGC and 329 healthy adults. Their age and gender distributions were different from each other as shown in Table II, however, neither gender or age affected the distribution of EBV subtype. Therefore, it was concluded that the gender or age difference between EBVaGC cases and healthy donors should not affect the comparisons in terms of EBV subtype distributions.

EBV type (type 1 and 2)

The amplification of U2 region encoding EBNA-3 gene was successful in 70 out of 73 (96%) EBVaGC and 279 out of 329 (85%) healthy donors. The distribution of types 1 and 2 between case and healthy donors groups is summarized in Table II. Type 1 accounted 59 (81%) of 73 EBVaGC and 257 (78%) of 329 healthy donors (p = 0.045). This difference was not statistically significant when corrected for age distribution (p = 0.22). The distribution of type 1 and 2 in the 2 countries did not differ significantly.

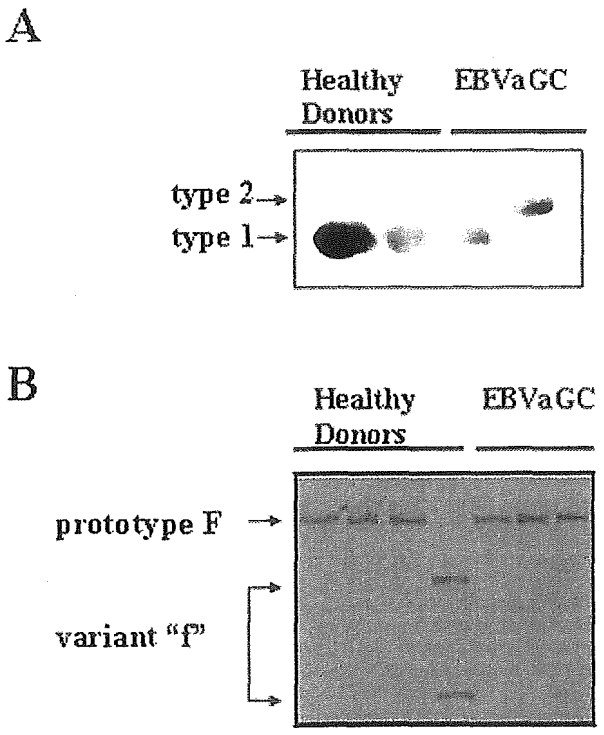

FIGURE 1 – Gentoypes of Epstein-Barr virus in Healthy donors and Epstein-Barr-associated gastric carcinomas (EBVaGC). (*a*) Southern blot analysis after PCR amplification and hybridization with specific probes for type 1 and 2 strains. (*b*) Polyacrylamide gel after PCR amplification and digestion with BamHI restriction enzyme for RFLP at BamHI-F region.

Representative examples of EBV types 1 and 2 in EBVaGC and healthy donors are shown in Figure 1*a*.

BamHI-F region (types F and "f")

The BamHI-F region could be amplified in 69 (95%) EBVaGC and in 284 (86%) healthy adults. The distribution of prototype F and "f" variant between EBVaGC and healthy adults is summarized in Table II. All of the 69 EBVaGC harbor the prototype F EBV. However, among healthy adults, prototype F and "f" variant were found in 267 (81%) and 17 (5%), respectively. This difference in cases and controls was statistically significant (p = 0.037), but not in age-corrected distribution (p = 0.053). Among 267 prototype F EBV, types 1 and 2 numbered 227 and 19, respectively. In the remaining 21 specimens, EBNA-3 region could not be amplified. Similarly, all but 1 of the 17 "f" variants obtained from healthy donors were identified as type 1 (data not shown). The distribution of prototype F and variant "f" in the 2 countries did not differ significantly. Representative examples of BamHI-F RFLP in healthy donors and EBVaGC are shown in Figure 1*b*.

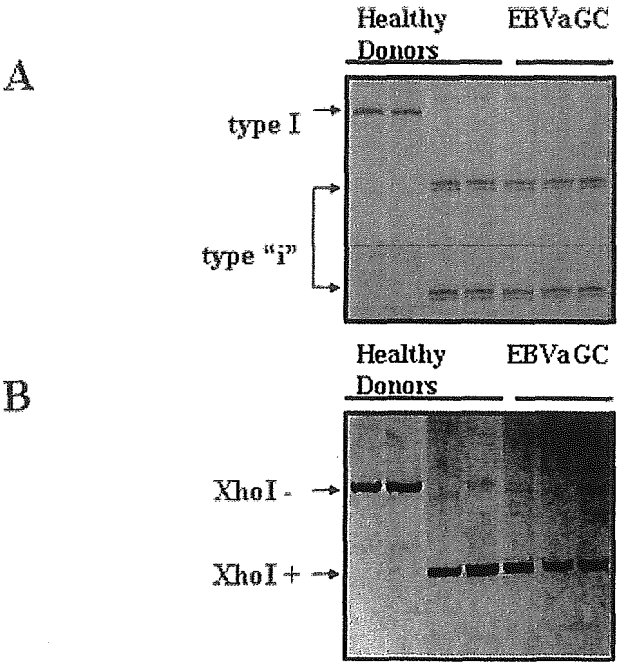

FIGURE 2 – Genotypes of Epstein-Barr virus in Healthy donors and Epstein-Barr-associated gastric carcinomas (EBVaGC). (*a*) Polyacrylamide gel after PCR amplification and digestion with BamHI restriction enzyme at BamHI W1/I1boundary region. (*b*) Polyacrylamide gel after PCR amplification and digestion with XhoI restriction enzyme at XhoI site polymorphism. The second fragment after XhoI digestion (46-bp fragments) for the kept-type is not seen in the gel.

BamHI W1/I1 boundary (types I and "i")

The BamHI-W1/I1-boundary restriction site was successfully amplified in 64 (88%) of 73 EBVaGC and in 255 (78%) of 329 healthy adults. The distribution of types I and "i" between EBVaGC and healthy adults is summarized in Table II. Only 2 (3%) EBVaGC harbor type I EBV while 62 (85%) out of 73 EBVaGC harbor type "i" EBV. Among healthy adults, type I and "i" were found in 85 (26%) and 170 (52%), respectively. This difference was highly significant ($p < 0.001$). Among 170 type "i" strain, types 1 and 2 numbered 149 and 11, respectively. In the remaining 10, EBNA-3 region could not be amplified. Among 85 type I healthy donors, 73 were type 1, 5 were type 2 and EBNA3A could not be amplified in the remaining 7 subjects. The distribution of type I and "i" in both countries did not differ significantly. Representative examples of type I and "i" in EBVaGC and healthy donors are shown in Figure 2*a*.

XhoI restriction site polymorphism at exon 1 of the LMP1 gene

A successful analysis of XhoI restriction site polymorphism was performed in 64 (88%) of 73 EBVaGC and in 234 (71%) of 329 healthy controls. The distribution of XhoI + and − between EBVaGC and healthy adults is summarized in Table II. Sixty (82%) out of 73 EBVaGC harbor XhoI + EBV and only 4 (5%) individuals harbor type XhoI −. However, among healthy adults, XhoI + and − were found in 142 (43%) and 92 (28%), respectively. This difference was highly significant ($p < 0.001$). The distribution of XhoI + and − in both countries did not differ significantly. Representative examples of XhoI + and − in EBVaGC and healthy donors are shown in Figure 2*b*.

Cosegregation of XhoI restriction site polymorphism with BamHI W1/I1 boundary RFLP

Since it is known that XhoI restriction site polymorphism co-segregate with the BamHI W1/I1 boundary RFLP,[21] we next analyzed the frequency of co-segregation between these 2 polymorphism in EBVaGC and healthy donors (Table III). Among 62 type "i" EBVaGC, 59 (95%) harbor XhoI + strain and a recombinant type "i"/XhoI − strain was found in 3 (5%) individuals. Among 143 type "i" healthy adults, XhoI + type was found in 83 (58%) cases and the recombinant strain type "i"/XhoI − was present in 60 (42%) of subjects. Among type I EBVaGC, XhoI + and − was present in 1 patient respectively. In 69 type I healthy donors, XhoI + and − was found in 46 (67%) and 23 (33%) of individuals, respectively. The proportion of type "i"/XhoI + in EBVaGC was significantly higher than the proportion of 58% observed among healthy donors (Odds ratio 9.0; 95% Confidence interval 1.2–69, Table III)

Correlations of EBV genotypes in both tumor samples and throat washing from EBVaGC cases

In 5 additional EBVaGC cases, both tumor samples and throat washing were available for genotyping analysis. Genotyping analysis of these cases reveals the presence of unique strain type 1, type F, type "i" and XhoI + in tumor sample. Corresponding throat washing of these cases reveals same genotype in 4 out of 5 cases. The remaining case was type 1, type F, type "i" but XhoI − in gargles. Representative examples are shown in Figure 3.

TABLE III – COSEGREGATION ANALYSIS OF BamHI-W1/I1 BOUNDARY REGION AND XhoI RESTRICTION SITE IN LMP1 GENE IN EPSTEIN-BARR-VIRUS-ASSOCIATED GASTRIC CARCINOMA AND THROAT WASHING OF HEALTHY ADULTS

BamHI-W1/I1	XhoI site	EBVaGC[1]	TW[2]	OR (95% CI)[3]	Age-adjusted OR (95% CI)[4]
I	−	1	23	1.0 (reference)	1.0 (reference)
	+	1	46	0.5 (0.01–40.9)	0.58 (0.004–78.1)
i	−	3	60	1.2 (0.09–63.0)	0.85 (0.04–55.5)
	+	59	83	16.3 (2.5–685)	Inf (5.9 to +inf)

[1]Epstein-Barr-virus-associated gastric carcinoma.–[2]Throat washing of healthy adults.–[3]OR and 95% CI were calculated by logistic regression model.–[4]OR and 95%CI were obtained by exact method.

Sequencing of XhoI restriction site polymorphism in cases of EBVaGC

Finally, the presence of the distinctive XhoI + strain in EBVaGC cases was confirmed by sequence of XhoI region in 5 cases of EBVaGC. All 5 cases show the presence of XhoI restriction site. Representative sequence data is shown in Figure 4.

Discussion

In the present study, we conducted an EBV genotyping analysis in EBVaGC and healthy adults in 2 Latin American countries with high incidence and mortality rate for gastric cancer. Our results show that a distinctive viral genotype is present in EBVaGC, characterized as type "i" at the BamHI W1/I1 boundary region and XhoI + at XhoI restriction site polymorphism in exon 1 of the LMP1 gene. However, among healthy adults, these polymorphism were as common as other 3 genotypes (type I/XhoI − and the recombinants type "i"/XhoI − and type I/XhoI +). These observed differences cannot be attributed to detection rates of EBV, since our detection rates were comparable or even better than that reported by other researchers.[24,36–38] Thus, these differences suggest that a particular polymorphism (type "i"/XhoI +) is predominant in EBVaGC eventhough several strains are found in healthy donors. In addition, although in a small subset of cases, most of EBVaGV harbour the same strain in both stomach and throat washings. Taken together, our findings are similar to that of NPC in Southern China, where the presence of the "f" variant was found in most NPC cases and in a small proportion of healthy Chinese.[24] Additionally, our findings suggest that the proposed hypothesis that EBV strains are geographically but not disease-restricted[22] might not be true for EBVaGC in Latin America.

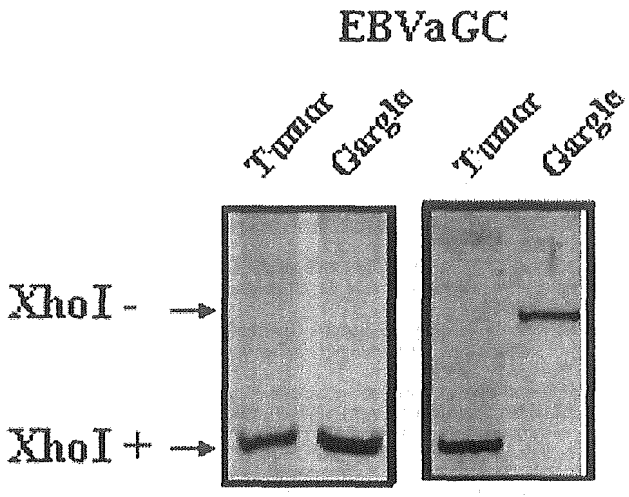

FIGURE 3 – Polyacrylamide gel after PCR amplification and digestion with XhoI restriction enzyme at XhoI site polymorphism in tumor and throat washing of Epstein-Barr-associated gastric carcinomas (EBVaGC).

The finding of a distinctive genotype in EBVaGC cases in comparison with healthy donors suggest that the expression of particular EBV gene(s) with transforming capacity might be encoded in the vicinity of BamHI W1/I1 boundary and XhoI restriction site polymorphism. There are at least 3 candidate genes involved in transformation and immortalization located in this region. The first is the BARF-1 gene, which is frequently expressed in the type I latent infection and reported to have a unique transcription pattern in EBVaGC.[39] The BARF-1 gene is able to immortalize primary monkey and human epithelial cells *in vitro*,[40,41] and transfection of BARF-1 into the rodent fibroblast cell line BALB/c 3T3 or into the EBV-negative B cell line, Loukes, resulted in tumorigenic transformation.[42,43] The second candidate is EBER-1 and/or -2 gene, which are by far the most abundant viral transcripts in latently EBV-infected cells and confer colony formation in soft agarose, tumorigenicity in immunodeficient mice, and resistance to apoptosis in Burkitt lymphoma cells.[44] Interestingly, recently it has been found that EBER-1 induce the secretion of IGF-I as an autocrine growth factor in EBVaGC,[45] and sequences containing a consensus ATF site upstream of the EBER-1 gene are important for EBER-1 expression.[46] The third candidate is the LMP2A gene. Comparison between EBVaGC and healthy donors have found that strains detected in EBVaGC tend to have LMP2A gene with threonin substitution at codon 348, which corresponds to HLA A-11 restricted CTL epitope.[47] This substitution may confer an advantage for viral persistence in tumor cells. Thus, polymorphisms or differences in expression of BARF-1, EBER's or LMP2A gene(s) might be associated with specific genotype that contribute to the EBVaGC carcinogenetic process.

EBV type 1 was identified in most of EBVaGC and healthy donors. The predominance of this strain is in agreement with its worldwide distribution.[18,19,36,38,44,48] The prototype F at BamHI-F region was the most common finding in our healthy donors as well as EBVaGC, a finding also in agreement with previous studies in Asian countries.[24,25] In this study, we found 2 recombinant strains among healthy donors (type I/XhoI + and type "i"/XhoI −). These findings might be explained by total or partial recombination during replication of the 2 wild-type EBVs (type I/XhoI − and type "i"/XhoI +).[49] Since type I/XhoI − is mainly from Asian origin and type "i"/XhoI + is common in Western countries, our findings correlate with ethnic distributions in our study area.[50] Thus, the generation of these new EBV recombinants might be due to the presence and mixing of different ethnic populations infected with EBV in this region. In this context, we cannot rule out the presence of more than 1 strain in healthy donors, since it has been described in up to 23% of normal individuals.[51,52] However, our PCR assay was not designed for detecting multiple strains and probably underestimates a more complex spectrum of EBV strains present in the throat of some healthy control individuals.

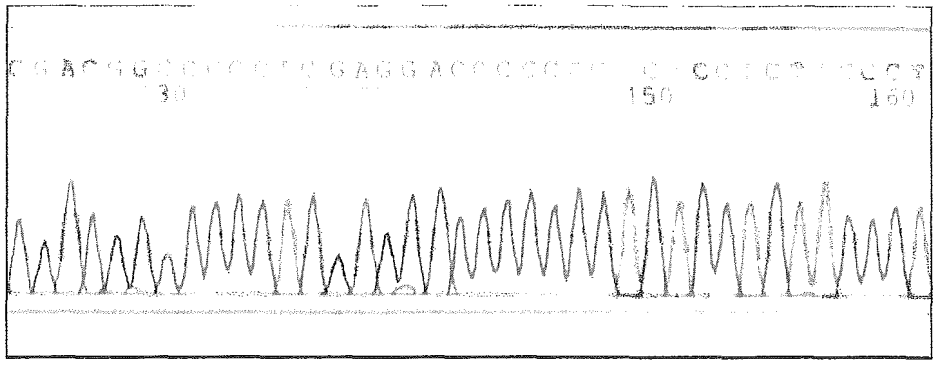

FIGURE 4.

In summary, the present study showed that the most of EBVaGC patients harbor a distinctive EBV strain (type "i"/XhoI +), but in healthy donors, this strain was as common as other 3 strains. In addition, although in a small subset of cases, the virus harbored in the orophaynx in cases of EBVaGC is the same to the virus seen in the stomach. Our findings identified a healthy population group that share the same strain that predominate in EBVaGC cases. It could be of interest to carry a extensive cohort studies following these individuals longitudinally to evaluate the risk to develop EBVaGC.

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Epstein-Barr Virus Detection in Tumors of Upper Gastrointestinal Tract. An in Situ Hybridization Study in Pakistan

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To examine the potential role of Epstein-Barr virus (EBV) in the carcinogenesis of upper gastrointestinal tract, we conducted an *in situ* hybridization assay for EBV- encoded small RNA (EBER) expression in the tumors of 56 oral and 50 esophageal squamous cell carcinoma (SCC) cases, and 52 stomach adenocarcinoma cases diagnosed in the King Edward Medical College and Allama Iqbal Medical College Lahore, Pakistan between 1996-2002. There were no malignancies with positive EBER expression in oral and esophageal SCC. Only one out of the 52 gastric adenocarcinoma cases (1.9%) was positive for EBER expression, and this frequency was relatively low as compared to cases reported worldwide. The case was a 42 year-old male patient and histologically classified as moderately differentiated tubular adenocarcinoma. 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.

Key Words: Epstein-Barr virus, Oral cavity carcinoma, Esophageal carcinoma, Squamous cell carcinoma, Gastric adenocarcinoma, Pakistan

More than 90% of adults in the world are infected by the 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 carcinomas (1). In 1992, Shibata and Weiss (2) reported the presence of Epstein-Barr virus (EBV) genome in 16% of gastric adenocarcinomas in a small North American series, using an *in situ* hybridization (ISH) technique to detect EBV-encoded small RNA (EBER) genome in gastric cancer tissue. They reported that EBER ISH staining was observed in all carcinoma cells of an EBV-associated gastric carcinoma (EBV-GC) but was not detected in adjacent normal epithelial cells and surrounding lymphocytes. In all types of latent infection, EBERs are always expressed in addition to EBNA1 and BARF0. In addition, EBERs are now suspected to play important roles in carcinogenesis. For example, recent studies have elucidated that EBERs activate insulin-like growth factor (IGF)-1 in EBV-GCs (3), and induce not only interleukin (IL)-10 expression but also confer resistance to interferon (IFN)-α-induced apoptosis in Burkitt's lymphoma (4). Recently, however, the hypothesis that the EBV infection may represent a late event in gastric carcinogenesis was also reported (5).

A large-scale study in Japan, published in 1993, also showed the presence of EBER in 7% of gastric carcinomas (6). This study has shown that EBV-GC is more frequently observed in lymphoepithelioma-like carcinomas or adenocarcinomas with the histological type of moderately differentiated tubular adenocarcinomas, or solid poorly differentiated adenocarcinomas (2). Male predominance is a well-known feature of EBV-GC, suggesting the involvement of environmental factors (7). Geographically, the proportion of EBV-GCs differs from country to country, and ranged form 2-17% (8). Apparently, countries in the American Continents have a relatively high EBV-GC frequency and countires in the Eurasian Continent have relatively low frequencies. Studies in Papua New Guinea and the United Kingdom reported an EBV-

GC frequency of less than 5% (8,9). It should be pointed out, however, that the data in South and Southeast Asian countries are quite limited.

The association of EBV with the development of oral squamous cell carcinoma (OSCC) has been suspected because of the presence of the EBV receptor, CD21, in normal and malignant oral epithelium (10). However, the close proximity of the esophagus to the nasopharynx raises the possibility that EBV may be involved in the carcinogenesis of esophageal cancer. Many investigators have attempted to clarify a role of EBV in the carcinogenesis of oral (9,11-16) and/or esophageal cancer (9, 17-22). However, their results remain controversial and the conflicting published data may be partially explained by the methodology employed and/or by the specimens analyzed.

In the present study, we examined the presence of EBV in cancers of the stomach, esophagus and oral cavity, using the ISH assay for EBER expression since the ISH techniqueis better than the PCR for specifying the the viral localization in the tumors.

Materials and Methods

Subjects and Specimen Collection. We examined a total number of 158 cancer cases of the upper gastrointestinal tract diagnosed in Pathology Departments of King Edward Medical College and Allama Iqbal Medical College Lahore, Pakistan. Paraffin-embedded formalin-fixed tissues of the following cancers were examined by ISH: 56 cases of the OSCC diagnosed during the period between 2001-2002; 50 cases of esophageal squamous cell carcinoma (ESCC) and 52 cases of gastric cancer diagnosed during the period between 1996 and 2002. Clinical information on age, sex, date of diagnosis, location of tumors, and place of residence was obtained from biopsy reports and computerized records were obtained for the patients diagnosed before the year 2000 and after 2000, respectively.

Histological classification. Histological classifications of oral and esophageal cancers were made following the guidelines of Japan Society for Head and Neck Cancer (23), and Japanese Society for Esophageal Diseases, respectively (24). Gastric carcinomas were classified as the intestinal- and diffuse-type of Lauren classification (25), and subclassified according to the Japanese Classification of Gastric Carcinoma of Japanese Research Society for Gastric Cancer (26). 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). The tumor location, defined as the predominant location of the tumor, was divided into the following three locations: cardia or upper third part, middle part of the stomach, and antrum or lower third part according to the guidelines of the Japanese Research Society for Gastric Cancer (27).

In situ hybridization. The presence of EBV was identified by the expression of EBER-1, the most abundant viral product in latently infected cells (28, 29). ISH assay of paraffin-embedded tissue samples obtained from the main tumor was conducted using a digoxigenin-labeled EBER-1 oligonucleotide probe as described before (30, 31). In brief, the tissue sections were deparaffinized, hydrated and predigested with pronase. After that, the tissue sections were hybridized overnight at 37°C with a concentration of 500 ng/ml of digoxigenin (DIG)-labeled antisense EBER-1 probe (5'-agacaccgtcctcaccaccc gggacttgta-3'). The hybridization signal was detected using DIG Nucleic Acid Detection Kit (Borhringer Mannheim GmbH, Germany) according to the instructions of the manufacturer. A case was considered to be EBER-1 positive based on a positive signal under microscopy. Lymph node section from a patient with infectious mononucleosis was used as positive control, and a sense probe for EBER-1 was used as negative control in every assay. In the present study, cases with EBER-1-positive tumor cells but not in the surrounding normal epithelial cells were determined to be EBV positive cases, and we defined cases with EBER-1-negative tumor cells as EBV-negative cases. Cases with EBER-1-negative tumor cells but EBER-1-positive lymphocytes around the carcinoma were also determined to be EBV-negative cases.

Results

Age and gender distributions by histological type of OSCC and ESCC are shown in Table I. There were twenty-four (43%) female OSCC patients. Forty-two (75%) of the 56 OSCC cases were well differentiated carcinomas. Nine (16%) and five (9%) cases were moderately and poorly differentiated carcinomas, respectively. Poorly differentiated carcinoma cases

Table I - Age and gender distribution by histology in Oral and Esophageal squamous cell carcinoma cases

	Males				Females			
	N	Age Mean	SD*1	Range	N	Age Mean	SD*1	Range
OSCC*2								
Well differentiated	26	48.5	14.6	12-70	16	53.1	12.9	30-70
Moderately differentiated	4	54.0	12.1	41-70	5	54.6	10.2	40-65
Poorly differentiated	2	40.0	–	–	3	34.0	14.4	22-50
Total	32	46.7	13.6	12-70	24	51.6	14.6	22-70
ESCC*3								
Well differentiated	9	52.1	8.3	40-60	0	–	–	–
Moderately differentiated	13	49.1	9.0	34-65	11	58.6	12.0	38-75
Poorly differentiated	6	53.2	17.5	28-70	11	53.4	13.8	25-70
Total	28	51.9	11.2	28-70	22	56.0	12.9	25-75

**1 The standard deviation and/or range of age was not calculated when there were less than 3 cases with information on age.*
**2 Oral squamous cell carcinoma.*
**3 Esophageal squamous cell carcinoma.*

showed the lowest mean age both in males and females. The most frequent location of the OSCC was the tongue (29/56), followed by the floor of the mouth (4/56), lip (3/56), and palate (3/56). There were no OSCC case with EBER expression.

In the ESCC cases, the proportion of female patients was 44% (22/50), and histological distribution differed by gender. In males, nine (32%) out of 28 ESCC cases were the well differentiated type of carcinomas. However, there were no cases with the well differentiated type in females. Although the information on tumor location for thirty-five (70%) cases was irretrievable, the most frequent location was the upper third of esophagus (11/50). The EBER-ISH assay revealed that there was no EBER-positive ESCC case.

The age and gender distributions by tumor location and histology for gastric adenocarcinomas are shown in Table II. The ratio of males to females was 2.5 among gastric adenocarcinoma cases. There was no significant difference in the distributions by pathological features between males and females. Among the 52 gastric adnocarcinomas, only a 42-year-old male case was positive for EBER expression (1.9%). Figure 1 shows the EBER-1positive case and an EBER-negative case that had positive signals for EBER in infiltrating lymphocytes. The case was histologically classified as moderately differentiated tubular adenocarcinoma as from the Japanese histological classification. There was no EBER-positive cases in tumors with diffuse type of Lauren's classification.

Discussion

We found only one EBER-positive case with gastric adenocarcinoma, histologically classified as moderately differentiated tubular adenocarcinoma from the Japanese histological classification. No lymphoepithelioma-like carcinoma was identified in this study. The proportion of EBV-GCs observed in the present study was 1.9% (1/52), which is lower than that observed among Indian patients (32) and among the lowest proportion found in literatures (8,9). It should be noted, however, that the observed propor-

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-

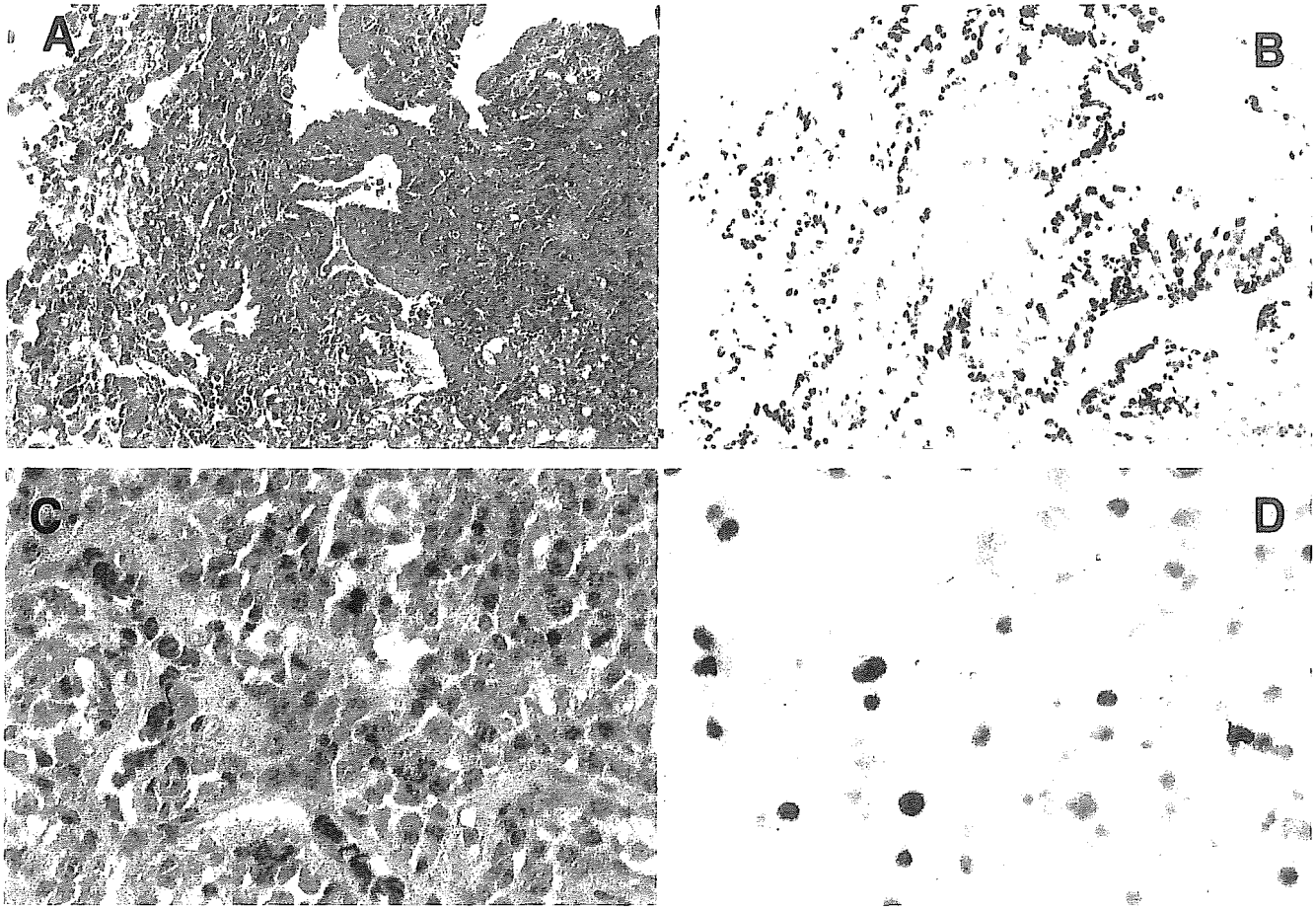

Fig. 1 - The EBER-positive and –negative gastric adenocarcinoma cases - **(A)** Hematoxylin and eosin staining for the EBER-positive case - **(B)** EBER-1 positive in tumors by ISH - **(C)** Hematoxylin and eosin staining for the EBER-negative case - **(D)** EBER-1 negative in tumors but positive in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

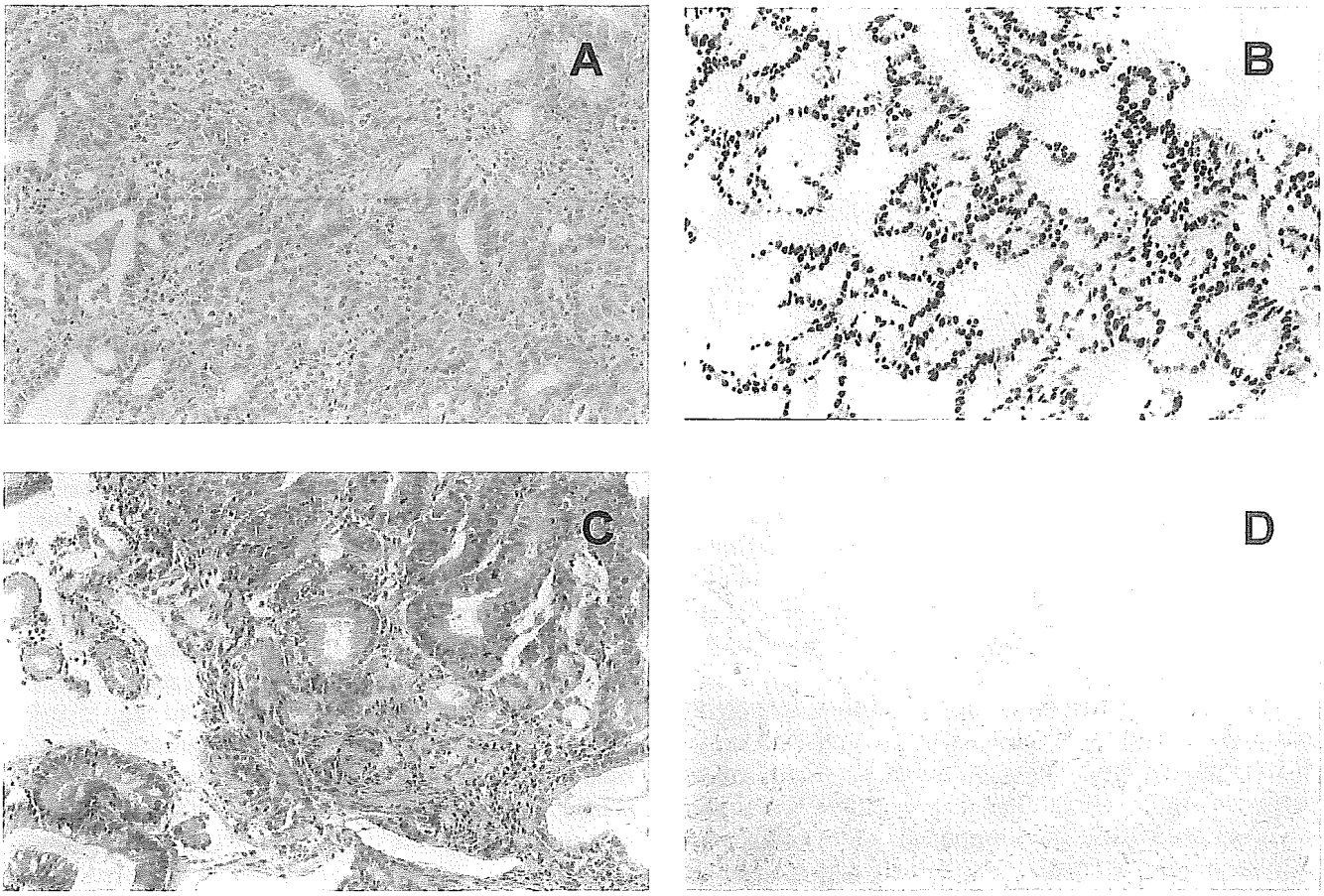

Fig. 1 - A case of suspected double carcinoma (Case 5 in Table II)
A. Hematoxylin and eosin staining (x10) **B.** EBER-1 in situ hybridization (x10, EBER-1 positive).
C. Hematoxylin and eosin staining (x10) **D.** EBER-1 in situ hybridization (x20, EBER-1 negative).