Identification of The Distinctive Type i/*Xho*I+ Strain of Epstein-Barr Virus in Gastric Carcinoma in Peru

PAULA ORDONEZ[1†], CHIHAYA KORIYAMA[1], SHAN DING[1‡], ELENA YOSHIWARA[2],
ALEJANDRO H. CORVALAN[3], JUAN TAKANO[2], JESUS L. CHIRINOS[4], JOSE WATANABE[2],
JUAN MIYAGUI[2], HERIBERTO HIDALGO[5], PEDRO CHACON[6],
VICTOR LINARES[7], YOSHITO EIZURU[8] and SUMINORI AKIBA[1]

[1]*Department of Epidemiology and Preventive Medicine, Kagoshima University
Graduate School of Medical and Dental Sciences, Kagoshima, Japan;*
[2]*Department of Pathology, Peruano Japones Polyclinic, Lima, Peru;*
[3]*Department of Hematology-Oncology, Pontificia Universidad Catolica de Chile, Santiago, Chile;*
[4]*Academic Department of Public Health and Epidemiology, Faculty of Public Health,
Universidad Peruana Cayetano Heredia, Lima, Peru;*
[5]*Hermilio Valdizan Medrano Regional Hospital, Huanuco, Peru;*
[6]*Edgardo Rebagliati Martins Hospital, Lima, Peru;*
[7]*Chiclayo Regional Hospital, Peru;*
[8]*Division of Oncogenic and Persistent Viruses, Center for Chronic Viral Diseases,
Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan*

Abstract. *Aim: To clarify the reason for the low frequency of Epstein-Barr virus-associated gastric carcinoma (EBVaGC) in Peru, despite the high frequency reported in neighboring countries, the distribution of the distinctive EBV (type i/XhoI+) strain in EBVaGC and a healthy population was examined. Materials and Methods: EBV polymorphisms in BamHI W1/I1 and XhoI restriction site of the latent membrane protein 1 gene (LMP1) were examined among 11 EBVaGCs and 172 healthy controls from Peru, and these frequencies were compared with those in a previous study of Chile and Colombia (n=303). Results: The frequency of the distinctive EBV strain in EBVaGCs (55%) was significantly higher than that in controls (7%). Furthermore, the frequency of this EBV type in Peruvian controls was significantly lower than that in controls from Chile and Colombia (27%, p<0.001). Conclusion: The low frequency of the distinctive EBV strain among the Peruvian population might be a reason for the lower incidence of EBVaGC in Peru, as compared with neighboring countries.*

Epstein-Barr virus (EBV) is associated with lymphoid malignancies and epithelial malignancies, including some gastric carcinomas (1, 2). Previous studies have revealed that the proportion of EBV-associated gastric carcinoma (EBVaGC) was different from country to country, ranging from 2 to 17% (1, 3-10), and the EBVaGC frequency was relatively higher in the American continent than in European and Asian countries. In Latin America, a low frequency of EBVaGC was reported in Lima, Peru (4%) (9), although relatively high frequencies of EBVaGC were observed in the neighboring countries, Chile (17%) and Colombia (16%) (3, 8).

Genetic polymorphisms of prevailing EBV differ among countries. Recently, a significantly high frequency of the distinctive EBV strain type i/*Xho*I+ in EBVaGC cases from Chile and Colombia was reported in comparison with healthy populations (11). This strain is determined by polymorphisms of *Bam*HI W1/I1 boundary regions and exon 1 of the latent membrane protein 1 gene (*LMP1*). There are two polymorphisms at *Bam*HI W1/I1 boundary regions: types I and i. Type I, without a *Bam*HI restriction site, predominated among healthy carriers and EBV-associated disease in Japan and China (12, 13). On the other hand, type i, with a *Bam*HI

Table I. *List of primers used in the present study.*

		Sequence 5'-3'	Reference
*Bam*HI-W1/I1	Sense	ACCTGCTACTCTTCGGAAAC	Lung *et al*. (12, 25)
	Antisense	TCTGTCACAACCTCACTGTC	
*Xho*I site in *LMP1*	Sense	GGAACACGACCTTGAGAGG	Sandvej *et al*. (26)
	Antisense	AACAGCAGCGCCAAGAGGAG	

restriction site, prevailed in healthy donors and EBV-associated disease in Western countries (14). The polymorphism in the *Xho*I restriction site at exon 1 of the *LMP1* gene is indicated by a G→T mutation resulting in loss of an *Xho*I restriction site. These genotypes are denoted as *Xho*I+ and *Xho*I– (also known as *Xho*I kept and *Xho*I loss), respectively. The *Xho*I+ variant is frequently observed in Western countries (14), while the *Xho*I– variant is common in Asia (15).

In EBVaGC, at least five EBV genes are expressed in carcinoma cells: EBV-encoded RNAs (EBERs), Epstein-Barr nuclear antigen 1 (*EBNA1*), latent membrane protein 2 (*LMP2*), *Bam*HI A rightward transcript 0 (*BARF0*), and *BARF1* (16). Although EBV was able to immortalize human gastric primary epithelial cells (17), *LMP1*, a well-known viral oncogene in other EBV-related malignancies, was only rarely expressed in EBVaGC (18, 19). Recent studies have proposed that EBER1 (20), LMP2A (21), and BARF1 (22) play important role(s) in the carcinogenesis of the stomach. Interestingly, these potentially oncogenic viral products are encoded by neighboring regions of the *Bam*HI W1/I1 boundary region and exon 1 of the *LMP1* gene.

These findings suggest that the geographical variance of EBVaGC frequency may be explained by different distributions of EBV variants around the world. In the present study, to clarify the reason for the low frequency of EBVaGC in Peru, the prevalence of the distinct EBV strain, type i/*Xho*I+, in patients with EBVaGC and non-cancer controls in Peru was examined. In addition, frequencies of this distinctive EBV strain were compared with controls from Peru and the neighboring countries, Colombia and Chile.

Materials and Methods

Patients and samples. Paraffin-embedded tumor specimens obtained from 11 EBVaGC cases (6 males and 5 females) were used. The EBV presence was examined previously (9) using an *in situ* hybridization assay with oligonucleotide probes specific to detect EBER1 in gastric tissue, as described before (23). In 2006, throat-washing specimens were obtained from 196 non-cancer individuals, with informed consent, in Lima, Peru. Thirteen were excluded since demographic information was missing. DNA specimens of the other 183 participants were subjected to viral genotype analysis. However, 11 were excluded because of low quality and/or an insufficient amount of DNA for viral typing. Thus, the number of controls was

172, aged between 20 and more than 80 years old (64 males and 108 females). Throat-washing samples were collected by gargling with 15 ml of phosphate-buffered saline (PBS), which was then stored at –20˚C until use. The Institutional Review Boards of Kagoshima University Graduate School of Medical and Dental Sciences, the Peruano Japones Polyclinic, Hermilio Valdizan Medrano Regional Hospital, Edgardo Rebagliati Martins Hospital and Chiclayo Regional Hospital previously approved this study.

DNA extraction. Paraffin-embedded specimens were cut into 10-μm-thick slices, treated with xylene and ethanol and centrifuged at $22,000 \times g$ for 20 min. The resulting pellets were re-suspended in 100 μl of extraction buffer following a method described before (24). Throat-washing specimens were centrifuged at $16,000 \times g$ for 50 min, and the pellets re-suspended in 100 μl of extraction buffer (1 M Tris, pH 8.0, 50 mM EDTA, 0.5% Tween 20). The suspended samples were treated with 200 μg ml^{-1} of Proteinase K at 37˚C overnight and boiled at 100˚C for 10 min for Proteinase K inactivation. Samples were then subjected to phenol/chloroform extraction and ethanol precipitation. Finally, the extracted DNA was dissolved in 50 μl of TE buffer [10 mM Tris/HCl (pH 8.0) and 1 mM EDTA] and kept at –20˚C until amplification.

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The polymorphisms of *Bam*HI W1/I1 boundary region and *Xho*I restriction site at exon 1 of the *LMP1* gene were investigated as reported previously (11). In brief, a 205-bp fragment of the *Bam*HI W1/I1 boundary region was amplified using the primers reported by Lung *et al*. (25). Analysis of *Xho*I restriction site polymorphism in exon 1 of *LMP1* gene was performed with a primer set to amplify a 113-bp fragment (26). The sequences of primers are shown in Table I. The *Bam*HI W1/I1 boundary region and *Xho*I restriction site of *LMP1* were amplified by PCR using 2-5 μl of DNA template in a 25 μl reaction mixture of PCR buffer, Ampdirect Plus (Shimazu, Japan) (10 mM Tris-HCl pH 8.0, 35 mM KCl, 1.5 mM MgCl$_2$), containing 200 μM dNTPs, 1 μM of each primer and 1.25 U NovaTaq Polymerase (Shimazu, Japan). Amplification cycles for these two regions were performed as described before (11). Restriction enzyme digestion was performed in 20 μl volumes with 10 U of restriction enzyme for 1 h at 37˚C. After *Bam*HI restriction enzyme digestion, type i was determined by the presence of *Bam*HI restriction site, producing fragments of 130-bp and 75-bp length. Digestion with *Xho*I restriction enzyme resulted in 67- and 46-bp fragments for the *Xho*I+ type, and the undigested 113-bp PCR product indicated the *Xho*I– type. Whole and digested PCR products were confirmed by electrophoresis in a 3% agarose gel and by staining with 0.5 μg ml^{-1} of ethidium bromide. The B95-8 cell line, initiated by exposing marmoset blood leukocytes to EBV extracted from a human leukocyte line, served as positive control for type I and *Xho*I+ strain. A DNA

Table II. *Age, gender and Epstein-Barr virus genotype distribution in EBVaGC cases and non-cancer controls.*

	EBVaGC (N=11)		TW (N=135)		*p*-Value*
	N	%	N	%	
Gender					
Female	5	45	82	61	0.320
Male	6	55	53	39	
Age (years)					
40-49	1	9	15	11	0.114
50-59	3	27	25	19	
60-69	4	36	15	11	
70-79	2	18	65	48	
80+	1	9	15	11	
*Bam*HI W1/I1					
I	5	45	101	75	0.001
i	6	55	17	13	
ND	0	0	17	13	
*Xho*I site					
–	2	18	105	78	<0.001
+	9	82	17	13	
ND	0	0	13	10	
Combination of *Bam*HI W1/I1 & *Xho*I site					<0.001
I/–	2	18	83	61	
I/+	3	27	5	4	
i/–	0	0	8	6	
i/+	6	55	9	7	
ND	0	0	30	22	

EBVaGC: Epstein-Barr virus-associated gastric carcinoma; TW: throat washing of non-cancer controls; ND: not determined. *Obtained by χ^2 test.

Table III. *Co-segregation analysis of BamHI-I and XhoI restriction sites in LMP1 gene in EBVaGC cases and non-cancer controls.*

*Bam*HI-W1/I1	*Xho*I site	OR (95% CI)*	Adjusted OR (95% CI)[†]
I	–	1.0 (reference)	1.0 (reference)
	+	25 (3.4-185)	29 (3.3-263)
i	–	-	-
	+	28 (4.8-158)	39 (4.7-320)

OR: Odds ratio; CI: confidence interval. *OR and 95% CI calculated by logistic regression model. [†]OR and 95% CI obtained by exact method after adjusting for the effects of age and sex.

sample of EBVaGC, whose genotype was determined in the previous study, was used as control for type i and *Xho*I–.

Statistical analysis. For a case–control comparison in Peru, non-cancer controls were limited to those aged 40 or over, since all cases with EBVaGC were over 40. Furthermore, distributions of EBV polymorphisms in all Peruvian controls were compared with the healthy controls from Chile and Colombia reported in the previous study (11). The associations between EBV polymorphisms and the risk of EBVaGC were analyzed using the chi-square test and logistic regression models. Maximum likelihood estimates of odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were obtained using a logistic regression model. A *p*-value less than 0.05 was considered to be statistically significant.

Results

There was no statistically significant differences in gender and age distributions between EBVaGC cases and non-cancer controls (Table II). Polymorphisms of *Bam*HI W1/I1 region were determined in all EBVaGCs and 118 controls (87%), and the frequency of type i was significantly higher in

EBVaGC (55%) than in controls (13%). Regarding the presence of *Xho*I restriction site in *LMP1*, the polymorphism in all EBVaGC cases and 122 controls (90%) was determined. The frequency of EBV with *Xho*I+ was significantly higher in EBVaGC (82%) than in controls (13%). The sequence of the *Xho*I variants was further analyzed in all EBVaGC cases by direct sequencing of the 113-bp amplified fragment of the *Xho*I region in exon 1 of *LMP1*. All *Xho*I+ variants showed the *Xho*I restriction site by sequencing (data not shown).

In a case–control comparison of the combination of these polymorphisms, the frequency of type i/*Xho*I+ strain in EBVaGC (55%) was significantly higher than that of controls (7%). This frequency was not changed when controls under 40 were included (7%). After adjusting for the effects of age and gender distributions, the EBVaGC risk for carrying this distinct strain was statistically significant (Table III). The OR of type I/*Xho*I+ was also significantly high in reference to the EBVaGC risk of type I/*Xho*I–, which was the most frequent strain among controls.

The frequency of EBV polymorphisms in all Peruvian non-cancer controls (n=172) was compared with those from Chile and Colombia (Table IV). In this comparison, 26 controls were excluded because the quality and quantity of DNA was not guaranteed for viral typing. Thus, 303 controls from Chile and Colombia were used in the present analysis. Among Peruvian controls, the most prevalent type was type I/*Xho*I– (52%). The frequency of EBV type i/*Xho*I+ was the lowest (7%). On the other hand, the distinctive type i/*Xho*I+ (27%) was as common as other types among Chilean and Colombian controls. Note that the distribution pattern of EBV type varies across age groups, and type i/*Xho*I+ tends to prevail among younger individuals in both populations (Table V). The difference in the frequency of this distinctive type between controls from Peru (15%) and Chile/Colombia (29%) still existed when the data were limited to those younger than 50 (*p*=0.043 by Chi-square test). In contrast, EBV type I/*Xho*I– was the most frequent type among older individuals, over 50 years of age, in Peru. Although the

Table IV. *Age, gender and Epstein-Barr virus genotype distribution in non-cancer subjects from Peru, Chile and Colombia.*

	Chile & Colombia* (N=303)		Peru (N=172)		p-Value[†]
	N	%	N	%	
Gender					
Female	159	52	108	63	0.029
Male	144	48	64	37	
Age (years)					
20-29	203	67	26	15	<0.001
30-39	50	17	11	6	
40-49	32	11	15	9	
50-59	15	5	25	15	
60-69	3	1	15	9	
70-79	0	0	65	38	
80+	0	0	15	9	
*Bam*HI W1/I1					
I	85	28	116	67	<0.001
i	169	56	24	14	
ND	49	16	32	19	
*Xho*I site					
−	92	30	118	69	<0.001
+	140	46	38	22	
ND	71	23	16	9	
Combination of *Bam*HI W1/I1 & *Xho*I site					<0.001
I/−	23	8	89	52	
I/+	46	15	13	8	
i/−	60	20	12	7	
i/+	82	27	12	7	
ND	92	30	46	27	

ND: Not determined. *Reported by Corvalan *et al*. (11). [†]Obtained by χ^2 test.

number of controls over 50 years old was limited in Chile and Colombia, the EBV type I/*Xho*I− has the lowest frequency (one out of 18, 6%). After adjusting for age and gender distributions, the heterogeneity of the four combinations of EBV polymorphisms between Peru and Chile/Colombia was statistically significant (p<0.001). When non-type i/*Xho*I+ was used as a reference group, however, the difference in the frequency of the distinctive strain was not statistically significant (p=0.483).

Discussion

In the present study, genetic polymorphisms of EBV in *Bam*HI W1/I1 boundary regions and the *Xho*I restriction site at exon 1 of the *LMP1* gene were examined among 11 EBVaGC tissue specimens and 172 throat-washing samples obtained from non-cancer individuals in Peru, where the prevalance of EBVaGC is lower than in other Latin

American countries (3, 8, 9). As was reported in the previous study (11), the frequency of EBV type i/*Xho*I+ in EBVaGC cases from Colombia and Chile (55%) was higher than that of non-cancer controls (7%). More recently, a high frequency of this strain was also reported in Iranian EBVaGC (five out of nine EBVaGC), although its prevalence in the general population of Iran is unknown (10). On the other hand, this EBV type was not detected in a recent study of EBVaGC conducted in Guangzhou, Southern China. The EBV type I/*Xho*I− was the most predominant type in Chinese EBVaGC cases (75.6%) (27). In the present study, the frequency of EBV type I/*Xho*I+ in Peruvian EBVaGC (27%) was also significantly higher than that of non-cancer controls (4%). However, as the frequency of this strain in EBVaGC was very low (1.6%) in the previous study (11), and the sample size in this study is small, this result should be interpreted cautiously.

Although the findings in the present study support the hypothesis, proposed by Corvalan *et al*. (11), that the EBV type i/*Xho*I+ is related to the risk of EBVaGC, there are some discrepancies between the results of the two studies. Among Chilean and Colombian EBVaGC, most of the cases (92%; 59 out of 64 determined) harbored the distinctive EBV strain (type i/*Xho*I+). In the present study, however, only six out of nine EBVaGC cases had this type. The difference was statistically significant, even after adjusting for the effects of age and gender distributions (p=0.019). This was true in a comparison with non-cancer controls (p<0.001). The EBV type I/*Xho*I− was the most frequent type among older Peruvians, those over 50 years old, but the frequency of this type was the lowest in older Chilean and Colombian controls (Table V). The point to notice here is that there are 1 to 12-year intervals between the study periods of EBVaGC cases and controls. In the case of Peru, the intervals were 5-12 years, since the sampling periods of EBVaGC cases and throat washing specimens were 1994-2001 (9) and 2006, respectively. Since the mean age of Peruvian EBVaGC cases was 62.5 years (95% CI, 53.8-71.1 years), the corresponding background populations are approximately 70 years old. In a similar way, the intervals of sampling in Chile and Colombia were 1-8 years. In these countries, the sampling periods of EBVaGC cases and throat-washing specimens were 1993-1999 (3, 8) and 2000-2001, respectively. The distribution pattern of EBV type in individuals in their early sixties should be regarded as background populations because the mean age of EBVaGC cases in Chile and Colombia was 58.1 years (95% CI, 54.8-61.4 years). Thus, the distribution pattern of EBV type in control populations significantly differed between Peru and Chile/Colombia, even taking account of sampling intervals. This might be one of the reasons for the low EBVaGC frequency in Peru. This hypothesis should be confirmed in other cohorts with different incidence rates of EBVaGC.

Table V. *Distribution patterns of the four EBV types, determined in BamHI-I and XhoI restriction site in LMP1 genes, by age in non-cancer controls from Peru, and Chile and Colombia.*

Country	Age group (years)	N	EBV genotype (%)				
			Type I/*Xho*I–	Type I/*Xho*I+	Type i/*Xho*I–	Type i/*Xho*I+	ND
Peru	20-29	26	4 (15)	8 (31)	2 (8)	0	12 (46)
	30-49	26	5 (19)	1 (4)	4 (15)	8 (31)	8 (31)
	50-69	40	28 (70)	1 (3)	4 (10)	3 (8)	4 (10)
	70+	80	52 (65)	3 (4)	2 (3)	1 (1)	22 (28)
Chile & Colombia	20-29	203	11 (5)	31 (15)	34 (17)	73 (36)	54 (27)
	30-49	82	11 (13)	13 (16)	19 (23)	9 (11)	30 (37)
	50-69	18	1 (6)	2 (11)	7 (39)	0	8 (44)
	70+	0	-	-	-	-	-

ND: Not determined.

The observations of the present and previous studies suggest a possibility that particular EBV strains, variants in neighboring regions of *Bam*HI W1/I1 and *LMP1*, may be related to EBVaGC development, transformation ability or cytotoxic immune responses. Note that the polymorphisms examined in this study are located in regions near genes encoding potential oncogenic viral products: *LMP1, LMP2A, BARF1,* and EBERs. Although LMP1 is an important EBV oncoprotein in epithelial malignancies (28, 29), it is rarely expressed in EBVaGC (18).

An *Xho*I restriction site is also located in the intron between exons 1 and 2 of *LMP2A*. Tanaka *et al*. (30) reported that EBV strains in EBVaGC tended to have *LMP2A* gene with threonine substitution at codon 348, which corresponds to an HLA A-11-restricted cytotoxic T lymphocyte (CTL) epitope, in comparison with EBV strains in healthy individuals. This substitution may confer an advantage for viral persistence in tumor cells. LMP2A up-regulates the cellular survivin gene, through the NF-κB pathway (21), and DNA methyltransferase 1 (31), which leads to anti-apoptotic effects and an increase in methylation of the phosphatase and tensin homolog gene (*PTEN*) promoter, respectively. Recently, a possible sequence of events within the epithelial mucosa was proposed in which EBV infection is followed by expression of viral latent genes, abnormal signal pathway in host cells, DNA methylation-mediated repression of tumor suppressor genes, and the growth of the predominant clone by the interaction with other etiologic factors (32).

BARF1 is frequently expressed in EBVaGC (16, 33), and is able to immortalize primary monkey and human epithelial cells *in vitro* (34, 35). Transfection of *BARF1* into the rodent fibroblast cell line BALB/c 3T3, or into the EBV-negative B-cell line, Louckes, resulted in tumorigenic transformation (36, 37). In EBVaGC, BARF1 was reported to play an anti-apoptotic role (22), and to induce cyclin D1 overexpression (38).

EBERs are widely expressed in EBVaGC, suggesting an important role in the development and maintenance of this carcinoma. According to the previous studies, EBERs played roles in colony formation and tumorigenicity in immunodeficient mice, had anti-apoptotic effects in Burkitt lymphoma cells (39), and caused induction of an anti-inflammatory cytokine IL-10 through retinoic acid-inducible gene I-mediated IRF-3 (40). Iwakiri *et al*. (41) reported that EBER1 induced the secretion of the insulin-like growth factor 1, which plays a role as an autocrine growth factor in EBVaGC.

The associations between cancer risk and EBV polymorphisms in these regions, *Bam*HI W1/I1 and *Xho*I restriction site at exon 1 of the *LMP1* gene, were also reported in other types of EBV-related cancer. The polymorphism in the *Xho*I restriction site of *LMP1* is strongly suspected to relate to the etiology of nasopharyngeal carcinoma (42). EBV with *Xho*I loss has been found in 97-100% of Chinese nasopharyngeal carcinoma, whereas such a subtype was detected in only 30-40% of the throat-washing specimens obtained from healthy Chinese (43, 44). NK/T-cell lymphoma, an aggressive type of non-Hodgkin's lymphoma, is also associated with EBV infection, and striking geographical variations are known. Recently, Cabrera *et al*. (45) suggested an association between a recombinant EBV strain (type i/*Xho*I–) and the risk of nasal NK/T-cell lymphoma in Chile.

These findings suggest a possibility that either variant of *Bam*HI W1/I1 or *Xho*I restriction site at exon 1 of *LMP1* can increase the risk of EBV-related malignancies. In fact, an increase of the EBVaGC risk in those with EBV type I/*Xho*I+ was observed in this study (Table III). Interestingly, EBVaGC cases with either EBV variant were younger than those with the prototype of EBV, type I/*Xho*I–. For Peruvian cases, the average/median age of EBVaGC cases with type I/*Xho*I–, type I/*Xho*I+, and type i/*Xho*I+ was 68/68, 62/66, and 61/56 years, respectively. The average/median age of non-EBVaGC

cases was 65/67 years. Although this age difference was not statistically significant (*p*=0.350, non-EBVaGC *vs*. EBVaGC with either variant type), similar trends were also observed for Chilean and Colombian gastric cancer cases (data not shown). Thus, these findings suggest that EBV types with either or both variants of *Bam*HI W1/I1 and *Xho*I restriction site at exon 1 of the *LMP1* may increase the risk of early development of EBVaGC.

In a recent study conducted by Chen *et al*. (27), although the most prevalent EBV type in Chinese EBVaGC cases was type I/*Xho*I– (75.6%) non-variant EBV, they found a new hotspot mutation in the *Bam*HI W1/I1 boundary region (148,972 T C) in 86.7% of the tumors. This result suggest the implication of a different EBV strain in EBVaGC, however, it supports the involvement of EBV variants in neighboring regions of *Bam*HI W1/I1 and/or *LMP1* in carcinogenesis of EBVaGC. It is worth investigating this mutation among EBVaGC cases with neither variant of *Bam*HI W1/I1 (type i) nor *Xho*I restriction site at exon 1 of LMP1 (*Xho*I+).

Some of the individuals were examined for other EBV polymorphisms in *EBNA3C* and *Bam*HI F regions. The two major types of EBV, type 1 and 2, were determined in the sequence of *EBNA3C*. Type 1 was more prevalent in both EBVaGC (9 out of 11, 82%) and controls (57 out of 58, 98%). In the *Bam*HI F region, the prototype F and f variants were determined, and the prototype F was the major type in both EBVaGC (11 out of 11, 100%) and non-cancer controls (108 out of 113, 96%).

In conclusion, as in the previous studies of individuals in Chile and Colombia, the distinctive EBV strain, type i/*Xho*I+, was also predominant among EBVaGC cases in Peru. However, the frequency of this type among populations at risk of gastric cancer in Peru was significantly lower than that in neighboring countries, which might explain the low incidence of EBVaGC in Peru.

Acknowledgements

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