without HTLV-I infection, we could not conclude if HTLV-I carriers have more subclinical neurologic abnormalities than uninfected people do in this study. The Miyazaki Cohort Study reported a lack of evidence for a role of HTLV-I infection in the occurrence of subclinical HAM/TSP based on self-reported symptoms such as paresthesia (13). It is possible that HTLV-I–infected individuals with hyperreflexia do not complain of paresthesia. In this study, detailed clinical findings were examined by three board-certified neurologists and analyzed with laboratory findings. In the HTLV-I–infected subjects with hyperreflexia in the lower limbs, the $CD4^+$ T-cell percentage tended to decrease, the $CD8^+$ cell percentage significantly increased, and the $CD4^+$ cell/$CD8^+$ cell ratio was significantly decreased compared with those carriers with no physical examination findings. In addition, four of these seven carriers with hyperreflexia in the lower limbs had an HTLV-I provirus load of >400 copies/10,000 PBMCs (4% of PBMCs). In the Retrovirus Epidemiology Donor Study, HAM/TSP symptoms were more frequently observed in HTLV-I–infected blood donors than in donors not infected with HTLV-I (22). These results suggest that immunologic changes occur in HTLV-I–infected subjects with hyperreflexia in the lower limbs and that these symptoms are associated with HTLV-I infection rather than occur by chance.

As regards the rate of transmission of HTLV-I infection from husband to wife, it was lower in our study than in a previous study. It has been estimated that the HTLV-I infection rate from husband to wife is 60% in 10 years (23). In our study, of the 17 families in which the husband was infected with HTLV-I, only two wives were infected with HTLV-I. It is still possible that these wives had already been infected from their mothers. The mean age of the 17 husbands was 43 years, and most of them had been married for >10 years. The average age of these couples was relatively low, and it is possible that the rate of transmission increases among older couples. The Miyazaki Cohort Study reported that the rate of seroconversion among wives of HTLV-I–positive husband was higher when the age of the husband was older than 60 years (12). The presence of antibody to Tax protein was suggested to be an age-dependent risk factor for male-to-female HTLV-I transmission (24). It is possible that husbands in our study had low levels of antibody to Tax protein, although we did not test this. Another possible explanation is the era and the area of study. The previous study was done in Okinawa in 1986 (23), and our study was done in 1999–2001 in Kagoshima. There could be differences in lifestyle or behavior. We could not clearly explain this discrepancy. A larger study in this area should be done for a clearer result.

Finally, as regards the HTLV-I subgroup, we previously reported that infection with HTLV-I containing the *tax* A sequence is associated with a higher risk of HAM/TSP than infection with HTLV-I containing the *tax* B sequence (9). In that previous study, we showed that *tax* A was present in 15.6% of patients with HAM/TSP and 7% of healthy carriers (9). In this study, HTLV-I *tax* A infection was observed only in six (5.4%) of 111 subjects, and this result is consistent with our previous observation. In our previous study, we did not check the physical examination findings for healthy carriers. Here, we extended our former study to investigate whether HTLV-I–associated symptoms were more frequent in HTLV-I *tax* A–infected subjects than in HTLV-I *tax* B–infected subjects. The HTLV-I subgroup in each subject was unknown at the time of physical examination, because blood specimens were obtained after physical examination. In this study, three (50%) of six HTLV-I *tax* A–infected blood donors had HTLV-I–related symptoms (such as hyperreflexia in the lower limbs, urinary frequency in the night, and history of uveitis) in contrast to the lower prevalence of these symptoms among HTLV-I *tax* B–infected blood donors (10 [9.5%] of 105); this difference was significant (Table 3). These results suggest that the HTLV-I *tax* gene subgroup was associated with a different risk of HTLV-I–related inflammatory symptoms in HTLV-I–positive blood donors. The higher incidence of subclinical symptoms that we observed among HTLV-I *tax* A–infected people compared with HTLV-I *tax* B–infected people is consistent with the higher incidence of HAM/TSP in countries where Cosmopolitan A group HTLV-I infection is endemic, such as Colombia (25) and the United Kingdom (26). We are currently examining the difference in Tax function and in immune reaction to HTLV-I peptides between *tax* A and *tax* B sequences.

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Trends in smoking by birth cohorts born between 1900 and 1977 in Japan

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Abstract

Background. The present study aimed to elucidate the changing patterns of smoking among successive birth cohorts in Japan.

Methods. Birth-cohort-specific smoking prevalence was estimated for birth cohorts born from 1900 to 1952, using data pooled from four prospective studies (242,330 men and 274,075 women), and for birth cohorts born from 1925 to 1977, using National Nutrition Survey data.

Results. For men, two peaks were observed in smoking prevalence, in the 1925 and late-1950s birth cohorts, while a trough was observed for the 1938 birth cohort. For women, ever smoking prevalence was lowest among the 1930s birth cohorts. After the female 1940s birth cohorts, no peak was observed until the end of our observations, the 1970s birth cohorts. Although Japanese women have historically tended to start smoking at later ages, recently, smoking habits have widely expanded among females in young birth cohorts.

Conclusions. Smoking trends in Japanese men and women vary by birth cohorts. Smoking cessation should continue to be strongly promoted among men, although the younger generation has widely adopted a nonsmoking lifestyle. For women, efforts for preventing the onset of smoking, while necessary among the younger generation, should even be enhanced among middle-aged women.

Keywords: Smoking prevalence; Birth cohort; Pooled analysis; Japan

Introduction

Cigarette smoking is a major public health problem and is known to cause premature death. In Japan, estimates have indicated that among men 22% of all deaths, 25% of all cancer deaths, and 17% of all deaths from circulatory system diseases may be attributable to smoking (Hara et al., 2002), while, among women, these figures are 5%, 3%, and 11%, respectively (Hara et al., 2002).

Trends in smoking prevalence according to age group have been monitored by annually repeated cross-sectional surveys conducted by Japan Tobacco Industry, Inc., and the National

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Nutrition Survey (NNS). Although several studies in the United States and European countries (Harris et al., 1983; Burns et al., 1997; Kenun, 2001) have suggested that smoking patterns differ across birth cohorts, no previous analysis regarding smoking prevalence by birth cohort has been conducted in Japan.

In the present study, in order to clarify the changing patterns of cigarette smoking among successive Japanese birth cohorts, we analyzed baseline data from four large prospective studies. Pooling data from these four studies allowed us to estimate smoking patterns across a wide range of birth cohorts. In addition, using data from the NNS facilitated the assessment of the smoking prevalence among more recent birth cohorts. These observations provide data that are beneficial to smoking-control efforts in Japan.

Materials and methods

Study population

The present study was conducted using data pooled from three ongoing prospective studies in Japan: (1) The Three-prefecture cohort study (3-pref study), (2) The Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, sponsored by the Japanese Ministry of Education, Science, Sports and Culture (JACC study) (Ohno and Tamakoshi, 2001), and (3) The Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Disease (JPHC study) (Table 1) (Tsugane et al., 1999; Sobue et al., 2002). The JPHC study consists of JPHC-I and II, which have different baseline years. From the subjects originally enrolled in each study, we excluded those who were born before 1900, those aged less than 40 or more than 80 at baseline, those for whom smoking data were incomplete, and birth cohorts with less than 100 individuals (i.e., men born in 1903–1904 and women born in 1903 for the 3-pref study and men and women born in 1907–1908 and 1951–1952 for the JACC study). We also excluded JPHC study participants who resided in Tokyo (JPHC-I) and Osaka prefecture (JPHC-II) because different definitions of the study population were applied in these regions (Sobue et al., 2002). In addition to data from these three prospective studies, we prepared a summary table of data from a baseline survey, the Six-prefecture study (6-pref study), which is a large scale population-based prospective study started in 1965 (Hirayama, 1990). In total, data from 516,405 subjects (242,330 men and 274,075 women) were pooled in this analysis (Table 1). The present study was approved by the institutional review board of the National Cancer Center, Japan.

Smoking assessment

Smoking habits were assessed by self-administered questionnaires in the 3-pref, JACC, and JPHC studies and by interviews using a simple questionnaire in the 6-pref study. Although the style of the questions differed slightly among the studies, all studies included questions about current smoking status, age at initiation of smoking, average number of cigarettes smoked per day, and age at cessation of smoking for past smokers. Smoking habits were classified into never smoker, past smoker, and current smoker including occasionally smokers. Ever smokers were defined as current plus past smokers. Never smokers were defined as nonsmoking for the entire years from their birth to the baseline year. Former smokers were defined as smoking from their age of initiation of smoking until their age of cessation. Current smokers were defined as smoking from their age of initiation of smoking until the baseline year.

Statistical analysis

Study-specific smoking prevalence at baseline, according to sex and calendar year of birth, was analyzed for each of the four studies. Smoking prevalence according to birth cohorts from their birth year to the baseline year was reconstructed from the 3-pref, JACC, and JPHC studies. Each subject was classified as either a nonsmoker or a smoker for each calendar year from his or her birth year to the baseline year. Because the baseline year differed among the studies, we calculated smoking prevalence only until 1980. Subjects were categorized according to their birth year into 1 of 11 birth cohorts: 1900–04, 1905–09, 1910–1914, 1915–1919, 1920–1924, 1925–1929, 1930–1934, 1935–1939, 1940–1944, 1945–1949, and 1950–1954. For each birth cohort group, the annual prevalence of smoking was calculated. Subjects who did not indicate their age at initiation of smoking or age at cessation (among former smokers) were excluded from this analysis. The mean age at initiation of smoking was calculated for each birth cohort. In addition, a pooled summary estimate of smoking prevalence was calculated using a random effects model (DerSimonian and Laird, 1986). The study-specific prevalence of each birth cohort was weighted by the inverse of its variance. The heterogeneity among studies was tested using the Q-statistic (DerSimonian and Laird, 1986).

Table 1
Characteristics of the Japanese cohort studies included in the pooled analysis of smoking prevalence

Study	Baseline year	Sex	Cohort size		Birth year range	Smoking status at baseline (%)		
			At baseline	After exclusion[a]		Current	Past	Nonsmoker
Three-prefecture study	1983–1985, 1990	M	49,114	44,311	1905–1950	25,634 (57.9)	11,125 (25.1)	7552 (17.0)
		W	55,763	43,675	1904–1950	5182 (11.9)	1631 (3.7)	36,862 (84.4)
JACC study	1988–1990	M	46,465	44,057	1909–1950	23,382 (53.1)	11,649 (26.4)	9026 (20.5)
		W	64,327	55,389	1909–1950	3094 (5.6)	962 (1.7)	51,333 (92.7)
JPHC-I study	1990	M	23,571	20,569[b]	1930–1949	10,941 (53.2)	4681 (22.8)	4947 (24.1)
		W	26,646	22,403[b]	1930–1949	1284 (5.7)	393 (1.8)	20,726 (92.5)
JPHC-II study	1993–1994	M	29,780	24,574[b]	1923–1952	12,637 (51.4)	5934 (24.2)	6003 (24.4)
		W	33,412	27,214[b]	1923–1952	1644 (6.0)	296 (1.1)	25,274 (92.9)
Six-prefecture study	1965	M	121,760	108,819	1900–1926	85,889 (78.9)	4081 (3.8)	18,849 (17.3)
		W	143,310	125,394	1900–1926	15,023 (12.0)	494 (0.4)	109,877 (87.6)
Total								
Three studies[c]		M		133,511		72,594 (54.4)	33,389 (25.0)	27,528 (20.6)
		W		148,681		11,204 (7.5)	3282 (2.2)	134,195 (90.3)
All studies		M		242,330		195,953 (80.9)		46,377 (19.1)
		W		274,075		30,003 (10.9)		244,072 (89.1)

JACC study, The Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, sponsored by the Japanese Ministry of Education, Science, Sports and Culture; JPHC Study, The Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Disease.

[a] Excluded were subjects born before 1900, those aged <40 or 80+ at baseline, those for whom smoking data were incomplete, or those in a birth cohort of less than 100 persons.

[b] Further excluded were subjects residing in Tokyo for the JPHC-I study and in Osaka for the JPHC-II study because of different definitions of the study subjects.

[c] Three-prefecture study, JACC study, and JPHC study.

Because smokers had a reduced survival rate compared to nonsmokers, a major problem in the present research was the underestimation of the actual past prevalence of smoking (Harris et al., 1983; Brenner, 1993; LaVecchia et al., 1986). The correction equation for excess mortality among smokers proposed by Harris et al. (1983) is given in Eq. (1):

$$Ptt = (Ptu/Stu)/[Ptu/Stu + (1 - Ptu)/Ntu] \quad (1)$$

where *Ptt* is the contemporary prevalence of smoking after adjustment for excess mortality among smokers at age *t*. Ptu is the prevalence of smoking at age *t* among respondents alive at age *u*, where *u* is greater than or equal to *t*. Stu is the proportion of individuals regularly smoking at age *t* who survive to age *u*. Ntu is the corresponding survival probability among those not smoking at age *t*. Estimates of Stu and Ntu were derived from follow-up data collected for the 3-pref, JACC, and JPHC studies. From death rates of smokers and nonsmokers according to sex and 5-year attained age groups, we then calculated smokers' and nonsmokers' cumulative survival rates for 40–79, 45–79, 50–79, 55–79, 60–79, 65–79, 70–79, and 75–79 years (data not shown) and applied Eq. (1) to these data. Age-range correction using this procedure was limited to ages from 40 to 75.

The National Nutrition Survey (NNS)

We obtained smoking prevalence data for the years 1989 to 2001 from the NNS, which is an annual nationwide cross-sectional survey (Ministry of Health Law, 1991–2003). Based on the prevalence of current and past smokers, according to sex and 10-year age groups from 20 to 69 years of age, we calculated the prevalence of ever smokers. We assumed that the recorded prevalence represented that of an individual in the middle of the 10-year age band and plotted the prevalence as a single point on a graph. The data were smoothed using a B-spline nonparametric regression model. A cubic polynomial function was used as the basis function. The best smoothing parameter and basis function number, between 4 and 10, were identified using a cross-validation criterion (Imoto and Konishi, 1999).

Results

A consistent trend was observed in the study-specific prevalences among male ever smokers in successive birth cohorts (Fig. 1). The prevalence of ever smokers increased with subsequent cohorts born after 1900. The peak in the prevalence of ever smokers was observed among men born in the mid-1920s (approximately 90%). The prevalence of ever smokers declined, showing a trough in the late 1930s birth cohort and subsequently showed an increasing trend among the 1940s birth cohorts. Smoking was widely prevalent by 1965, the baseline year of the 6-pref study. Approximately 25–30% of men born from 1900 to 1925 had quit smoking between 1965 and 1983 to 1994.

Smoking prevalence among Japanese women was considerably lower than that of men (Fig. 2). Ever smoking prevalence was lower among the 1930s birth cohorts compared to adjacent birth cohorts; however, the observed pattern was not as consistent among the four studies as the pattern among men.

Annual smoking prevalence within successive 5-year birth cohorts were reconstructed from birth to 1980 by calculating

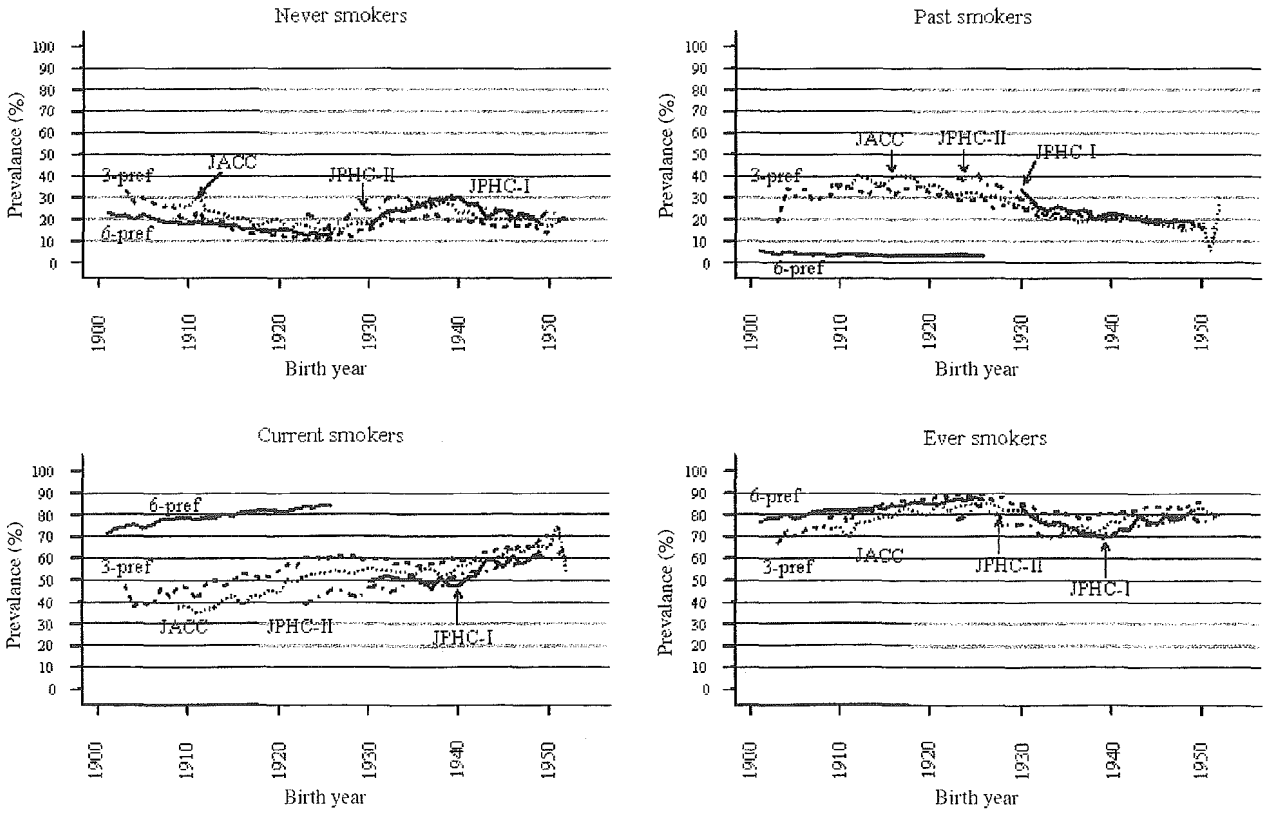

Fig. 1. Study-specific prevalence of never, past, current and ever smokers by birth cohort (1900–1952) among Japanese men. Survey years for each study were 1965 for 6-pref Study, 1983–1985 and 1990 for 3-pref Study, 1988–1990 for JACC study, 1990 for JPHC-I study, and 1993 and 1994 for JPHC-II study. JACC Study, The Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, sponsored by the Japanese Ministry of Education, Science, Sports and Culture; JPHC study, The Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Disease.

pooled summary estimates from the 3-pref, JACC, and JPHC studies. For men (Fig. 3), smoking was most prevalent among the 1920–1929 birth cohorts in the early 1950s, with a prevalence of 80% at age 30. Within the increasing trend at age 20, there was a clear dip in the 1950s among the 1930–1939 birth cohorts in comparison to the adjacent birth cohorts. The gradual steepening of the curve for younger birth cohorts indicates that more men had recently stopped smoking at an earlier age. For women, while smoking prevalence at age 20 was extremely low (less than 5% for all birth cohorts), it gradually decreased in successive birth cohorts after 1920, until increasing steeply among women born in the 1940s (Fig. 4). This finding indicates that the mean age of smoking initiation among women declined remarkably and converged with the level among men in the younger birth cohort (Fig. 5). Among individuals in the birth cohorts between 1900 and 1924, smoking prevalence continuously increased with age into their late 50s or 60s. For any age in the observed birth cohorts, smoking prevalence reached the lowest point between birth cohorts from 1925 to 1939. By 1980, a peak had not yet been observed among birth cohorts born after 1930.

After adjusting for excess mortality among smokers, the prevalence among the 1900–1904 male birth cohort reached approximately 70% (Fig. 6). Although the magnitude of the adjustment was larger for older birth cohorts and for male cohorts, the overall trends in smoking prevalence did not differ materially.

Fig. 7 shows the ever smoking prevalence estimates from the NNS in conjunction with ever smoking prevalence of study-specific and pooled summary estimates from the four prospective studies. After applying the B-spline nonparametric regression model, we obtained basis functions of 6 for men and 10 for women and smoothing parameters of $3.81 * 10^{-6}$ for men and $6.81 * 10^{-3}$ for women. For men, the pooled summary estimates showed a peak in ever smoking prevalence at around the 1925 birth cohort [87%, 95% confidence interval (CI): 84–88%] and a trough at around the 1938 birth cohort [73%, 95% CI: 68–77%]. This trough was similar to the one observed in the NNS data. After the 1938 birth cohort, smoking prevalence increased again, with a peak observed for the late 1950s birth cohorts. For women, smoking prevalence increased continuously after the 1930s birth cohorts and exceeded 20% at around the 1973 birth cohort.

Discussion

In the present study, smoking patterns among Japanese successive birth cohorts were described based on the analysis of data pooled from four prospective studies and the NNS. The new data on smoking prevalence according to birth cohorts are relevant for the development of anti-smoking measures in Japan. For men, ever smoking prevalence showed two peaks, one at around 1925 and one in the late 1950s birth cohorts. Male smoking prevalence showed a

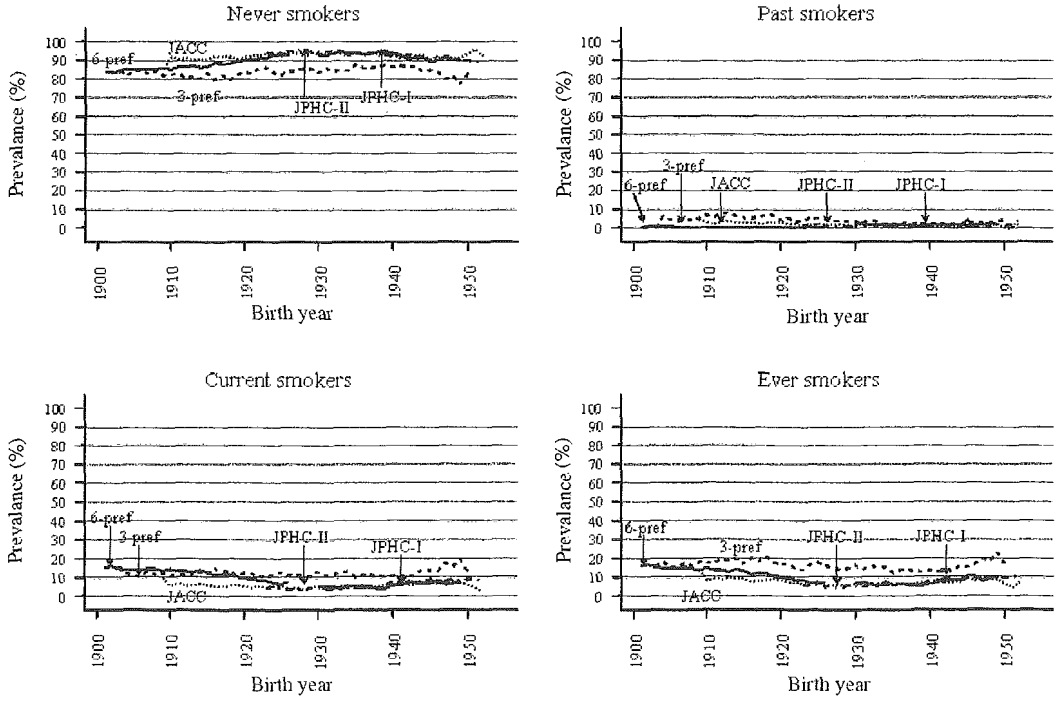

Fig. 2. Study-specific prevalence of never, past, current and ever smokers by birth cohort (1900–1952) among Japanese women. Survey years for each study were 1965 for 6-pref study, 1983–1985 and 1990 for 3-pref study, 1988–1990 for JACC study, 1990 for JPHC-I study, and 1993 and 1994 for JPHC-II study. JACC Study, The Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, sponsored by the Japanese Ministry of Education, Science, Sports and Culture; JPHC Study, The Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Disease.

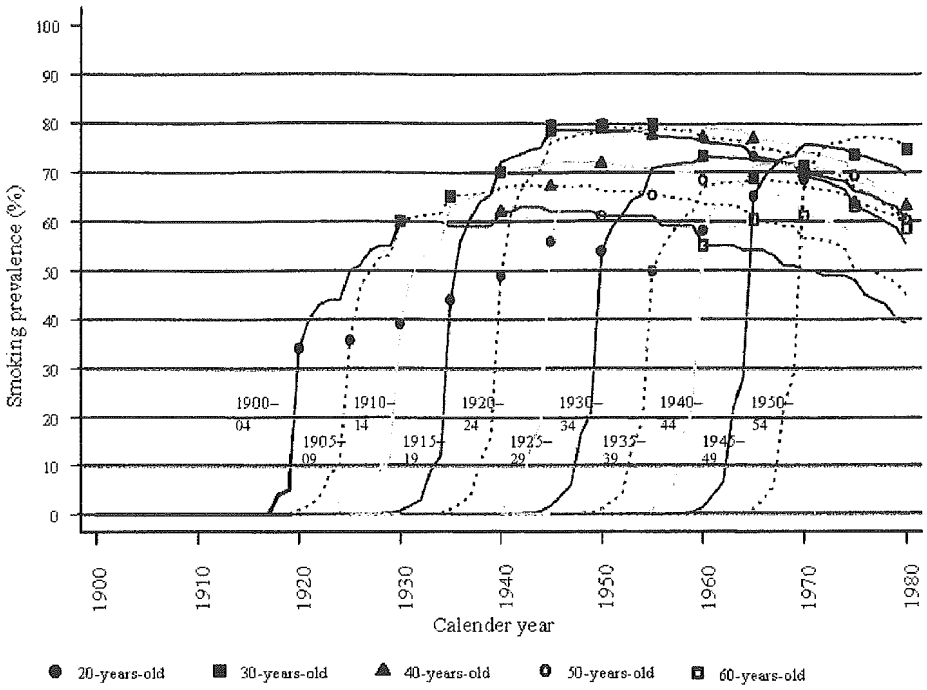

Fig. 3. Smoking prevalence by 5-year birth cohort according to calendar year among Japanese men.

trough at the 1938 birth cohort. The increase in smoking prevalence observed among males in the most recent birth cohorts, born after 1975, might be a result of random variation. Among young women, smokers were comparatively rare among those born in the first half of the 20th century. Ever smoking prevalence continuously decreased after the 1900 birth cohort and was followed by a trough among the 1930s birth cohorts. After the 1940 birth cohort, however, a steady increase in ever smoking prevalence, surpassing 20%, was observed among women. This trend had not reached a peak by the late 1970s birth cohorts. While some women tended to start smoking later in life, the mean age of smoking initiation declined remarkably among younger female generations. These observations differed from those in other developed countries. In many European countries and in North America, smoking trends among birth

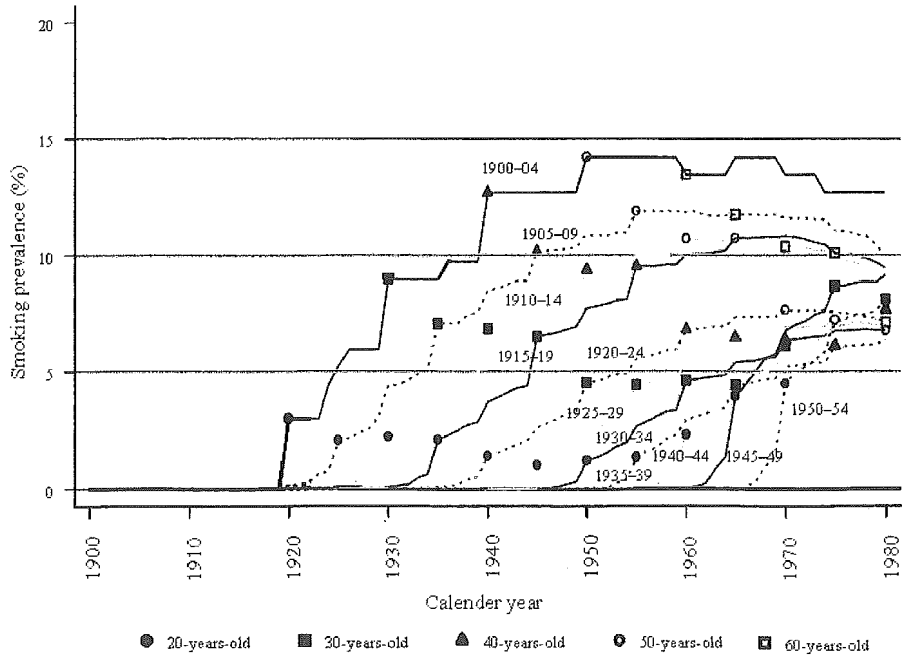

Fig. 4. Smoking prevalence by 5-year birth cohort according to calendar year among Japanese women.

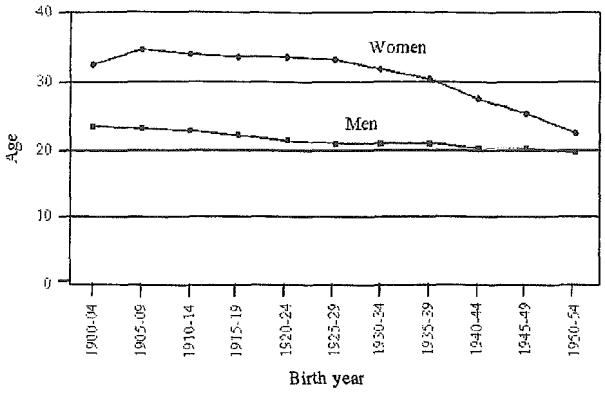

Fig. 5. Initiation of smoking by 5-year birth cohort among Japanese men and women.

cohorts showed a single peak, with the peak for men being generally 10 to 30 years earlier than the peak for women (Harris et al., 1983; Burns et al., 1997; Kemm, 2001). However, few similar studies have been conducted in Asian countries.

The period effect, as well as the birth cohort effect, appeared in smoking cessation trends. Among male smokers, more recent birth cohorts tended to quit earlier. This tendency might be important for further reducing the exposure period among Japanese men, who still have a high smoking prevalence.

As the number of subjects included in the present study was large, we believe that our observations represent the actual smoking prevalence of the Japanese population. Smoking patterns according to birth cohorts among the four prospective cohort studies, which each had a different study area and a different baseline year, were relatively consistent across all birth cohorts, especially among men. However, for women, the results were not as consistent across studies because a higher smoking prevalence was observed among participants in the 3-pref study. This difference may be a result of the fact that the 3-pref study was conducted in both urban and rural areas, while the other studies were conducted mainly in rural areas. Generally, smoking prevalence among women in urban areas tended to be higher (data not shown). However, the changing pattern of smoking prevalence across birth cohorts did not differ materially between the 3-pref and the other studies.

In the present study, we avoided any possible variation due to a small sample in a birth cohort by excluding birth cohorts of less than 100 individuals in any of the four studies. Pooling data from four large cohort studies allowed us to analyze a wide range of birth cohorts. Recall bias among smokers in the older birth cohorts was ruled out because the ever smoking prevalence of the 1900 to 1925 birth cohorts in the 6-pref study, conducted in 1965, was consistent with that in the 3-pref and JACC studies, conducted 20–25 years later. In contrast, because smoking patterns were reconstructed from a single questionnaire, recall bias for the starting and cessation dates among older individuals was likely and may have confounded inter-cohort comparisons. However, we had no method for assessing such a bias. Finally, excess mortality among smokers, in comparison to never smokers, may have resulted in underestimation of the prevalence of smoking among the older

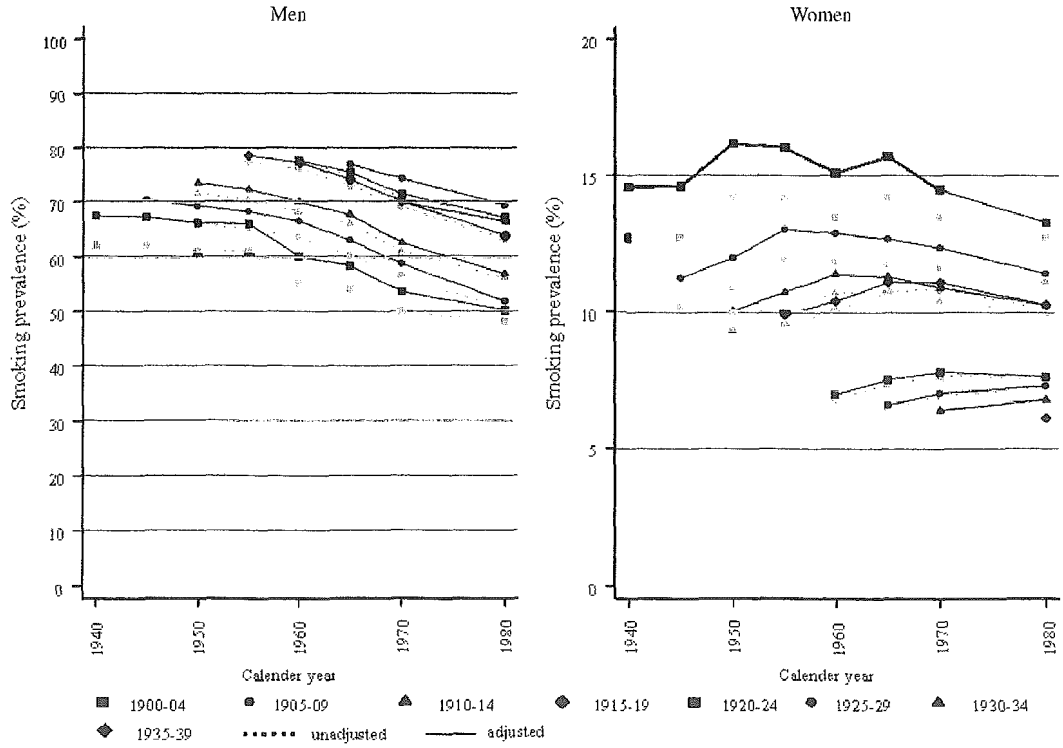

Fig. 6. Adjusted and unadjusted smoking prevalence by 5-year birth cohort according to calendar year among Japanese men and women.

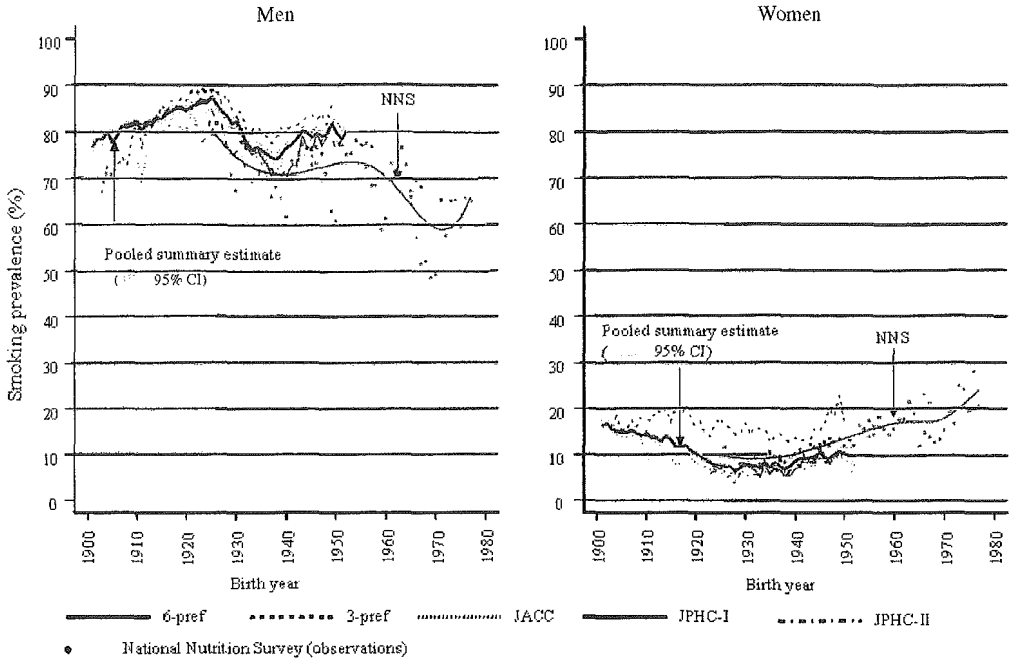

Fig. 7. Ever smoking prevalence by birth cohort among Japanese men and women, according to study-specific pooled summary estimate and 95% confidence intervals (1900–1952 birth cohorts) and the National Nutrition Survey (NNS, 1925–1977 birth cohorts). JACC study, The Japan Collaborative Cohort Study for 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 1	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.

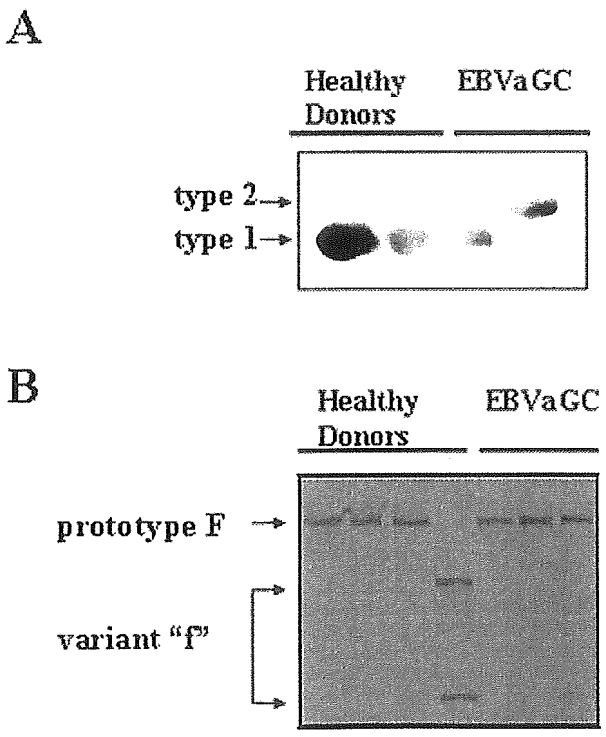

FIGURE 1 – Gentoypes of Epstein-Barr virus in Healthy donors and Epstein-Barr-associated gastric carcinomas (EBVaGC). (*a*) Southern blot analysis after PCR amplification and hybridization with specific probes for type 1 and 2 strains. (*b*) Polyacrylamide gel after PCR amplification and digestion with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

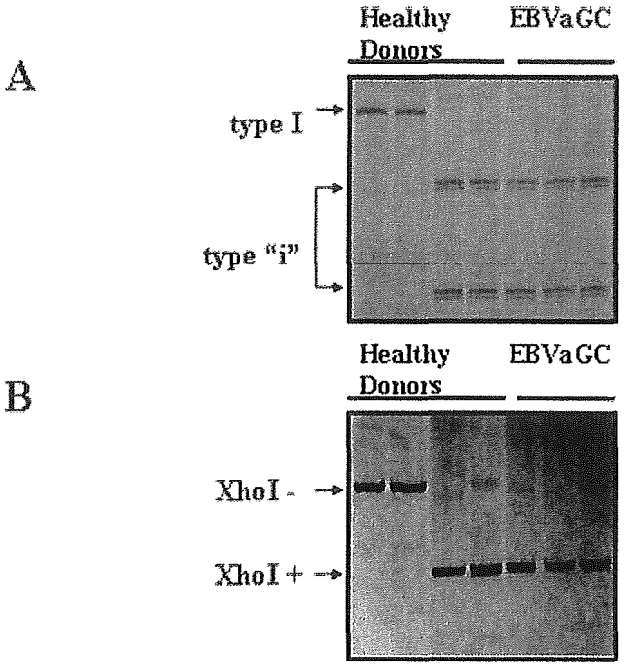

FIGURE 2 – Gentoypes of Epstein-Barr virus in Healthy donors and Epstein-Barr-associated gastric carcinomas (EBVaGC). (*a*) Polyacrylamide gel after PCR amplification and digestion with BamHI restriction enzyme at BamHI W1/I1boundary region. (*b*) Polyacrylamide gel after PCR amplification and digestion 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.

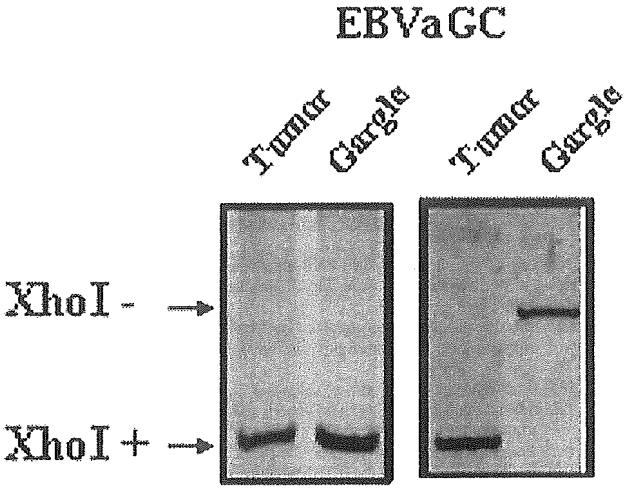

FIGURE 3 – Polyacrylamide gel after PCR amplification and digestion with XhoI restriction enzyme at XhoI site polymorphism in tumor and throat washing of Epstein-Barr-associated gastric carcinomas (EBVaGC).

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, Louckes, 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.

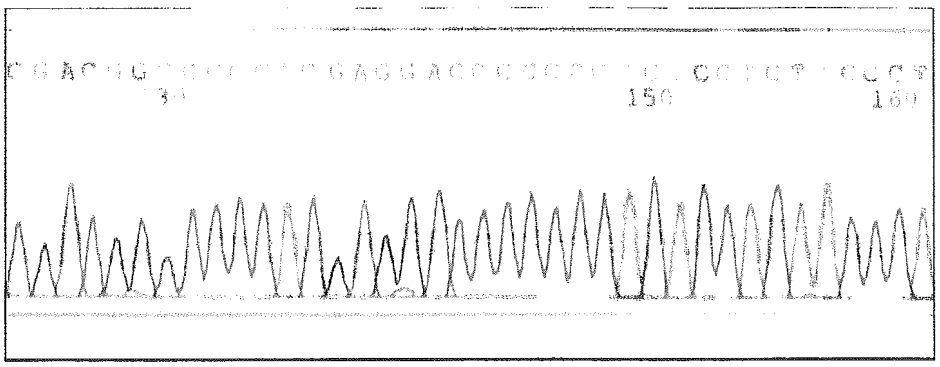

FIGURE 4.

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-