Independent Effect of EBV and Cigarette Smoking on Nasopharyngeal Carcinoma: A 20-Year Follow-Up Study on 9,622 Males without Family History in Taiwan

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Abstract

This study aimed to assess independent effects of EBV and cigarette smoking on nasopharyngeal carcinoma, which have never been assessed in long-term follow-up studies. A cohort of 9,622 men was enrolled from 1984 to 1986. Blood samples collected at study entry were tested for antibodies against EBV antigens (anti-EBV) viral capsid antigen immunoglobulin A and DNase. The cigarette smoking habit was inquired through questionnaire interview. Newly developed nasopharyngeal carcinoma cases were ascertained through computerized linkage with national cancer registry profile. Cox's proportional hazard regression analysis was used to estimate multivariate-adjusted hazard ratio with its 95% confidence interval (95% CI). During the follow-up of 173,706 person-years, 32 pathologically confirmed nasopharyngeal carcinoma cases were identified >1 year after recruitment. Increasing serum levels of anti–EBV viral capsid antigen immunoglobulin A and DNase were significantly associated with nasopharyngeal carcinoma risk in a dose-response relationship. The multivariate-adjusted hazard ratio (95% CI) of developing nasopharyngeal carcinoma for low and high antibody levels compared with seronegatives was 9.5 (2.2-40.1) and 21.4 (2.8-161.7), respectively, for anti–EBV viral capsid antigen immunoglobulin A ($P < 0.001$ for trend), and 1.6 (0.5-4.6) and 16.0 (5.4-47.1), respectively, for anti–EBV DNase ($P < 0.001$ for trend). The shorter the time interval between study entry and nasopharyngeal carcinoma diagnosis, the higher was the proportion of anti–EBV viral capsid antigen immunoglobulin A among nasopharyngeal carcinoma patients. The multivariate-adjusted hazard ratio (95% CI) was 3.0 (1.3-7.2) for ≥30 pack-years of cumulative cigarette smoking compared with <30 pack-years as the reference. The longer and heavier the cigarette smoking habit, the higher was the nasopharyngeal carcinoma risk. Anti–EBV viral capsid antigen immunoglobulin A, anti–EBV DNase, and long-term heavy cigarette smoking are independent nasopharyngeal carcinoma risk predictors. (Cancer Epidemiol Biomarkers Prev 2009;18(4):1218–26)

Introduction

Nasopharyngeal carcinoma is rare in most populations around the world with an incidence rate generally <1/100,000 person-years, but it occurs at relatively high rates in Southern China and Southeast Asia (1). The annual age-adjusted incidence rates for males and females in 2003 were 8.42/100,000 and 3.08/100,000, respectively, in Taiwan.[7]

EBV has been suggested as a major risk factor for the development of nasopharyngeal carcinoma. EBV is known to infect most adults worldwide, usually with lifelong persistence (2). Most EBV infections are asymptomatic, but the virus is associated with rare malignant transformations in lymphoid cells or epithelial tissue (3). Malignancies linked to EBV infection include nasopharyngeal carcinoma, and a variety of methods have been used to detect antibodies against EBV antigens (anti-EBV) in patients with the disease (2, 4). Numerous studies have shown diagnostic and prognostic utility of anti-EBV for nasopharyngeal carcinoma (5-8). Anti-EBVs are found frequently in sera from nasopharyngeal carcinoma patients, and they may be used as a diagnostic tool (9-12).

Most previous studies were based on cross-sectional observations, which cannot resolve the critical issue of causal temporality, that is, the appearance of serologic markers of EBV before the development of nasopharyngeal carcinoma. In our previous cohort study, participants seropositive for either one or both anti-EBV markers, anti–EBV viral capsid antigen immunoglobulin

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A and anti–EBV DNase, had an increased nasopharyngeal carcinoma risk, showing multivariate-adjusted hazard ratios [95% confidence interval (95% CI)] of 4.0 (1.6-10.2) and 32.8 (7.3-147.2), respectively, compared with the seronegatives of both markers (13). Elevated serum level of various anti-EBVs may reflect the repeated reactivation of EBV (14). Although anti-EBV seropositivity is closely associated with nasopharyngeal carcinoma, only few case-control studies have evaluated the biological gradient of nasopharyngeal carcinoma risk with increasing serum levels of anti-EBVs (15-18). The dose-response relationship between serum anti-EBV levels and nasopharyngeal carcinoma risk has never been examined through a prospective cohort study.

Although some studies implicated cigarette smoking to be a risk factor for nasopharyngeal carcinoma (17-26), others have failed to replicate this finding (27-32). Because most studies were case-control studies, the potential of recall bias in cigarette smoking duration and quantity might be one possible reason for this inconsistency. A recent cohort study conducted in Singapore reported that cigarette smoking for ≥40 years was significantly associated with nasopharyngeal carcinoma risk, showing a hazard ratio of 2.0 (95% CI, 1.2-3.3; ref. 22). Unfortunately, most previous studies did not adjust for the impact of EBV infection seromarkers.

We have examined the association between anti-EBV seromarkers and nasopharyngeal carcinoma previously (13). However, there were only 22 nasopharyngeal carcinoma cases ascertained through computerized linkage with National Cancer Registry profile up to September 30, 2000, in the previous report. Because of the small number of nasopharyngeal carcinoma cases and short follow-up period, we did not examine the dose-response relationship with nasopharyngeal carcinoma risk for anti-EBV seromarkers and cigarette smoking habit. In this further study with a 20-year follow-up period, we aimed (*a*) to assess the dose-response relationship between serum anti-EBV levels and nasopharyngeal carcinoma risk, (*b*) to compare the relative importance of anti-EBV seromarkers in the prediction of nasopharyngeal carcinoma risk during different periods after enrollment, and (*c*) to evaluate the independent effect of cigarette smoking on nasopharyngeal carcinoma after adjustment for anti-EBV seromarkers.

Materials and Methods

Cohort Recruitment. The recruitment of study cohort has been described previously (13). Briefly, the study participants were recruited between 1984 and 1986 from six townships (Kuanhsi, Hsinpu, Hengshan, Yuanshan, Chutien, and Checheng) where age-standardized mortality rates of nasopharyngeal carcinoma were highest in Taiwan (33). A total of 9,699 male residents who were 30 y of age or older participated in this study. Seven of them had been affected with nasopharyngeal carcinoma before enrollment, and 67 had nasopharyngeal carcinoma family history. There was only one nasopharyngeal carcinoma case diagnosed within 1 y after enrollment among 67 participants with a family history of nasopharyngeal carcinoma. The number of participants with family history was too small to adjust for its confounding effect in the Cox's regression model. They were thus excluded from this analysis. Each participant provided written informed consent for the personal interview based on a structured questionnaire, the collection of blood specimen for various serologic testing, including anti–viral capsid antigen immunoglobulin A and anti–EBV DNase antibodies, and the otorhinolaryngologic examination and medical consultation by physicians of the National Taiwan University Hospital. This study was approved by the institutional review board of the College of Public Health National Taiwan University.

Questionnaire Interview. All participants were personally interviewed at local research centers using a structured questionnaire at recruitment. The information obtained from questionnaire interview included sociodemographic characteristics, habits of cigarette smoking and alcohol drinking, dietary intake of various food items, personal history of sinusitis or other nasal disease, and family history of nasopharyngeal carcinoma. Duration and quantity of cigarette smoking and alcohol consumption were collected. Cigarette smoking was defined as having smoked at least 1 cigarette/d for at least 6 mos, whereas alcohol drinking habit was defined as having drunk alcohol at least 3 d/wk for at least 6 mos. The cumulative exposure to cigarette smoking was defined in pack-years by the product of multiplying the pack of cigarette smoked per day by the year of smoking cigarettes. For comparison of differences in nasopharyngeal carcinoma risk in various cigarette smoking categories, quantity and duration of cigarette smoking were categorized in to three groups (never, <1, and ≥1 pack/d for smoking quantity and never, <30, and ≥ 30 y for smoking duration). The cumulative exposure to cigarette smoking was categorized as never, <30, and ≥ 30 pack-years.

Serum Collection and Serologic Examination. Serum samples were taken at the time of enrollment and stored at −30°C on the day of collection. They were then transported to the central laboratory and stored at −80°C until assay. The sera were tested for levels of anti–EBV viral capsid antigen immunoglobulin A and anti–EBV DNase by indirect immunofluorescence assay and neutralization assay (10, 34), respectively. An anti–EBV viral capsid antigen immunoglobulin A titer <1:10 was regarded as seronegative. One unit of DNase activity was defined as the amount of enzyme required to convert 1 μg of double-stranded DNA to acid-soluble material in 10 mins at 37°C (34). Seronegativity was defined as when 1 mL serum could neutralize <2 units of DNase activity.

Ascertainment of Newly Developed Nasopharyngeal Carcinoma. Newly diagnosed nasopharyngeal carcinoma cases were ascertained through computerized linkage with the National Cancer Registry profile between January 1, 1984, and December 31, 2006. Taiwan Cancer Registry was established in 1979, with a reporting completeness >90% in 2001 and a proportion of death certification only <5% in 1999.[7] A total of 35 newly diagnosed nasopharyngeal carcinoma cases were identified. Three of them were diagnosed within 1 y after enrollment and excluded from this analysis because they were much likely to be prevalent rather than incident

Table 1. Incidence and hazard ratio (95% CI) of developing nasopharyngeal carcinoma by sociodemographic characteristics at study entry

Variable	Participant number (%)	Person-years	Nasopharyngeal carcinoma		
			Case no.	Incidence rate*	Hazard ratio (95% CI)
Age (y)					
<40	1,871 (19.5)	39,014.4	8	20.5	1.0 (reference)
40-49	2,344 (24.4)	47,090.3	8	17.0	0.8 (0.3-2.2)
50-59	2,700 (28.0)	50,048.3	8	16.0	0.8 (0.3-2.1)
≥60	2,707 (28.1)	37,552.7	8	21.3	1.1 (0.4-3.1)
Ethnicity					
Fukkienese	3,226 (33.5)	55,368.8	7	12.6	1.0 (reference)
Hakka	6,395 (66.5)	118,315.6	25	21.1	1.7 (0.7-3.9)
Missing	1	21.3	0	0	
Years of schooling					
<1	1,047 (11.1)	14,905.0	2	13.4	1.0 (reference)
1-6	5,336 (56.7)	95,176.6	17	17.9	1.3 (0.3-5.4)
≥7	3,036 (32.2)	60,046.8	11	18.3	1.3 (0.3-5.7)
Missing	203	3,577.3	2	55.9	

*Per 100,000 person-years.

case. Consequently, a total of 9,622 male participants with 32 new nasopharyngeal carcinoma cases were included in the present analysis.

Statistical Analysis. The number of person-years of follow-up for each subject was calculated from the date of enrollment to the date of the diagnosis of newly developed nasopharyngeal carcinoma, the date of death, or the date of last linkage data from the National Cancer Registry (December 31, 2006), whichever came first. Incidence rates were calculated by dividing the number of incident cases of nasopharyngeal carcinoma by the number of person-years of follow-up. Cox's proportional hazards regression analyses were used to assess the multivariate-adjusted hazard ratio of developing nasopharyngeal carcinoma with its 95% CI for anti-EBV seromarkers and cigarette smoking, independently and in combination. The associations between anti-EBV seromarkers and nasopharyngeal carcinoma risk were first analyzed with adjustment for age and cumulative exposure of cigarette smoking (<30 and ≥30 pack-years). The associations between cigarette smoking and nasopharyngeal carcinoma risk were then analyzed with adjustment for age and combination of two anti-EBV seromarkers. The modifying effects of cigarette smoking on the association between anti-EBV seromarkers and nasopharyngeal carcinoma risk were further examined through stratification analyses with adjustment for age. All models met the Cox assumption of proportionality. The statistical significance for dose-response relationships between nasopharyngeal carcinoma risk and the biological gradient of anti-EBVs was

Table 2. Incidence and adjusted hazard ratio (95% CI) of developing nasopharyngeal carcinoma by seromarkers of antibodies against EBV at study entry

Variable	Participant number (%)	Person-years	Nasopharyngeal carcinoma		
			Case no.	Incidence rate*	Hazard ratio (95% CI)†
Anti–EBV VCA IgA (titer)					
Seronegative (<1:10)	9,460 (98.9)	171,052.5	27	15.8	1.0 (reference)
1:10	87 (0.9)	1,333.9	2	149.9	9.5 (2.2-40.1)‡
≥1:40	22 (0.2)	316.3	2	632.3	21.4 (2.8-161.7)‡
Missing	53	1,002.9	1	99.7	
P value for trend					<0.0001
Anti–EBV DNase (unit)					
Seronegative (<2)	8,420 (88.0)	152,853.8	23	15.0	1.0 (reference)
2-5	1,032 (10.8)	18,000.7	4	22.2	1.6 (0.5-4.6)
≥6	116 (1.2)	1,833.6	4	218.2	16.0 (5.4-47.1)‡
Missing	54	1,017.5	1	98.3	
*P*value for trend					<0.0001
Combination of VCA IgA/DNase					
<1:10 and <2	8,357 (87.3)	151,867.2	21	13.8	1.0 (reference)
1:10 and/or 2-5	1,078 (11.3)	18,748.4	4	21.3	1.6 (0.5-4.7)
≥1:40 or ≥6	132 (1.4)	2,050.2	6	292.7	19.0 (7.0-51.5)‡
Missing	55	1,039.8	1	96.2	
P value for trend					<0.0001

Abbreviation: IgA, immunoglobulin A; VCA, viral capsid antigen.
*Per 100,000 person-years.
†Adjustment for age and cumulative exposure to cigarette smoking (pack-years).
‡$P < 0.01$.

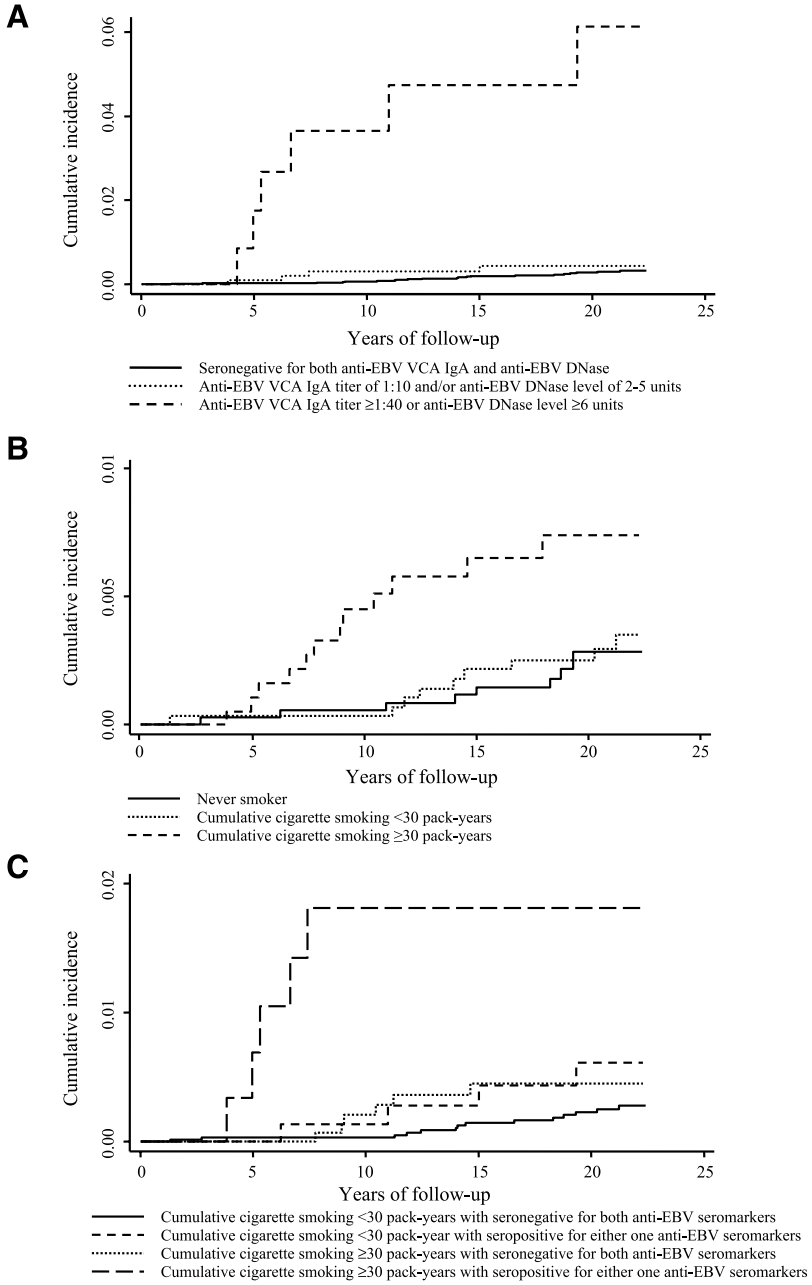

Figure 1. A. Cumulative incidence of nasopharyngeal carcinoma during follow-up among 9,622 males in Taiwan, according to the titers of anti–EBV viral capsid antigen immunoglobulin A and the levels of anti–EBV DNase at enrollment. **B.** Cumulative incidence of nasopharyngeal carcinoma during follow-up among 9,622 males in Taiwan, according to the cumulative exposure of cigarette smoking. **C.** Cumulative incidence of nasopharyngeal carcinoma during follow-up among 9,622 males in Taiwan, according to the combination of the cumulative cigarette smoking and anti-EBV seromarkers.

evaluated by the Mantel-Haenszel χ^2 test. The levels of anti-EBVs were transferred to ordinal scale (0, 1, and 2), and missing data (<4%) was simply excluded from analysis. Cumulative incidence of nasopharyngeal carcinoma by follow-up year was derived using the Nelson-Aalen method. The statistical significance of the interaction term was tested through the comparison of the likelihood of Cox's regression models with and without the interaction term. To compare the seropositive rate of anti-EBV among nasopharyngeal carcinoma patients affected in different periods of follow-up, test for trend of the seropositive rates for those who developed nasopharyngeal carcinoma during the periods of 1 to 5, 6 to 10, 11 to 15, and >15 y after enrollment was carried out. All statistical tests were two tailed.

Table 3. Incidence and adjusted hazard ratio (95% CI) of developing nasopharyngeal carcinoma by cigarette smoking habit

Variable	Participant number (%)	Person-years	Nasopharyngeal carcinoma		
			Case no.	Incidence rate*	Hazard ratio (95% CI) †
Cigarette smoking					
Never	3,316 (34.6)	61,461.9	9	14.6	1.0 (reference)
Ever	6,279 (65.4)	111,737.6	23	20.6	1.2 (0.6-2.6)
Missing	27	506.2	0	0	
Quantity of cigarette smoking (pack/d)					
Never	3,316 (35.1)	61,461.9	9	14.6	1.0 (reference)
<1	2,641 (28.0)	46,703.4	6	12.8	
≥1	3,491 (36.9)	62,473.6	17	27.2	1.7 (0.9-3.5)
Missing	174	3,066.7	0	0	
Duration of cigarette smoking (y)					
Never	3,316 (35.6)	61,461.9	9	14.6	1.0 (reference)
<30	3,008 (32.3)	59,944.4	7	11.7	
≥30	2,990 (32.1)	47,072.8	14	29.7	3.2 (1.2-8.8) ‡
Missing	308	5,226.6	2	38.3	
Cumulative exposure to cigarette smoking (pack-years)					
Never	3,316 (35.8)	61,461.9	9	14.6	1.0 (reference)
<30	3,883 (41.9)	72,923.6	9	12.3	
≥30	2,074 (22.3)	33,382.1	12	35.9	3.0 (1.3-7.2) ‡
Missing	349	5,938.0	2	33.7	

*Per 100,000 person-years.
†Adjustment for age, anti–EBV viral capsid antigen immunoglobulin A, and anti–EBV DNase.
‡$P < 0.05$.

Results

A total of 32 cases of nasopharyngeal carcinoma was diagnosed >1 year after recruitment with 173,705.7 person-years of follow-up. The mean duration of follow-up was 18.1 years with an SD of 6.0 years. The overall incidence rate was 18.4/100,000 person-years. Table 1 shows the nasopharyngeal carcinoma incidence and hazard ratio of developing nasopharyngeal carcinoma by sociodemographic characteristics at study entry. More than half of study participants were ≥50 years old at study entry. Whereas 66.5% of participants were Hakka whose ancestors migrated to Taiwan from Guangdong of China, where a high nasopharyngeal carcinoma incidence has been reported, 33.5% of participants were Fukkienese whose ancestors migrated to Taiwan from Fukkien of China, where the nasopharyngeal carcinoma incidence was reported to be lower than that in Guangdong. More than half of study participants had 1 to 6 years of schooling. No significant association with nasopharyngeal carcinoma was observed for age and years of schooling. The Hakka had a higher nasopharyngeal carcinoma incidence than the Fukkienese, but the difference was not statistically significant.

Table 2 shows the incidence rate and multivariate-adjusted hazard ratio of developing nasopharyngeal carcinoma by anti-EBV seromarkers. Serum levels of anti–EBV viral capsid antigen immunoglobulin A and anti–EBV DNase were significantly associated with the increasing risk for nasopharyngeal carcinoma, and it shows a striking dose-response relationship after adjustment for age and cumulative exposure to cigarette smoking ($P < 0.0001$ for trend). Compared with the seronegative for anti–EBV viral capsid antigen immunoglobulin A as the reference group, participants with anti–EBV viral capsid antigen immunoglobulin A titer of 1:10 and ≥1:40 had a multivariate-adjusted hazard ratio (95% CI) of 9.5 (2.2-40.1) and 21.4 (2.8-161.7), respectively. Compared with the seronegative for anti–EBV DNase as the reference group, participants with anti–EBV DNase level of 2 to 5 units and ≥6 units had a multivariate-adjusted hazard ratio (95% CI) of 1.6 (0.5-4.6) and 16.0 (5.4-47.1), respectively. In the analysis combining two anti-EBV seromarkers, there was an increasing nasopharyngeal carcinoma risk with serum levels of antibodies ($P < 0.0001$). Compared with individuals seronegative for both EBV markers, the multivariate-adjusted hazard ratio (95% CI) of developing nasopharyngeal carcinoma was 1.6 (0.5-4.7) for participants with anti–EBV viral capsid antigen immunoglobulin A titer of 1:10 and/or anti–EBV DNase level of 2 to 5 units, and 19.0 (7.0-51.5) for those anti–EBV viral capsid antigen immunoglobulin A titer of ≥1:40 or anti–EBV DNase level of ≥6 units. The cumulative incidence of nasopharyngeal carcinoma is shown in Fig. 1A for these three groups. The cumulative incidence was similar for participants seronegative for anti-EBV markers and participants with anti–EBV viral capsid antigen immunoglobulin A titer of 1:10 and/or anti–EBV DNase level of 2 to 5 units. Participants with anti–EBV viral capsid antigen immunoglobulin A titer of ≥1:40 or anti–EBV DNase level of ≥6 units had a much higher cumulative incidence of nasopharyngeal carcinoma than the other two groups. The longer the follow-up period, the greater was the difference in cumulative incidence.

We analyzed nasopharyngeal carcinoma risk in relation to environmental factors, including cigarette smoking, alcohol consumption, vegetarian habit, and consumption frequency of liver, salted food, fermented food, and fresh vegetables. Only cigarette smoking was associated with a significantly elevated risk for nasopharyngeal carcinoma. The cumulative incidence of nasopharyngeal carcinoma by cumulative cigarette smoking

Table 4. Combined and stratified analysis of nasopharyngeal carcinoma risk by seromarkers of antibodies against EBV with cigarette smoking habit

Cigarette smoking	Combination of VCA IgA/DNase	Participant number (%)	Person-years	Nasopharyngeal carcinoma			
				Case no.	Incidence rate*	Combined effect hazard ratio (95% CI)†	Stratum-specific effects hazard ratio (95% CI)†
Quantity (pack/d)							
<1	Both negative	5,212 (55.5)	95,442.2	13	13.6	1.0 (reference)	1.0 (reference)
<1	Any positive	708 (7.5)	12,005.3	2	16.7	1.2 (0.3-5.4)	1.3 (0.3-5.8)
≥1	Both negative	2,999 (31.9)	53,844.8	8	14.9	1.1 (0.5-2.6)	1.0 (reference)
≥1	Any positive	476 (5.1)	8,344.9	8	25.9	7.0 (2.9-17.0)‡	6.2 (2.3-16.7)‡
				Test for interaction: $P = 0.068$			
Duration (y)							
<30	Both negative	5,581 (60.3)	107,551.7	13	12.1	1.0 (reference)	1.0 (reference)
<30	Any positive	704 (7.6)	13,086.1	3	22.9	1.9 (0.6-6.8)	1.9 (0.6-6.8)
≥30	Both negative	2,516 (27.1)	39,920.1	7	17.5	2.5 (0.8-7.8)	1.0 (reference)
≥30	Any positive	461 (27.1)	6,941.8	6	86.4	12.5 (3.8-41.3)‡	5.0 (1.7-14.9)‡
				Test for interaction: $P = 0.263$			
Cumulative exposure (pack-years)							
<30	Both negative	6,315 (68.5)	118,635.2	14	11.8	1.0 (reference)	1.0 (reference)
<30	Any positive	840 (9.1)	14,939.4	4	26.8	2.3 (0.8-7.1)	2.3 (0.8-7.1)
≥30	Both negative	1,748 (19.0)	28,248.3	6	21.2	2.4 (0.8-6.7)	1.0 (reference)
≥30	Any positive	317 (3.4)	4,965.3	5	100.7	11.4 (3.7-35.1)‡	4.8 (1.5-15.9)‡
				Test for interaction: $P = 0.381$			

*Per 100,000 person-years.
†Adjustment for age.
‡$P < 0.01$.

exposure is shown in Fig. 1B. The cumulative incidence was similar for never-smokers and smokers with cumulative cigarette smoking exposure of <30 pack-years. Smokers with cumulative cigarette smoking exposure of ≥30 pack-years had a much higher cumulative incidence of nasopharyngeal carcinoma than the other two groups. The longer the follow-up period, the greater was the difference in cumulative incidence.

Table 3 shows the incidence rate and multivariate-adjusted hazard ratio of developing nasopharyngeal carcinoma by cigarette smoking. After adjustment for age, anti–EBV viral capsid antigen immunoglobulin A, and anti–EBV DNase, no significant association with nasopharyngeal carcinoma was observed for cigarette smoking habit (multivariate-adjusted hazard ratio, 1.2; 95% CI, 0.6-2.6) and quantity of cigarette smoking (multivariate-adjusted hazard ratio, 1.7 for ≥1 versus <1 pack daily; 95% CI, 0.9-3.5). Compared with the cigarette smoking duration of <30 years as reference group, the multivariate-adjusted hazard ratio (95% CI) was 3.2 (1.2-8.8; $P = 0.025$) for the cigarette smoking duration of ≥30 years. Compared with cumulative cigarette smoking of <30 pack-years as the reference, the multivariate-adjusted hazard ratio (95% CI) after adjustment for age was 3.0 (1.3-7.2; $P = 0.014$) for ≥30 pack-years of cumulative cigarette smoking.

We also examined the associations between anti-EBV seromarkers and other nasopharyngeal carcinoma risk factors, including age, residential township, ethnicity, history of sinusitis, family history of nasopharyngeal carcinoma, cigarette smoking, alcohol consumption, and dietary intake. Only age and cigarette smoking were consistently associated with an increased prevalence of both anti-EBV seromarkers.

Figure 1C shows the cumulative incidence of nasopharyngeal carcinoma by the combination of cumulative cigarette smoking exposure and anti-EBV seromarkers. The highest nasopharyngeal carcinoma risk was observed for anti-EBV–seropositive cigarette smokers with a cumulative cigarette smoking exposure of ≥30 pack-years compared with other three groups. The associations with nasopharyngeal carcinoma for the

Table 5. Seropositivity of anti–EBV viral capsid antigen immunoglobulin A and anti–EBV DNase at study entry for 31 newly developed cases of nasopharyngeal carcinoma stratified by period after enrollment

Years after enrollment	Total (*n*)	Anti–EBV VCA IgA		Anti–EBV DNase	
		Negative [*n* (%)]	Positive [*n* (%)]	Negative [*n* (%)]	Positive [*n* (%)]
1-5	5	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)
6-10	7	6 (85.7)	1 (14.3)	4 (57.1)	3 (42.9)
11-15	10	9 (90.0)	1 (10.0)	9 (90.0)	1 (10.0)
>15	9	9 (100.0)	0 (0.0)	7 (77.8)	2 (22.2)
P value based on trend test			$P = 0.046$		$P = 0.257$

NOTE: Anti-EBV seromarkers for one nasopharyngeal carcinoma case were not available.

combination of cigarette smoking and anti-EBV seromarkers after adjustment for age are shown in Table 4. Compared with participants seronegative for both anti-EBV seromarkers with low cigarette smoking exposure (<1 pack/d; <30 years or <30 pack-years) as the reference, the age-adjusted multivariate-adjusted hazard ratio (95% CI) was 7.0 (2.9-17.0; $P < 0.001$) for anti-EBV seropositives with cigarette smoking quantity of ≥1 pack/d, 12.5 (3.8-41.3; $P < 0.001$) for anti-EBV seronegatives with cigarette smoking duration of ≥30 years, and 11.4 (3.7-35.1; $P < 0.001$) for anti-EBV seropositives with cumulative cigarette smoking exposure of ≥30 pack-years. The nasopharyngeal carcinoma risk was highest for anti-EBV seropositives with heavy and/or long-term cigarette smoking. However, the interaction terms between cigarette smoking and anti-EBV seromarkers were not statistically significant partly because of the small number of nasopharyngeal carcinoma cases. In the stratification analyses, the nasopharyngeal carcinoma risks associated with anti-EBV seromarkers were more pronounced in heavy and/or long-term cigarette smokers than light and/or short-term smokers.

Table 5 shows the seropositivity of anti-EBV seromarkers at study entry for nasopharyngeal carcinoma cases stratified by the period after enrollment. There were 5, 7, 10, and 9 newly diagnosed nasopharyngeal carcinoma cases, respectively, during the periods of 1 to 5, 6 to 10, 11 to 15, and >15 years after enrollment. The seropositive percentage for nasopharyngeal carcinoma cases developed in 1 to 5, 6 to 10, 11 to 15, and >15 years after enrollment declined from 40.0, 14.3, and 10.0 to 0 for anti–EBV viral capsid antigen immunoglobulin A ($P = 0.046$ based on test for trend) and from 40.0, 42.9, and 10.0 to 22.2 for anti–EBV DNase ($P = 0.257$ based on test for trend). In other words, the shorter the time interval between study entry and diagnosis of newly developed nasopharyngeal carcinoma, the higher was the seropositive proportion of anti–EBV viral capsid antigen immunoglobulin A.

Discussion

This study had several strengths over previous studies evaluating the association between various risk factors and nasopharyngeal carcinoma. This study was a population-based cohort study with a long follow-up that yielded a number of newly developed cases of nasopharyngeal carcinoma after initial enrollment. All blood samples were collected and tested before the diagnosis of nasopharyngeal carcinoma. EBV and cigarette smoking have been found to be associated with nasopharyngeal carcinoma, but their independent effects have never been examined in a cohort study previously. However, there are some limitations in our study. Only 32 nasopharyngeal carcinoma cases were detected with follow-up of 173,706 person-years. Despite the small numbers due to the low incidence of nasopharyngeal carcinoma in Taiwan, we still observed that anti-EBV seromarkers were significantly associated with the increasing risk for nasopharyngeal carcinoma, which is consistent with findings of previous case-control studies. Because only the data at one time point were available for both EBV serology and cigarette smoking, it was not possible to analyze fluctuations in anti-EBV seromarkers and cigarette smoking over time. If there were changes in anti-EBV serology and/or cigarette smoking habit after study entry, the relative risk for nasopharyngeal carcinoma associated with anti-EBV serology and cigarette smoking would have been underestimated. In other words, the relative risks observed in this study were conservative estimates.

The seropositivity of anti–EBV viral capsid antigen immunoglobulin A and anti–EBV DNase has been documented as a strong risk predictor of nasopharyngeal carcinoma (13). Although the biological gradient of nasopharyngeal carcinoma risk with the increasing serum level of anti-EBVs has been reported in previous case-control studies (15-18), the dose-response relationship between serum levels of anti-EBVs and nasopharyngeal carcinoma risk has never been examined in any cohort study. Findings of this study provide clear evidence for the significant association between nasopharyngeal carcinoma risk and two anti-EBV seromarkers in a dose-response relationship. EBV viral capsid antigen and DNase antigens are expressed in the lytic phase of EBV infection (4). Elevated serum levels of antibodies against these two antigens may reflect the reactivation of EBV in humans (14). The nasopharyngeal carcinoma risk was significantly associated with anti–EBV viral capsid antigen immunoglobulin A at low and high serum levels but with anti–EBV DNase only at high level. The seropositive proportion of anti-EBV, especially anti–EBV viral capsid antigen immunoglobulin A, among newly developing nasopharyngeal carcinoma cases decreased with the time interval between study entry and diagnosis of newly developed nasopharyngeal carcinoma. These findings suggest that EBV serology plays a more important role for the prediction of nasopharyngeal carcinoma that occurred during the short follow-up period in comparison with that that occurred long after enrollment.

In several prognosis studies, the anti-EBVs declined significantly after nasopharyngeal carcinoma treatment and elevated again when nasopharyngeal carcinoma relapsed (5-8). In our previous report from this cohort study, the hazard ratios associated seropositivity of anti–EBV viral capsid antigen immunoglobulin A and anti–EBV DNase were higher for the nasopharyngeal carcinoma developed <5 years than that developed ≥5 years after recruitment (13). Ji et al. (12) also observed that the seropositive proportion of anti–EBV viral capsid antigen immunoglobulin A among nasopharyngeal carcinoma cases is higher at the time of diagnosis than those before diagnosis. In this analysis based on a longer follow-up period, the significantly decreasing seropositive proportion of anti–EBV viral capsid antigen immunoglobulin A among nasopharyngeal carcinoma patients with increasing follow-up time suggests anti–EBV viral capsid antigen immunoglobulin A may be a short-term risk predictors of nasopharyngeal carcinoma. It also implies that EBV may play a more important role in the late rather than early stage of nasopharyngeal carcinogenesis.

The mechanisms of the EBV-induced carcinogenesis of nasopharynx remain to be elucidated. EBV may induce related malignancy through the immortalization of target cells by latent genes. Some *EBV* latent genes, including

EBER and *EBNA1*, are consistently expressed in the nasopharyngeal carcinoma tumor. The latent membrane protein 2A can be detected in 50% of nasopharyngeal carcinoma and may have growth-promoting effects in epithelial cells. The latent membrane protein 1 is more difficult to detect, with only 30% of nasopharyngeal carcinoma, and considered the EBV oncogene. The latent infection may play an important role in the development of nasopharyngeal carcinoma (35-37). Genomic instability contributes to the development of human cancers, and molecular genetic studies have revealed genetic instability in nasopharyngeal carcinoma tissues (38, 39).

In our preliminary studies (40), EBV-harboring nasopharyngeal carcinoma cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate and sodium *n*-butyrate to induce the lytic phase of EBV. There was an increase in the formation of micronuclei and the accumulation of DNA strand breaks in the treated cells. In the repeatedly reactivated NA cells, there was also a profound increase in the carcinogenesis by invasiveness and tumorigenesis assays, as well as the genomic instability by comparative genomic hybridization. Furthermore, the expression of EBV DNase also increased the microsatellite instability and frequency of gene mutation in human epithelial cells. EBV reactivation seems to induce genomic instability through DNA damage and contribute subsequently to the tumorigenesis. In our previous case-control study on genetic susceptibility genes of nasopharyngeal carcinoma, significant associations were observed for polymorphisms of two DNA repair genes, *hOGG1* and *XRCC1* (41). EBV replication has been shown in oropharyngeal epithelial cells, suggesting that reactivation of EBV may occur after primary infection and possibly in human epithelial cells (42-44). It is conceivable that EBV reactivation reflected by the elevated serum anti-EBV levels may contribute to the development of nasopharyngeal carcinoma.

In this study, there was a significant association between long-term cigarette smoking and nasopharyngeal carcinoma. Our finding corroborates with results from most case-control studies and cohort studies (17-26). Furthermore, this association persisted even when EBV seromarkers were included in the multivariate analysis. Although some studies failed to detect such an association (27-32), the inconsistency may result from the recall bias from the retrospective studies, the difference in definition of long-term cigarette smoking, and pathologic types of nasopharyngeal carcinoma, as well as the control of confounding risk factors such as anti-EBV seromarkers, or not.

The interaction between anti-EBV seromarkers and cigarette smoking has been reported by Lin et al. (45). The highest nasopharyngeal carcinoma risk was observed in heavy smokers (quantity of cigarette smoking, ≥10 cigarettes/d) who were seropositive for anti-EBV seromarkers, but the interaction was not statistically significant. Similar finding was found in this study. In this study, long-term heavy cigarette smokers (cumulative cigarette smoking exposure, ≥30 pack-years) had a much higher anti-EBV seropositivity (15.4%) than never smokers (11.5%) and short-term light smokers (10.9%). Despite of the small number of newly developed nasopharyngeal carcinoma cases in this study, there is a hint on the importance of EBV–cigarette smoking interaction in the multifactorial etiology of nasopharyngeal carcinoma.

The nasopharynx is a site directly exposed to tobacco smoke from active cigarette smoking. Tobacco smoke is a complex mixture of >4,000 compounds, many of which are mutagenic or carcinogenic, including nitrosamine (46). Nitrosamine from dietary intake in childhood has been documented to be associated with an increased risk for nasopharyngeal carcinoma (47, 48). CYP2E1 is involved in the metabolic activation of nitrosamine, and the genetic polymorphism of CYP2E1 was documented to be associated with the risk for nasopharyngeal carcinoma (49). The etiologic link between cigarette smoking and nasopharyngeal carcinoma risk seems biologically plausible.

Because only long-duration exposure to tobacco smoke and dietary intake of nitrosamine in childhood are associated with the development of nasopharyngeal carcinoma, these chemical carcinogens may be considered as the long-term risk predictors of nasopharyngeal carcinoma. It is hypothesized that nasopharyngeal carcinogenesis may be initiated by the exposure to chemical carcinogens in childhood and early adulthood. Although the etiologic mechanism for EBV to induce nasopharyngeal carcinoma remains to be further elucidated, anti-EBV seromarkers may still be used as useful biomarkers for the risk prediction of nasopharyngeal carcinoma, especially among those who have the early exposure to chemical carcinogens, the family history of nasopharyngeal carcinoma, or the susceptible genotypes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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