# SEROLOGIC MARKERS OF EPSTEIN–BARR VIRUS INFECTION AND NASOPHARYNGEAL CARCINOMA IN TAIWANESE MEN

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## ABSTRACT

***Background*** It is probable but unproven that Epstein–Barr virus (EBV) has a role in nasopharyngeal carcinoma. We determined whether antibodies against EBV are present before the development of nasopharyngeal carcinoma.

***Methods*** A total of 9699 men were enrolled between 1984 and 1986. Blood samples were examined for IgA antibodies against EBV capsid antigen and neutralizing antibodies against EBV-specific DNase. During 131,981 person-years of follow-up, 22 pathologically confirmed new cases of nasopharyngeal carcinoma that were diagnosed more than one year after recruitment were ascertained through linkage with the National Cancer Registry of Taiwan.

***Results*** The cumulative risk of nasopharyngeal carcinoma per 100,000 person-years was 11.2 for subjects who tested positive for neither serologic marker, 45.0 for those who had one marker, and 371.0 for those who had both markers. After adjustment for age and the presence or absence of a family history of nasopharyngeal carcinoma, the relative risk of nasopharyngeal carcinoma was 32.8 for subjects with both markers (95 percent confidence interval, 7.3 to 147.2; $P<0.001$) and 4.0 for subjects with one marker (95 percent confidence interval, 1.6 to 10.2; $P=0.003$), as compared with subjects with neither marker. The longer the duration of follow-up, the greater the difference in the cumulative incidence of nasopharyngeal carcinoma between seropositive and seronegative subjects.

***Conclusions*** IgA antibodies against EBV capsid antigen and neutralizing antibodies against EBV DNase are predictive of nasopharyngeal carcinoma. (N Engl J Med 2001;345:1877-82.)



NASOPHARYNGEAL carcinoma is rare in most populations around the world but common in southern China and Southeast Asia.[1] It has been suggested that Epstein–Barr virus (EBV) infection may be a major risk factor for nasopharyngeal carcinoma, and a variety of methods have been used to detect antibodies against EBV antigens in patients with this disease.[2] In 1966, Old et al. found elevated levels of antibodies against EBV in patients with nasopharyngeal carcinoma,[3] and subsequently, titers of IgG antibodies against EBV capsid antigen, early antigen, and soluble complement-fixing antigen were shown to increase with progressive clinical stages of nasopharyngeal carcinoma.[4,5] IgA antibodies against EBV capsid antigen and early antigen were later found to be characteristic of the disease.[6,7]

Most residents of Taiwan are first infected with EBV in early childhood, and IgG antibodies against EBV capsid antigen persist for at least decades and probably for life.[8] IgG antibodies are serologic markers of past infection and are not specific for nasopharyngeal carcinoma, whereas the presence of IgA antibodies against EBV capsid antigen reflects frequent reactivation of latent EBV in B cells, repeated viral infection, or both. These IgA antibodies and the neutralizing antibodies against EBV DNase are highly specific markers for nasopharyngeal carcinoma.[9,10]

There are uncertainties regarding the direct carcinogenic effect of EBV, and it remains unclear at which stage in the pathogenesis of nasopharyngeal carcinoma the virus has a role.[1,11,12] Most previous studies were based on cross-sectional observations, which did not resolve the critical issue of whether serologic markers of EBV become detectable before or after the development of nasopharyngeal carcinoma. Previous studies had a short follow-up period or a small number of new cases of nasopharyngeal carcinoma.[13-17] Here we report the results of a 16-year follow-up study of the relation between the incidence of nasopharyngeal carcinoma and seropositivity for IgA antibodies against EBV capsid antigen, anti-EBV DNase antibodies, or both.

## METHODS

### Study Subjects

The study subjects were recruited between 1984 and 1986 from the six townships in Taiwan with the highest age-standardized rates of death due to nasopharyngeal carcinoma.[18] We invited all male residents of the six townships who were 30 years of age or older to participate in our study. For each participant, we provided free services, including serologic testing for IgA antibodies against EBV capsid antigen, anti-EBV DNase antibodies, and chronic hepatitis B virus infection, as well as otorhinolaryngologic examinations and medical consultations by physicians of the National Taiwan University Hospital. No monetary compensation was offered to participants. Since the serologic survey was being conducted for the first time in the townships participating in the study, none of the residents knew their serologic status before recruitment. A total of 9699 residents were enrolled after giving written informed consent.

All study subjects — of whom there were 1521 to 1710 men

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per township — were informed about the details of the study and interviewed at home by public health nurses from six local health centers. A standardized personal interview, based on a structured questionnaire, was used to collect information on sociodemographic characteristics, cigarette smoking, alcohol consumption, betel-nut chewing, diet, personal history of sinusitis or other nasal diseases, and family history of nasopharyngeal carcinoma. Blood samples were collected from each study subject at the time of enrollment. All serum samples were immediately stored at −30°C. They were later tested for IgA antibodies against EBV capsid antigen with the use of an indirect immunofluorescence assay and for anti-EBV DNase antibodies with the use of antigen prepared from iododeoxyuridine-treated P3HR-1 cells (EBV-positive lymphoblastoid cell lines derived from a patient with Burkitt's lymphoma) and a neutralization assay, as described previously.[10]

A total of 1176 study subjects tested positive for one or both serologic markers of EBV at the time of enrollment. They were told the results of these tests and referred to the special nasopharyngeal carcinoma clinic at the National Taiwan University Hospital, where they underwent fiberoptic endoscopy that was performed by otorhinolaryngologists. We identified seven subjects with cases that were in remission who had received a diagnosis of nasopharyngeal carcinoma before enrollment; four subjects had new cases diagnosed within one year after enrollment.[10] These 11 subjects were excluded from further analyses, leaving a total of 9688.

## Follow-up

We identified newly diagnosed cases of nasopharyngeal carcinoma by linkage with computerized profiles in the National Cancer Registry in Taiwan, which has a coverage rate of more than 90 percent; 95 percent of the registered cases of nasopharyngeal carcinoma were confirmed pathologically. To check the completeness of the ascertainment, we also reviewed the National Death Certification profiles but did not identify any cases that had not been recorded in the National Cancer Registry. The follow-up of newly diagnosed cases of nasopharyngeal carcinoma was considered complete and accurate and was similar regardless of whether the subject was seropositive or seronegative.

Only pathologically confirmed, newly diagnosed cases of nasopharyngeal carcinoma were included in the analysis. All but one medical chart of the 22 subjects with nasopharyngeal carcinoma that was diagnosed more than one year after enrollment were reviewed. One medical chart was not available because the subject had died. The pathological findings for that subject were available in the computerized profiles in the National Cancer Registry.

**TABLE 1.** PREVALENCE, ACCORDING TO RISK FACTORS, OF ANTIBODIES AGAINST EBV CAPSID ANTIGEN IgA AND ANTI-EBV DNASE ANTIBODIES IN A COHORT OF 9699 ADULT MALE RESIDENTS OF SIX AREAS OF TAIWAN WITH HIGH INCIDENCE OF NASOPHARYNGEAL CARCINOMA.*

| VARIABLE | ALL SUBJECTS | SUBJECTS WITH POSITIVE TESTS | | |
|---|---|---|---|---|
| | | IgA ANTIBODIES AGAINST EBV CAPSID ANTIGEN | ANTI-EBV DNASE ANTIBODIES | BOTH MARKERS |
| Age at enrollment — no. (%) | | | | |
| <40 yr | 1893 (19.5) | 14 (0.7) | 182 (9.6) | 191 (10.1) |
| 40–49 yr | 2362 (24.4) | 12 (0.5) | 233 (9.9) | 241 (10.2) |
| 50–59 yr | 2722 (28.1) | 38 (1.4) | 360 (13.2) | 383 (14.1) |
| ≥60 yr | 2722 (28.1) | 50 (1.8) | 386 (14.2) | 416 (15.3) |
| P value | | <0.001 | <0.001 | <0.001 |
| Township — no. (%) | | | | |
| Yuanshan | 1694 (17.5) | 26 (1.5) | 191 (11.3) | 209 (12.3) |
| Hsinpu | 1710 (17.6) | 29 (1.7) | 154 (9.0) | 173 (10.1) |
| Kuanhsi | 1525 (15.7) | 14 (0.9) | 196 (12.9) | 204 (13.4) |
| Hengshan | 1521 (15.7) | 14 (0.9) | 195 (12.8) | 203 (13.3) |
| Chutien | 1693 (17.5) | 15 (0.9) | 219 (12.9) | 230 (13.6) |
| Checheng | 1556 (16.0) | 16 (1.0) | 206 (13.2) | 212 (13.6) |
| P value | | 0.11 | <0.001 | 0.008 |
| Ethnic group — no. (%) | | | | |
| Fukkienese | 3248 (33.5) | 42 (1.3) | 398 (12.3) | 422 (13.0) |
| Hakka | 6450 (66.5) | 72 (1.1) | 763 (11.8) | 809 (12.5) |
| P value | | 0.47 | 0.59 | 0.56 |
| Personal history of sinusitis — no. (%) | | | | |
| Yes | 419 (4.3) | 7 (1.7) | 53 (12.6) | 55 (13.1) |
| No | 9203 (94.9) | 107 (1.2) | 1102 (12.0) | 1170 (12.7) |
| P value | | 0.35 | 0.70 | 0.82 |
| Family history of nasopharyngeal carcinoma — no. (%) | | | | |
| Yes | 68 (0.7) | 2 (2.9) | 10 (14.7) | 12 (17.6) |
| No | 9544 (98.4) | 111 (1.2) | 1137 (11.9) | 1204 (12.6) |
| P value | | 0.17 | 0.46 | 0.22 |

*Data on ethnic background were missing for 1 subject, data on personal history of sinusitis were missing for 77 subjects, and data on family history of nasopharyngeal carcinoma were missing for 87 subjects. For each test category, P values are for the comparison among subgroups for each risk factor, with the use of the chi-square test. EBV denotes Epstein–Barr virus.

### Statistical Analysis

We used the chi-square test to determine the statistical significance of the differences in prevalence of the two serologic markers among different groups defined according to age, township, ethnic background, presence or absence of a personal history of sinusitis, and presence or absence of a family history of nasopharyngeal carcinoma. The duration of follow-up for each subject was calculated from the date of recruitment to the date of the diagnosis of newly developed nasopharyngeal carcinoma, the date of death, or the last date for which linked data were available from the National Cancer Registry (September 30, 2000), whichever came first.

We used the life-table method to derive the cumulative incidence of nasopharyngeal carcinoma for each year of follow-up among subjects who were seropositive at the time of enrollment and among those who were seronegative at the time of enrollment. We used Cox proportional-hazard models to analyze the risk of nasopharyngeal carcinoma associated with the two serologic markers and other factors. We used relative risks to assess the magnitude of the associations between the risk factors and nasopharyngeal carcinoma, and we calculated the 95 percent confidence intervals of the relative risks. All statistical tests were two-tailed.

Titers of IgA antibodies against EBV capsid antigen and anti-EBV DNase antibodies were dichotomized into binary variables (positive or negative). A titer of less than 1:10 was considered to be negative for the IgA antibodies. The level of anti-EBV DNase antibodies was expressed in terms of the units of DNase activity neutralized by 1 ml of serum. A serum sample neutralizing less than 2 units of DNase activity was considered negative. In the multivariate Cox regression analysis, age was controlled for as a continuous variable in order to ensure tight control of its possibly confounding effect.

## RESULTS

Table 1 shows the prevalence of IgA antibodies against EBV capsid antigen and anti-EBV DNase antibodies at the time of enrollment, according to age, township, ethnic background, presence or absence of a personal history of sinusitis, and presence or absence of a family history of nasopharyngeal carcinoma. The overall prevalence was 12 percent for anti-EBV DNase antibodies and 1.2 percent for IgA antibodies against EBV capsid antigen. The prevalence of IgA antibodies against EBV capsid antigen, of anti-EBV DNase antibodies, and of both types of antibodies was highest among subjects older than 50 years of age ($P<0.001$ for each comparison). The prevalence of the two se-

**TABLE 2.** CHARACTERISTICS OF 22 SUBJECTS WITH NASOPHARYNGEAL CARCINOMA DIAGNOSED MORE THAN ONE YEAR AFTER RECRUITMENT.*

| SUBJECT NO. | DATE OF RECRUITMENT | DATE OF DIAGNOSIS | IgA ANTIBODIES AGAINST EBV CAPSID ANTIGEN AT RECRUITMENT | ANTI-EBV DNASE ANTIBODIES AT RECRUITMENT | AGE AT DIAGNOSIS | HISTOLOGIC FEATURES | WHO TYPE† |
|---|---|---|---|---|---|---|---|
| | | | | | yr | | |
| 1 | 9/84 | 1/86 | − | − | 45 | Unclassified tumor | NA |
| 2 | 9/84 | 1/90 | + | − | 59 | Nonkeratinizing carcinoma | II |
| 3 | 9/84 | 12/90 | − | + | 59 | Squamous-cell carcinoma | NA |
| 4 | 9/84 | 3/95 | − | − | 70 | Keratinizing carcinoma | I |
| 5 | 9/84 | 9/98 | − | − | 62 | Undifferentiated carcinoma | III |
| 6 | 10/84 | 8/88 | + | + | 71 | Epithelial tumor | II |
| 7 | 10/84 | 7/92 | − | − | 63 | Unclassified tumor | NA |
| 8 | 10/84 | 10/95 | + | + | 82 | Epithelial tumor | NA |
| 9 | 10/84 | 3/97 | − | − | 63 | Undifferentiated carcinoma | III |
| 10 | 11/84 | 3/96 | − | − | 48 | Nonkeratinizing carcinoma | II |
| 11 | 12/84 | 3/89 | + | − | 75 | Squamous-cell carcinoma | NA |
| 12 | 12/84 | 5/92 | − | + | 69 | Nonkeratinizing carcinoma | II |
| 13 | 12/84 | 3/96 | − | − | 75 | Nonkeratinizing carcinoma | II |
| 14 | 12/84 | 12/99 | − | + | 51 | Nonkeratinizing carcinoma | III |
| 15 | 9/85 | 12/86 | − | + | 54 | Undifferentiated carcinoma | III |
| 16 | 9/85 | 5/92 | − | + | 67 | Nonkeratinizing carcinoma | II |
| 17 | 9/85 | 8/94 | − | − | 62 | Undifferentiated carcinoma | III |
| 18 | 9/85 | 9/94 | − | − | 63 | Nonkeratinizing carcinoma | II |
| 19 | 9/85 | 7/97 | − | − | 47 | Nonkeratinizing carcinoma | II |
| 20 | 9/85 | 10/99 | − | − | 50 | Squamous-cell carcinoma | III |
| 21 | 9/85 | 3/00 | − | − | 46 | Epithelial tumor | III |
| 22 | 1/86 | 10/88 | − | − | 47 | Unclassified tumor | III |

*Unclassified tumors were malignant tumors for which there was no detailed description of histologic characteristics. Plus signs indicate the presence of antibodies, and minus signs their absence. EBV denotes Epstein–Barr virus.

†World Health Organization (WHO) type I denotes keratinizing squamous-cell carcinoma, type II differentiated nonkeratinizing carcinoma, type III undifferentiated carcinoma, and NA not available.

rologic markers was similar regardless of subjects' history of sinusitis, and the differences between subjects with a family history of nasopharyngeal carcinoma and those without such a family history were not statistically significant.

A total of 22 cases of nasopharyngeal carcinoma were newly diagnosed more than one year after enrollment, during a follow-up period of 131,981 person-years, for an incidence of 16.7 per 100,000 person-years. Table 2 shows the dates of enrollment and diagnosis, serologic status, age at the time of diagnosis, and pathological type of nasopharyngeal carcinoma in the subjects with these 22 cases. The mean age at diagnosis for seropositive and seronegative patients was 62 and 57 years, respectively ($P=0.23$). The cumulative incidence of nasopharyngeal carcinoma per 100,000 person-years was 11.2 among subjects who had no serologic markers of EBV, 45.0 among those who had one marker, and 371.0 among those who had both markers (Table 3). The relative risk of nasopharyngeal carcinoma after adjustment for age and the presence or absence of a family history of nasopharyngeal carcinoma was 22.0 (95 percent confidence interval, 7.3 to 66.9; $P<0.001$) among subjects who tested positive for IgA antibodies against EBV capsid antigen, as compared with those who tested negative (Table 4). The multivariate-adjusted relative risk was 3.5 (95 percent confidence interval, 1.4 to 8.7; $P=0.006$) among subjects who were positive for anti-EBV DNase antibodies, as compared with those who tested negative for these antibodies, 32.8 (95 percent confidence interval, 7.3 to 147.2; $P<0.001$) among those who had both markers, as compared with those who had neither marker, and 4.0 (95 percent confidence interval, 1.6 to 10.2; $P=0.003$) among those who had one marker as compared with those who had neither marker. The multivariate-adjusted relative risk remained statistically significant in further analysis of the data with stratification according to the date of the diagnosis. Five cases were newly diagnosed between one and five years after recruitment, and 17 were found more than five years after recruitment. Among the subjects whose cases were diagnosed between one and five years after recruitment, the multivariate-adjusted relative risk was 85.3 (95 percent confidence interval, 7.4 to 978.4; $P<0.001$) for those who had both serologic markers, as compared with those who had neither marker. The corresponding figure for the subjects whose cases were diagnosed more than five years after recruitment was 20.7 (95 percent confidence interval, 2.6 to 162.0; $P=0.004$).

The cumulative incidence of nasopharyngeal carcinoma for each year of follow-up among subjects who were seronegative and those who had IgA antibodies against EBV capsid antigen, anti-EBV DNase antibodies, or both at the time of recruitment is shown in Figure 1. Seropositive subjects had a much higher cumulative incidence of nasopharyngeal carcinoma than seronegative subjects during the entire follow-up period, and the longer the follow-up, the greater the difference in cumulative incidence.

**TABLE 3.** CUMULATIVE RISK OF NASOPHARYNGEAL CARCINOMA AMONG 9688 SUBJECTS, ACCORDING TO THE PRESENCE OR ABSENCE OF ANTI-EBV CAPSID ANTIGEN IgA AND ANTI-EBV DNASE ANTIBODIES.*

| VARIABLE | NO. OF SUBJECTS | PERSON-YEARS OF FOLLOW-UP | NO. OF CASES OF NASOPHARYNGEAL CARCINOMA | CUMULATIVE RISK OF NASOPHARYNGEAL CARCINOMA PER 100,000 PERSON-YEARS |
|---|---|---|---|---|
| Entire follow-up period | 9688 | 131,981 | 22 | 16.7 |
| IgA antibodies against EBV capsid antigen | | | | |
| Negative | 9525 | 130,653 | 18 | 13.8 |
| Positive | 110 | 1,328 | 4 | 301.3 |
| Anti-EBV DNase antibodies | | | | |
| Negative | 8477 | 116,652 | 15 | 12.9 |
| Positive | 1157 | 15,314 | 7 | 45.7 |
| Both serologic markers | | | | |
| Neither positive | 8413 | 115,848 | 13 | 11.2 |
| Either positive | 1173 | 15,564 | 7 | 45.0 |
| Both positive | 47 | 539 | 2 | 371.0 |

*A total of 11 subjects were excluded from follow-up analyses: 7 had received a diagnosis of nasopharyngeal carcinoma before enrollment, and 4 received such a diagnosis within one year after enrollment. Data were not available for all subjects for all tests. EBV denotes Epstein–Barr virus.

## DISCUSSION

In this prospective cohort study, the presence of IgA antibodies against EBV capsid antigen and the presence of anti-EBV DNase antibodies were found to be strong predictors of the risk of nasopharyngeal carcinoma, even when the tumor developed more than five years after recruitment.

Our study has several advantages over previous studies of the association between serologic markers of EBV infection and nasopharyngeal carcinoma. It was a population-based cohort study with a long follow-up and a large number of newly developed cases of nasopharyngeal carcinoma. All blood samples were collected and tested before the diagnosis of nasopharyngeal carcinoma. Increased titers of IgA antibodies against EBV capsid antigen and anti-EBV DNase antibodies were observed long before the occurrence of nasopharyngeal carcinoma.

However, there are some limitations to our study. It is possible that tumors already existed in the seronegative subjects who were not examined clinically at the time of enrollment. We think this is unlikely because in our previous study of antibodies against EBV in subjects with newly diagnosed cases of nasopharyngeal carcinoma,[19] all subjects with the disease were positive for both IgA antibodies against EBV capsid an-

**TABLE 4.** MULTIVARIATE-ADJUSTED RELATIVE RISK OF NASOPHARYNGEAL CARCINOMA ACCORDING TO STUDY PERIOD.*

| VARIABLE | RELATIVE RISK OF NASOPHARYNGEAL CARCINOMA | | | | | |
|---|---|---|---|---|---|---|
| | ENTIRE STUDY PERIOD | P VALUE | 1–5 YR AFTER ENROLLMENT | P VALUE | >5 YR AFTER ENROLLMENT | P VALUE |
| | RR (95% CI) | | RR (95% CI) | | RR (95% CI) | |
| IgA antibodies against EBV capsid antigen | | | | | | |
| Negative | 1.0 | | 1.0 | | 1.0 | |
| Positive | 22.0 (7.3–66.9) | <0.001 | 55.5 (8.9–345.4) | <0.001 | 13.9 (3.1–61.7) | <0.001 |
| Anti-EBV DNase antibodies | | | | | | |
| Negative | 1.0 | | 1.0 | | 1.0 | |
| Positive | 3.5 (1.4–8.7) | 0.006 | 4.7 (0.8–28.5) | | 3.2 (1.1–9.2) | 0.03 |
| Both serologic markers | | | | | | |
| Neither positive | 1.0 | | 1.0 | | 1.0 | |
| Either positive | 4.0 (1.6–10.2) | 0.003 | 7.1 (1.0–50.6) | 0.05 | 3.5 (1.2–10.0) | 0.02 |
| Both positive | 32.8 (7.3–147.2) | <0.001 | 85.3 (7.4–978.4) | <0.001 | 20.7 (2.6–162.0) | 0.004 |

*The relative risks (RRs) have been adjusted for age and the presence or absence of a family history of nasopharyngeal carcinoma. P values for "either positive" and "both positive" are for the comparisons with "neither positive." CI denotes confidence interval, and EBV Epstein–Barr virus.

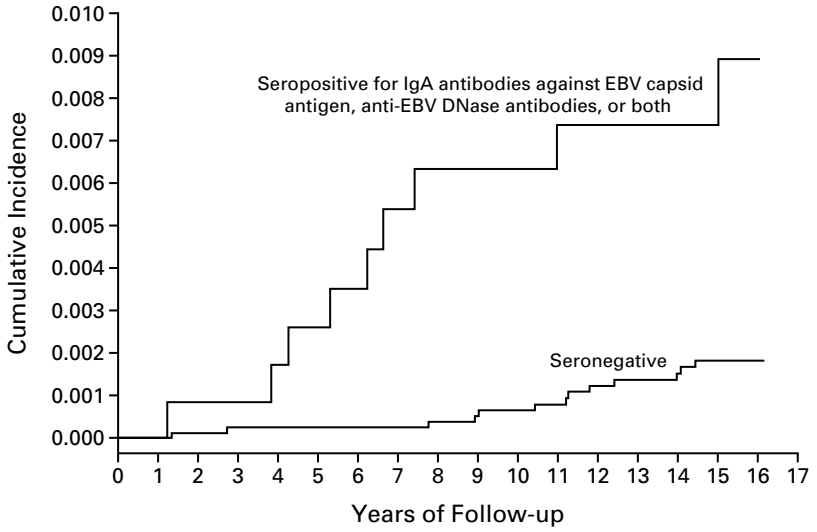


**Figure 1.** Cumulative Incidence of Nasopharyngeal Carcinoma during Follow-up among 9688 Study Subjects, According to Whether They Tested Positive or Negative for Either Serologic Marker of Epstein–Barr Virus (EBV) Infection (or Both) at the Time of Enrollment between 1984 and 1986.

tigen and anti-EBV DNase antibodies. In the present study, there were 13 newly diagnosed cases of nasopharyngeal carcinoma during the 16-year follow-up period among 8413 subjects who were seronegative at enrollment, and only 1 of these seronegative subjects had a case that was newly diagnosed within 2 years after enrollment.

Regardless of the presence or absence of serologic markers, patients with nasopharyngeal carcinoma are very unlikely to remain free of symptoms and signs for more than two years. National Health Insurance covers the health care costs of more than 99 percent of the residents of Taiwan, and otorhinolaryngologists are accessible to all patients with nasopharyngeal carcinoma. For these reasons, any early cases in seronegative subjects that were missed at enrollment would have been diagnosed later and reported to the National Cancer Registry.

Since we collected only one blood sample from each subject at the time of enrollment, it was not possible to analyze fluctuations in titers of antibodies against EBV. If seroconversion had occurred after recruitment, the relative risk of nasopharyngeal carcinoma associated with serologic markers might have been underestimated. The increase in the cumulative incidence among the seronegative subjects nine years after enrollment (Fig. 1) might be explained by seroconversion during the follow-up period.

In conclusion, we found strong evidence that two specific serologic markers of EBV infection can appear long before the development of nasopharyngeal carcinoma. Measurement of IgA antibodies against EBV capsid antigen and anti-EBV DNase antibodies may be useful for the early detection of nasopharyngeal carcinoma in high-risk populations.

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