



## Research Paper

# The Relationship Between Environmental Factors and the Profile of Epstein-Barr Virus Antibodies in the Lytic and Latent Infection Periods in Healthy Populations from Endemic and Non-Endemic Nasopharyngeal Carcinoma Areas in China



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## ABSTRACT

Our previous study found that smoking was associated with an elevated level of the antibody against VCA in the Epstein-Barr virus (EBV) lytic phase, which was an important predictive marker of the risk of nasopharyngeal carcinoma (NPC). It remained unknown whether environmental factors were associated with the levels of other EBV antibodies, such as Zta-IgA, EA-IgA, EBNA1-IgA, and LMP1-IgA, in the lytic and latent infection periods. We aimed to investigate the possible environmental inducers that could affect EBV antibody levels in two independent healthy male populations from endemic NPC areas in South China ( $N = 1498$ ) and non-endemic NPC areas in North China ( $N = 1961$ ). We performed ELISA and immunoenzymatic assays to test the levels of antibodies specific to the EBV antigens. The seropositive rates of antibodies against the antigens expressed in both the EBV latent and lytic infection periods, namely, LMP1-IgA, EBNA1-IgA, and Zta-IgA, in endemic areas (28.65%, 5.43% and 14.49%, respectively) were significantly higher than those in non-endemic areas (14.43%, 1.07% and 6.32%, respectively). Smoking was associated with higher seropositivity for EBNA1-IgA (OR = 1.47, 95% CI = 1.12–1.93) and Zta-IgA (OR = 1.28, 95% CI = 0.99–1.66), with dose-response effects, while not associated with the levels of LMP1-IgA. In conclusion, smoking was an important environmental factor, which associated with increased levels of EBNA1-IgA, and Zta-IgA.

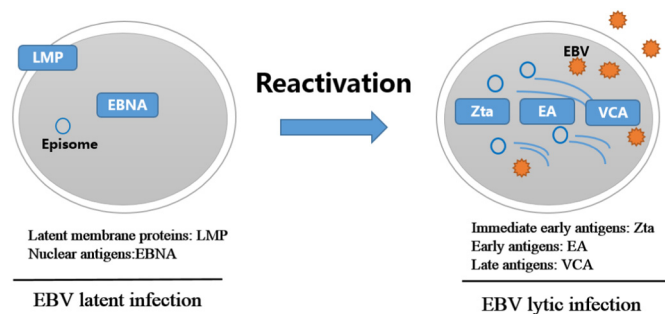
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## 1. Introduction

EBV is a widespread  $\gamma$ -herpesvirus that causes a life-long, asymptomatic infection in >95% of adults worldwide, remaining in resting memory B cells with a low copy number of episomal virus in the latent phase in healthy individuals (Babcock et al., 1998). EBV can be reactivated periodically and can switch from the latent to the lytic phase with elevated viral particle assembly and release, which could trigger an elevated immune response with higher antibody levels against specific antigens, such as BZLF1 transcription activator protein (Zta), early antigen (EA), and viral capsid antigen (VCA) (Fig. 1).

EBV, identified as a class I oncovirus by the WHO, is associated with the occurrence and development of nasopharyngeal carcinoma (NPC). NPC, which exhibits distinctive geographic and ethnic distributions globally, is rare in most areas of the world, while it is common in South China, where the risk can be 50-fold higher than that in non-endemic areas (Jia et al., 2006; Tang et al., 2016). Numerous studies have shown that NPC patients and individuals at high-risk for developing NPC exhibit distinctive anti-EBV antibody profiles, with elevated IgA antibodies against specific EBV antigens, and the most widely used serological biomarkers have been VCA-IgA, EA-IgA, Zta-IgA and EBNA1-IgA. Previous cohort studies in endemic areas of NPC in South China (Cao et al., 2011; Zeng et al., 1985; Ji et al., 2007; Liu et al., 2012) and Taiwan (Chien et al., 2001) have identified several serological biomarkers for screening and early-diagnosis in high-risk individuals, and those with higher antibody levels may have up to a 20-fold increased NPC risk compared with seronegative individuals. These cohort studies

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**Fig. 1. EBV antigens being expressed during life cycles in latent and lytic infection.** During EBV latent infection, a limited number of antigens were expressed, mainly was latent membrane proteins and nuclear antigens. The blue circle represents the episome of EBV. Once EBV was reactivated under certain conditions, large numbers of antigens were expressed, including the immediate early antigens of Zta, early antigens of EA and late antigens of VCA. The orange particles represent the replicated and released EBV particles.

have reported that elevated antibody levels precede the occurrence of NPC and that the serological windows can be detected several years prior to disease onset, which implies that EBV seroreactivity may have a causal role in the occurrence of NPC (Fang et al., 2009).

Although the cause of this phenomenon is not fully understood, identifying environmental inducing factors that trigger elevated levels of IgA antibodies to EBV specific antigens in vivo may be crucial for NPC primary prevention. In our previous molecular epidemiological study (Xu et al., 2012), we observed that among the putative NPC risk factors, smoking is an inducing factor. Smoking was associated with elevated levels of the IgA antibody against VCA, which is expressed in the late stage of lytic infection. However, it is unclear whether the potential environmental factors are associated with other EBV IgA antibodies, such as the antibody against EBNA1, which is expressed in both the latent and lytic stages of infection, or the antibodies against Zta and EA, which are expressed in the early stage of lytic infection. Additionally, few large-scale studies have focused on describing the distribution of the IgA antibody against latent membrane protein 1 (LMP1), which is specifically expressed in latent infections and is essential for EBV-mediated growth transformation.

We conducted this comprehensive study in 3459 males from areas with different NPC risk levels to describe the epidemiology of the profiles of antibodies against EBV antigens that are expressed at different stages of EBV infection, namely, EBNA1-IgA, Zta-IgA, EA-IgA and LMP1-IgA.

## 2. Materials and Methods

### 2.1. 21-RCCP Population in South China and Yangquan Population in North China

Two populations, one from South China, where NPC is endemic, and one from North China, where NPC is not endemic, were included in our study. These populations have been previously described in detail (Xu et al., 2012). Briefly, one population was from the areas of Guangdong Province where NPC is endemic. In total, 1498 males from 21 municipalities of Guangdong Province who attended a physical examination center between October 1, 2005 and October 1, 2007 were included in this study (this population is abbreviated as the 21-RCCP population). All participants were without any history of cancer or immunological disease. The other population came from areas of Shanxi Province where NPC is not endemic (this population is abbreviated as the North population). For the North population, 1961 healthy males were enrolled who visited the First General Hospital of Yangquan City between March 1 and August 1, 2010 for a health check-up. An informed consent was

signed by every subject before the interview, and the human ethics committee of the Sun Yat-Sen University Cancer Center approved our study.

### 2.2. Data Collection

In this study, the comprehensive face-to-face interviews were conducted by well-trained interviewers (Xu et al., 2012; He et al., 2015; Jia et al., 2010). The structured questionnaire was mainly based on the questionnaires issued by the University of Arizona Cancer Center (<http://uacc.arizona.edu/research/shared-resources/bmisr/questionnaires>). The information included demographic data (age, education level), lifestyle behaviors (cigarette smoking, alcohol consumption and consumption of preserved vegetables), and family history (family history of tumors and NPC). For the 21-RCCP population, we also investigated the prevalence of the Cantonese diet of salted fish, slow-cooked soup, tea, and herbal tea. For cigarette smoking, those who had smoked at least 100 cigarettes during their lifetime were considered smokers. Ex-smokers were those who had quit smoking at least 1 year before the interview. Detailed smoking data such as the age at which they started smoking, the number of years they smoked, the average number of cigarettes smoked per day and the type of smoking were also included for further analysis. Pack-years were calculated by multiplying the number of packs smoked per day by the years the person had smoked. Heavy smokers were those with no fewer than 20 pack-years, which was the median value of the smoking pack-years for the total population.

### 2.3. EBV Antibody Tests

A 5–10 ml blood sample was collected from each subject in the two populations (21-RCCP and North populations). The LMP1-IgA, EBNA1-IgA and Zta-IgA antibody levels were measured with commercial ELISA kits by the same technicians in the same laboratory of the Sun-Yat Sen University Cancer Center (SYSUCC) (Xu et al., 2012; Liu et al., 2012; Cao et al., 2011). LMP1 peptide was derived from Yeast-expressed EBV strain (GD1) 185-366aa; EBNA1 and Zta were produced with purified recombinant peptides specified by EBV BKR1F1 (72 kD), BZLF1 (36 + 38-kDa fine doublet) respectively. The serostatuses of LMP1-IgA, EBNA1-IgA and Zta-IgA were defined as seronegative or seropositive according to the ELISA OD values following the manufacturers' instructions (Shanghai Jining Shiye Co., Ltd., Shanghai City, China; Zhongshan Bio-technology Co., Ltd., Zhongshan city, China). For EBNA1-IgA, in addition to the seronegative and seropositive statuses, a weak seropositive status was also defined by the manufacturer's instructions. The EA-IgA antibody was detected by an immunoenzymatic assay performed according to the protocol of the SYSUCC clinical laboratory, which used the Raji cell line and determined the presence of the EA-IgA antibody by titration, with the cut-off value set at 1:10 (Chen et al., 2014). To evaluate the reproducibility for each assay, we randomly selected 90 samples to conduct a test-retest assay for each sero-marker.

### 2.4. Statistical Analysis

Multivariable unconditional logistic regressions were used to assess the associations between EBV serostatus and environmental factors by adjusting for age (years, continuous variable) and education levels (primary school or less, high school, and university or more). Ordered logistic regressions were used to assess the EBV seropositivity risk among different populations by dividing them into four levels according to the quartile of the corresponding OD (optical density) value of the total population for LMP1-IgA, EBNA1-IgA and Zta-IgA. Linear trend analyses for associations between exposures and EBV antibodies were conducted by treating the categorical variables as continuous variables, such as smoking and consumption of alcohol, tea, herbal tea, salted fish and preserved vegetables. A detailed subgroup analysis was performed

to explore the dose-dependent relationships between EBV seropositivity and smoking status with the respective median values as cutoff points. For EBNA1-IgA, the weakly seropositive and seropositive samples, which both had limited sample sizes, were merged together into one group to increase the statistical power. The possible synergistic effect of smoking status and living in endemic/non-endemic NPC areas to EBV seropositivity was evaluated by Rothman's additive interaction method by estimating the attributable proportion due to interaction (AP) (Rothman, 1976). STATA 10.0 (Stata Corp., College Station, TX) was used, and two-sided *P*-values < .05 were significant. The raw data in this paper has been successfully uploaded and locked onto Research Data Deposit with a RDD number of RDDA2018000551.

### 3. Results

We found that the risk of EBV seropositivity was significantly higher in the 21-RCCP population than in the North population. The seropositive rates of LMP1-IgA, EBNA1-IgA, and Zta-IgA in the population from the endemic area (28.65%, 5.43% and 14.49%, respectively) were significantly higher than those in the population from the non-endemic area (14.43%, 1.07% and 6.32%, respectively). Because the seropositive rate for EA-IgA was extremely low in the population from the endemic area (0.07%), it was not analyzed in the population from the non-endemic area. By dividing EBV antibody levels into different quartile subgroups ( $P_{25}$ ,  $P_{50}$ ,  $P_{75}$ ) according to the corresponding OD values of the total population, we found by ordered logistic regression that people in the endemic areas had 1.7 to 2.6-fold increased seropositivity for LMP1-IgA, EBNA1-IgA and Zta-IgA than those in the non-endemic areas (Table 1).

In the association analysis between the relevant factors and EBV antibody seropositivity, we found linear trends between age and the levels of antibodies of LMP1-IgA, EBNA1-IgA and Zta-IgA (Fig. 2). Among the putative risk factors, smoking was consistently associated with EBNA1-IgA seropositivity in the populations from both the endemic and non-endemic areas and was associated with Zta-IgA seropositivity in the population from the endemic area but not in the population from the non-endemic area. However, smoking was not associated with LMP1-IgA seropositivity in the populations from either the endemic areas or non-endemic areas. Additionally, alcohol consumption was negatively associated with the seropositivity of LMP1-IgA, Zta-IgA, and EBNA1-IgA in the pooled data, although it did not reach statistical significance in the separate population. Consumption of salted-fish and preserved vegetable was negatively associated with the seropositivity of LMP1-IgA and EBNA1-IgA in the pooled data (Table 2, Supplemental Tables 2 and Table 3).

We did multiple liner regression analysis and the relationships between environmental factors and the antibody levels of their original OD remain consistent. Smoking was still positively associated with higher antibody levels of EBNA1-IgA and Zta-IgA, and alcohol drinking

was associated with lower levels of anti-EBV antibodies. All of the results were quite consistent with previous results calculated by using categories data format for antibody level (Supplemental Table 4).

To further explore the dose-response effects of smoking on the seropositivity of EBNA1-IgA and Zta-IgA, we performed a subgroup analysis according to smoking-related factors, such as the age at which they started smoking, the number of years they smoked, the cumulative amount smoked and the type of smoking, with the cutoff points defined as the median values of the corresponding parameter in the total population. We found significant dose-response relationships between smoking and EBNA1-IgA and Zta-IgA seropositivity, especially in the population from the endemic areas. We also observed a similar significant dose-response relationship between smoking and EBNA1-IgA seropositivity in the North population, although it was not significant for Zta-IgA seropositivity (data not shown). Compared with non-smokers, smokers who started smoking at a younger age, had longer smoking durations, had greater cumulative amounts of smoking (pack-years) and practiced the deeply inhaled smoking type had higher risks of being EBNA1-IgA and Zta-IgA seropositive (Table 3 and Supplemental Table 5).

The ORs for EBNA1-IgA seropositivity (Fig. 3) and Zta-IgA seropositivity (Fig. 4) were markedly elevated in heavy smokers in endemic areas compared with non-smokers in non-endemic areas. We observed significant additive interactions of smoking status and having lived in different NPC risk areas to EBV seropositivity for both EBNA1-IgA and Zta-IgA. Additionally, the effect reached statistical significance when a multiplicative model was used for the seropositive risk of Zta-IgA, while no significant multiplicative interaction was observed for the seropositive risk of EBNA1-IgA (Table 4).

The CVs for the OD values of ELISA assays of LMP1-IgA, EBNA1-IgA and Zta-IgA were 2.41%, 1.03%, 1.80%, respectively. In test-retest analysis, 90 samples were randomly selected and ICCs (intraclass correlation coefficients) of LMP1-IgA, EBNA1-IgA, and Zta-IgA were 0.70, 0.97 and 0.92, respectively. The agreement percentage of LMP1-IgA, EBNA1-IgA, and Zta-IgA was 86.96%, 97.87% and 84.04%, respectively.

### 4. Discussion

This is a comprehensive epidemiological study to investigate the EBV serostatuses of general populations from different NPC risk areas in China by evaluating the seropositive rates of antibodies against antigens expressed in both the lytic and latent infection periods, such as Zta-IgA, EA-IgA, EBNA1-IgA, and LMP1-IgA, and identifying the potential risk factors that could affect EBV seroreactivity. We believe that understanding the epidemiology of EBV antibodies and identifying associated risk factors in healthy subjects is not only crucial for clarifying the mechanisms of EBV-associated diseases but also for the primary prevention of those diseases, such as NPC in South China.

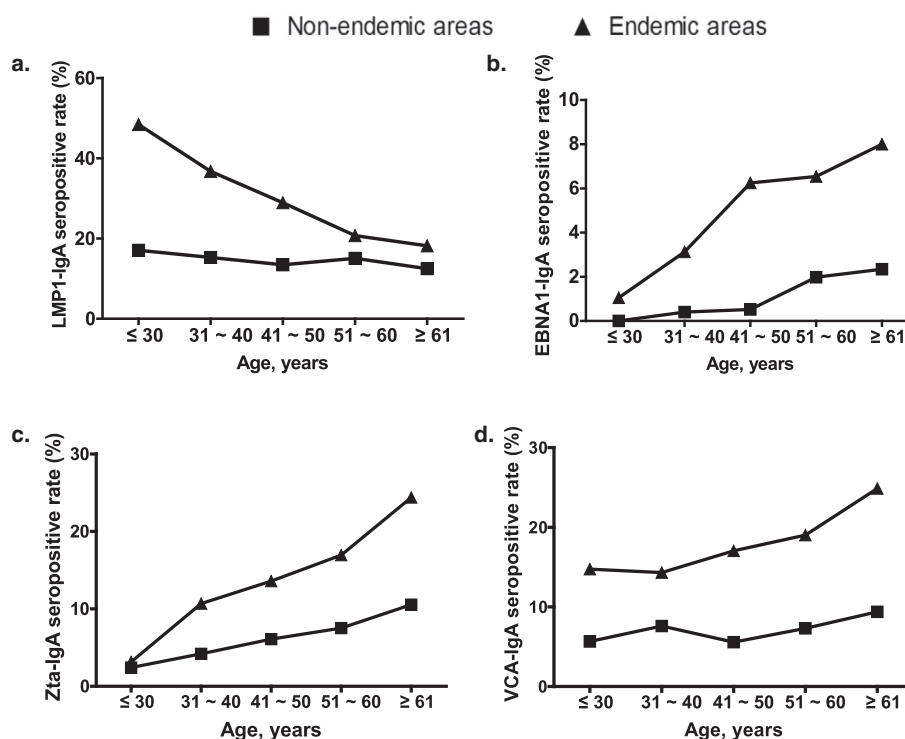
**Table 1**  
Comparison of EBV serum antibody levels of optical density values by ELISA in 3459 subjects from different NPC risk areas.

EBV-IgA <sup>a</sup>	Seropositive rate	OD < $P_{25}$ <sup>a</sup>	$P_{25} \leq OD < P_{50}$ <sup>a</sup>	$P_{50} \leq OD < P_{75}$ <sup>a</sup>	$P_{75} \leq OD$ <sup>a</sup>	OR (95%CI) <sup>b</sup>	<i>P</i>
LMP1-IgA							
Non-endemic areas <sup>c</sup>	14.43%	561 (28.71%)	587 (30.04%)	459 (23.49%)	347 (17.76%)	1.00 (reference)	/
Endemic areas <sup>c</sup>	28.65%	283 (19.03%)	283 (19.03%)	413 (27.77%)	508 (34.16%)	2.23 (1.97–2.53)	<0.001
EBNA1-IgA							
Non-endemic areas <sup>c</sup>	1.07%	627 (31.97%)	424 (21.62%)	523 (26.67%)	387 (19.73%)	1.00 (reference)	/
Endemic areas <sup>c</sup>	5.43%	236 (15.82%)	434 (29.09%)	381 (25.54%)	441 (29.56%)	1.70 (1.50–1.92)	<0.001
Zta-IgA							
Non-endemic areas <sup>c</sup>	6.32%	705 (35.95%)	460 (23.46%)	421 (21.47%)	375 (19.12%)	1.00 (reference)	/
Endemic areas <sup>c</sup>	14.49%	158 (10.55%)	420 (28.04%)	432 (28.84%)	488 (32.58%)	2.64 (2.33–2.99)	<0.001

<sup>a</sup> LMP1-IgA, EBNA1-IgA and Zta-IgA antibody levels were tested by ELISA according to the manufacture's protocols; OD is the abbreviation of optical density value by ELISA;  $P_{25}$ ,  $P_{50}$ ,  $P_{75}$  refers to the first quartile, median, third quartile of the OD values of LMP1-IgA, EBNA1-IgA and Zta-IgA of the total 3459 healthy males.

<sup>b</sup> Ordered logistic regression analyses were used to assess OR and 95%CI by adjusting age (continuous variables) and education (primary school or less, high school, university or more).

<sup>c</sup> Non-endemic areas represent non-endemic areas of NPC of North population and endemic areas represent endemic areas of NPC of 21RCCP population.



**Fig. 2.** The seropositive rate of LMP1-IgA, EBNA1-IgA, Zta-IgA and VCA-IgA in healthy males. a) It presents the seropositive rate of LMP1-IgA in the healthy males among non-endemic areas and endemic areas of NPC; b) It presents the seropositive rate of EBNA1-IgA in the healthy males among non-endemic areas and endemic areas of NPC; c) It presents the seropositive rate of Zta-IgA in the healthy males among non-endemic areas and endemic areas of NPC; d) It presents the seropositive rate of VCA-IgA in the healthy males among non-endemic areas and endemic areas of NPC.

We found significant differences in the LMP1-IgA, EBNA1-IgA and Zta-IgA seropositivity between the healthy populations from different NPC risk areas, revealing that the immune response against the antigens expressed in both the latent and lytic periods was higher in the NPC endemic area than in the non-endemic area. Our previous study similarly found VCA-IgA seropositivity levels was >2-fold greater in subjects in NPC endemic areas than in subjects in non-endemic areas (Xu et al., 2012). Numerous serological studies have indicated that those with higher EBV-IgA antibody levels may have a 20- to 30-fold increased risk for NPC compared with those who are seronegative and that EBV reactivation with subsequently increased antibody levels occurs several years prior to NPC onset (Chien et al., 2001; Cao et al., 2011; Raab-Traub and Flynn, 1986; Zeng et al., 1985; Ji et al., 2007). We suspected that EBV reactivation may be an early event in NPC and that the significant differences in EBV seropositivity status among different subpopulations may provide some evidence to help explain the distinctive distribution of NPC incidence and the underlying tumorigenesis mechanisms.

Our previous study found a positive relationship between smoking and VCA-IgA seropositivity and provided direct evidence that cigarette smoke extract could induce EBV reactivation in vitro by using cell biological assays (Xu et al., 2012). In this study, we further confirmed that smoking reactivates EBV because we found similar dose-response relationships between smoking and EBNA1-IgA and Zta-IgA seropositivity. Smoking was not associated with seropositivity for LMP1-IgA, which is an antibody against an antigen specifically expressed during EBV latent infection. This finding suggests that smoking is associated with antibodies against infection stage-specific antigens. Smoking is mainly associated with the antigens expressed in the lytic phase and not with the antigens specifically expressed in the latent phase, revealing that smoking potentially plays a positive role in EBV lytic reactivation. Furthermore, smokers have 5 to 6-fold increased risk of simultaneous seropositivity for EBNA1-IgA, Zta-IgA and VCA-IgA compared with non-smokers with all the 3 antibodies was seronegative (detailed in

Supplemental Table 6). Taken together, we believe that cigarette smoking might contribute to NPC risk in an indirect way by synergistically elevating anti-EBV IgA levels, in addition to contributing directly to NPC carcinogenesis by the introduction of multiple carcinogenic materials. However, the exact materials that result in higher EBV-IgA antibody levels and the detailed mechanisms by which cigarette smoking modulates EBV reactivation remain to be further elucidated.

Few studies have focused on the LMP1-IgA serostatus in either healthy subjects or NPC patients. In our study, we observed that smoking was related to serostatus of EBNA1 but not to LMP1-IgA, although both of them were expressed in latent stages of EBV. The quite different biological behaviors might result from the different expression phase and biological functions of the antigens. EBNA1 is the only nuclear protein expressed in both latent and lytic modes of infection, and exhibits two critical roles in the reactivation of latent EBV (1) In latently infected cells, EBNA1 showing a role in suppressing reactivation; (2) while when the lytic cycle was induced, EBNA1 positively contributed to lytic infection (Sivachandran et al., 2012). While for LMP1, it was the oncoprotein only involves in latent infection and the studies focusing on the antibody against LMP1 is very limited. Our study current provide an epidemiology evidence and the association is not clearly understood. Additionally, since the ELISA kit of LMP1-IgA is a newly marketed commercial kit and without available comparison with others, we recommend more studies to confirm this findings and explore the potential biological significance.

We did rank correlation analysis by using the OD values of the serological markers and found a weak correlation between the antibody levels of EBNA1-IgA and Zta-IgA ( $r = 0.29$ ). While, no significant correlation between LMP1-IgA and EBNA1-IgA, or between LMP1-IgA and Zta-IgA (data not shown), which reveals that these sero-markers may be independent in general population.

It is noteworthy that the consumption of alcohol was negatively associated with the seropositivity of LMP1-IgA, EBNA1-IgA, Zta-IgA in the pooled data of 3459 male subjects, although it was not statistically



**Table 2**  
Association between NPC risk factors and EBV antibodies of LMP1-IgA, EBNA1-IgA and Zta-IgA in 3459 healthy males.

Variables <sup>a</sup>	LMP1-IgA		EBNA1-IgA		Zta-IgA	
	± <sup>b</sup>	OR (95%CI) <sup>e</sup>	± <sup>c</sup>	OR (95%CI) <sup>e</sup>	± <sup>d</sup>	OR (95%CI) <sup>e</sup>
Age						
≤30	151/67	1.00 (reference)	209/10	1.00 (reference)	213/6	1.00 (reference)
31–40	663/216	0.76 (0.55–1.06)	826/57	1.36 (0.68–2.70)	822/62	2.58 (1.10–6.05)*
41–50	802/202	0.59 (0.42–0.82)**	916/91	1.88 (0.96–3.67)	915/94	3.42 (1.48–7.93)**
51–60	731/155	0.52 (0.37–0.73)**	781/107	2.31 (1.18–4.52)*	787/103	4.20 (1.81–9.75)**
≥61	386/68	0.43 (0.29–0.65)**	392/64	2.50 (1.24–5.05)*	381/76	6.09 (2.58–14.39)**
<i>P</i> <sub>trend</sub> <sup>f</sup>		<0.001		<0.001		<0.001
Education						
Primary school or less	261/41	1.00 (reference)	254/49	1.00 (reference)	252/52	1.00 (reference)
High school	1364/341	1.33 (0.93–1.90)	1521/191	0.80 (0.56–1.14)	1546/171	0.71 (0.50–1.02)
University or more	1079/316	1.41 (0.97–2.05)	1313/86	0.47 (0.31–0.70)**	1286/113	0.67 (0.45–0.99)*
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.118		<0.001		0.089
Cigarette smoking						
Never smoker	862/252	1.00 (reference)	1038/77	1.00 (reference)	1027/90	1.00 (reference)
Ever smoker	1871/456	0.88 (0.74–1.06)	2086/252	1.47 (1.12–1.93)**	2091/251	1.28 (0.99–1.66)
Alcohol drinking						
Nondrinker	808/221	1.00 (reference)	898/133	1.00 (reference)	902/132	1.00 (reference)
≤1 drink per day	1047/282	0.88 (0.72–1.08)	1221/116	0.77 (0.59–1.01)	1216/122	0.82 (0.62–1.07)
>1 drink per day	873/204	0.76 (0.61–0.95)*	1002/77	0.60 (0.44–0.81)**	995/86	0.68 (0.51–0.92)*
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.014		0.001		0.011
Tea intake						
Less than monthly	171/65	1.00 (reference)	197/41	1.00 (reference)	208/32	1.00 (reference)
Monthly	239/111	0.97 (0.66–1.42)	298/53	1.05 (0.66–1.65)	312/40	1.07 (0.64–1.79)
Weekly or more	646/249	0.96 (0.69–1.34)	722/175	1.25 (0.85–1.84)	756/144	1.40 (0.91–2.15)
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.844		0.175		0.067
Herbal tea intake						
Less than monthly	220/79	1.00 (reference)	237/62	1.00 (reference)	254/46	1.00 (reference)
Monthly	424/168	0.95 (0.69–1.31)	492/102	0.90 (0.63–1.28)	514/83	1.02 (0.68–1.52)
Weekly or more	412/178	1.13 (0.82–1.55)	488/105	0.88 (0.62–1.26)	508/87	1.05 (0.71–1.56)
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.331		0.527		0.790
Canton soup						
Less than monthly	50/24	1.00 (reference)	63/12	1.00 (reference)	67/8	1.00 (reference)
Monthly	102/46	0.85 (0.46–1.57)	119/30	1.47 (0.70–3.09)	123/26	1.95 (0.83–4.58)
Weekly or more	904/355	0.85 (0.51–1.42)	1035/227	1.13 (0.59–2.15)	1086/182	1.31 (0.61–2.79)
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.618		0.762		0.750
Salted fish						
Less than monthly	931/391	1.00 (reference)	1088/239	1.00 (reference)	1141/192	1.00 (reference)
Monthly	64/13	0.51 (0.27–0.94)*	61/16	1.13 (0.63–2.01)	70/7	0.55 (0.25–1.23)
Weekly or more	61/21	1.04 (0.61–1.78)	68/14	0.76 (0.42–1.39)	65/17	1.27 (0.72–2.25)
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.440		0.520		0.831
Preserved vegetable						
Less than monthly	2237/598	1.00 (reference)	2563/283	1.00 (reference)	2569/283	1.00 (reference)
Monthly	176/42	0.89 (0.62–1.27)	194/24	1.03 (0.66–1.62)	191/27	1.22 (0.80–1.88)
Weekly or more	315/67	0.81 (0.61–1.07)	364/19	0.44 (0.27–0.71)**	353/30	0.73 (0.49–1.09)
<i>P</i> <sub>trend</sub> <sup>f</sup>		0.115		0.002		0.251
Family history of tumor						
No	2152/556	1.00 (reference)	2476/242	1.00 (reference)	2462/261	1.00 (reference)
Yes	576/151	1.09 (0.89–1.34)	645/84	1.29 (0.99–1.69)	651/79	1.09 (0.83–1.43)
Family history of NPC						
No	2631/671	1.00 (reference)	3007/307	1.00 (reference)	2996/323	1.00 (reference)
Yes	53/20	1.51 (0.89–2.54)	62/11	1.73 (0.89–3.34)	61/12	1.82 (0.96–3.45)

<sup>a</sup> Cantonese dietary of salted fish, slow cooked soup, tea, herbal tea were only investigated for NPC endemic areas of 21RCCP.

<sup>b</sup> For LMP1-IgA antibody, “±” represents seronegative group vs. seropositive group with the reference cutoff defined by manufactures.

<sup>c</sup> For EBNA1-IgA antibody, “±” represents seronegative group vs. combined group which consist of weak seropositive and seropositive ones together with the reference cutoff defined by manufactures, since the limited sample size.

<sup>d</sup> For Zta-IgA antibody, “±” represents seronegative group vs. the seropositive group with the reference cutoff defined by manufactures.

<sup>e</sup> Logistic regression analyses were used to assess OR and 95%CI by adjusting age (continuous variables) and education (primary school or less, high school, university or more).

<sup>f</sup> Linear trends tests were performed by treating ordered categorical variables as continuous variables.

\* *P* < .05.

\*\* *P* < .01.

significant in the separate populations. The underlying reasons for the negative association between increased alcohol consumption and decreased anti-EBV IgA levels are largely unknown, and further studies are recommended. The consumption of salted fish and preserved vegetables, which are confirmed risk factors for NPC, were associated with

decreased LMP1-IgA and EBNA1-IgA seropositivity in the NPC endemic area, although the reasons for these associations are unknown. However, the present results may be biased, and we question the robustness of these relationships because of the limited sample size, which were 13 seropositive individuals who ate salted fish on a monthly basis and 19

**Table 3**  
The dose-response relationship between smoking and EBV-IgA antibody status in NPC endemic area.

Smoking status	EBNA1-IgA				Zta-IgA			
	Current smokers		Ex-smokers		Current smokers		Ex-smokers	
	± <sup>a</sup>	OR (95%CI) <sup>b</sup>	± <sup>a</sup>	OR (95%CI) <sup>b</sup>	± <sup>a</sup>	OR (95%CI) <sup>b</sup>	± <sup>a</sup>	OR (95%CI) <sup>b</sup>
<b>Age started smoking (years)</b>								
Never	429/66	1.00 (reference)	429/66	1.00 (reference)	443/54	1.00 (reference)	443/54	1.00 (reference)
≥20	335/66	1.11 (0.74–1.65)	109/28	1.31 (0.76–2.27)	349/52	1.01 (0.65–1.55)	116/21	1.28 (0.70–2.32)
<20	236/83	1.95 (1.31–2.90)**	107/28	1.47 (0.85–2.53)	253/69	1.97 (1.29–3.01)**	115/21	1.31 (0.71–2.40)
<i>P</i> -trend <sup>c</sup>		0.001		0.142		0.002		0.333
<b>Smoking duration (years)</b>								
Never	429/66	1.00 (reference)	429/66	1.00 (reference)	443/54	1.00 (reference)	443/54	1.00 (reference)
<25	265/44	1.12 (0.72–1.76)	149/37	1.40 (0.86–2.29)	276/34	1.16 (0.71–1.88)	161/25	1.16 (0.67–2.03)
≥25	307/106	1.73 (1.16–2.58)**	68/19	1.34 (0.70–2.56)	328/87	1.55 (1.01–2.37)*	71/17	1.57 (0.79–3.12)
<i>P</i> -trend <sup>c</sup>		0.008		0.221		0.048		0.209
<b>Cumulative amount (pack-years)</b>								
Never	429/66	1.00 (reference)	429/66	1.00 (reference)	443/54	1.00 (reference)	443/54	1.00 (reference)
<20	298/55	1.20 (0.80–1.81)	150/33	1.24 (0.75–2.05)	313/41	1.08 (0.69–1.69)	159/25	1.23 (0.71–2.13)
≥20	274/94	1.72 (1.15–2.57)**	66/23	1.72 (0.93–3.18)	290/80	1.72 (1.12–2.63)*	72/17	1.42 (0.72–2.82)
<i>P</i> -trend <sup>c</sup>		0.008		0.084		0.014		0.271
<b>Smoking type, inhaled or not</b>								
Never	429/66	1.00 (reference)	429/66	1.00 (reference)	443/54	1.00 (reference)	443/54	1.00 (reference)
Non-inhaled	327/88	1.46 (1.00–2.14)*	114/28	1.33 (0.78–2.29)	352/64	1.26 (0.83–1.92)	126/17	0.98 (0.52–1.85)
Inhaled	230/59	1.42 (0.93–2.16)	84/26	1.64 (0.93–2.90)	237/54	1.51 (0.97–2.37)	88/22	1.79 (0.98–3.27)
<i>P</i> -trend <sup>c</sup>		0.090		0.073		0.067		0.095

<sup>a</sup> For EBNA1-IgA antibody, “±” represents seronegative group vs. combined group which consist of weak seropositive and seropositive ones together with the reference cutoff defined by manufactures, since the limited sample size; for Zta-IgA antibody, “±” represents the number of seronegative group/seropositive group with the reference cutoff defined by manufactures.

<sup>b</sup> OR and 95%CI were calculated by adjusting age (continuous variables) and education (primary school or less, high school, university or more), alcohol drinking (non-drinker, ≤1 drink per day, >1 drink per day), tea consumption (less than monthly, monthly, weekly or more), herbal tea intake (less than monthly, monthly, weekly or more), canton soup consumption (less than monthly, monthly, weekly or more), salted-fish consumption (less than monthly, monthly, weekly or more), preserved vegetable consumption (less than monthly, monthly, weekly or more) and family history of NPC (yes or no).

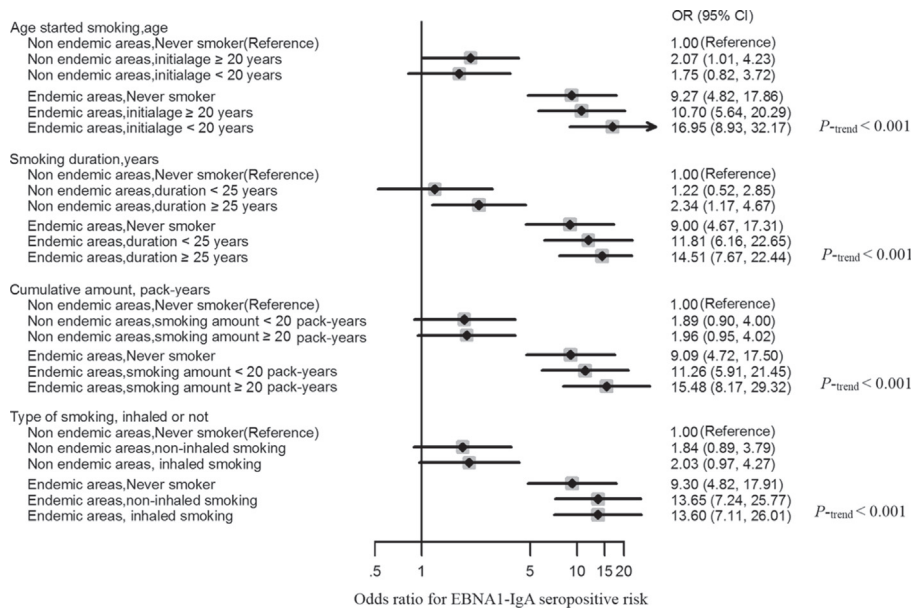
<sup>c</sup> Linear trends tests were performed by treating ordered categorical variables as continuous variables.

\* *P* < .05.

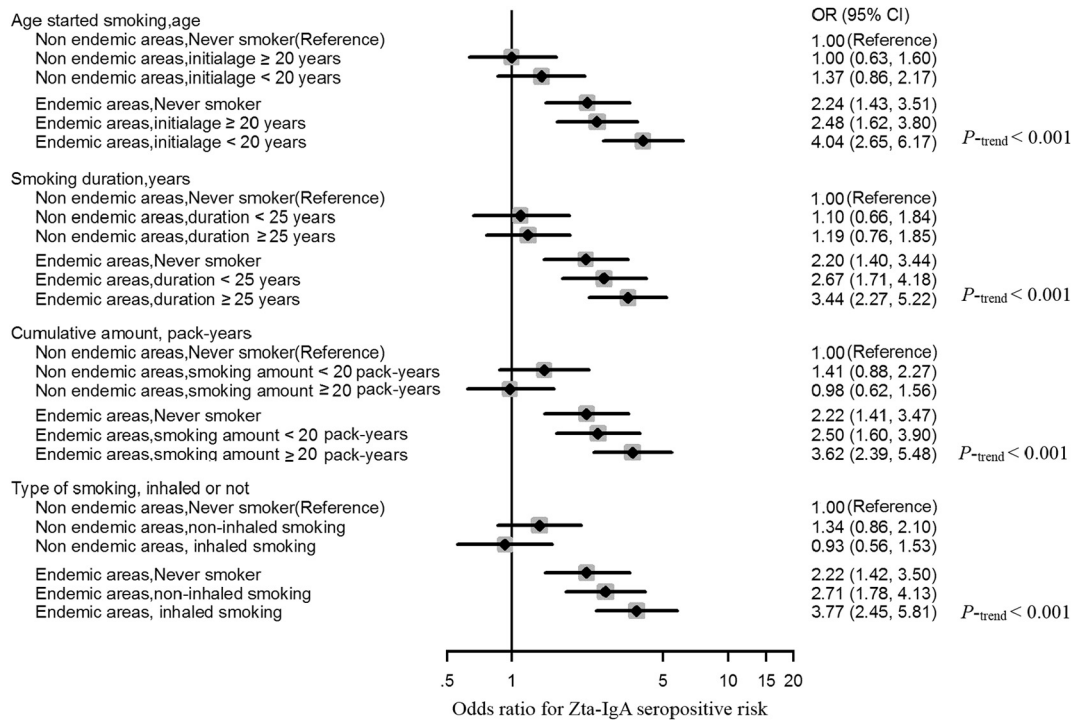
\*\* *P* < .01.

seropositive individuals who ate preserved vegetables on a weekly basis. We recommend more large-scale studies to explore the natural inhibitors of EBV reactivation, including dietary ingredients such as sulforaphane, fatty acids, valproic acid, and epigallocatechin gallate

(Gorres et al., 2014; Daigle et al., 2011; Wu et al., 2013; Chang et al., 2003). These large-scale studies would be helpful in guiding recommendations for diet and behavior modifications that could reduce EBV replication and transmission and prevent EBV-associated diseases.



**Fig. 3. The relationship between cigarette smoking and EBNA1-IgA antibody serostatus in different risk areas of NPC by smoking status.** Odds ratios (ORs) for EBNA1-IgA seropositive group (weak seropositive ones + seropositive ones) vs. seronegative group among different risk areas were calculated. We stratified smoking categories by medium values as the cutoff points for subgroup analysis: age started smoking (<20, ≥20 years), smoking duration years (<25, ≥25 years) and cumulative amount (<20, ≥20 pack-years). Both groups were adjusted for age (years, continuous variable) and education levels (primary school or less, high school, university or more) using logistic regression analysis with never smokers in non-endemic areas as the reference, and the tests were two-sided. Squares = study-specific odds ratios; horizontal lines = study-specific confidence intervals (CIs); solid vertical line = odds ratio of 1.0.



**Fig. 4. The relationship between cigarette smoking and EBV Zta-IgA antibody serostatus in different risk areas of NPC by smoking status.** Odds ratios (ORs) for the Zta-IgA seropositive group vs. seronegative group among different risk areas were calculated. We stratified smoking categories by medium values as the cutoff points for subgroup analysis: age started smoking (<20, ≥20 years), smoking duration years (<25, ≥25 years) and cumulative amount (<20, ≥20 pack-years). Both groups were adjusted for age (years, continuous variable) and education levels (primary school or less, high school, university or more) using logistic regression analysis with never smokers in low risk areas as the reference, and the tests were two-sided. Squares = study-specific odds ratios; horizontal lines = study-specific confidence intervals (CIs); solid vertical line = odds ratio of 1.0.

Because there is no international standard for the evaluation of EBV antibodies, we used the same batch of commercial ELISA kits among different populations for the detection of each EBV antibody, obtaining the semi-quantitative results of optical densities to ensure comparability within our study. The prevalence of current smoking for males (54.3%) in our study was quite close to the prevalence reported in China in the latest WHO report (52.9%) according to the Global Adult Tobacco Survey (GATS), indicating that our sample population was reliably representative, however, non-randomly selected individuals may result in biased results. To test the validity of the questionnaires, plasma cotinine levels, an objective indicator of smoking, were measured in 906 subjects of the 21-RCCP population. We found that the self-reported smoking status was highly concordant with plasma cotinine levels. The median and interquartile range for plasma cotinine was 0.99 (0.60–1.53), 1.44 (0.88–2.75) and 143.92 (73.01–228.33) for self-reported never smokers, ex-smokers and current-smokers, respectively. The analysis of the dose-

response relationships by smoking status subgroup was significant not only when the corresponding median values were used as cutoff points but also when other cutoff values were used (data not shown). We believe the associations between smoking and EBV-IgA seropositivity are genuine and consistent. Besides, due to its cross-sectional design, it is unclear whether study subjects with EBV seropositive will develop NPC in the future, precluding us to investigate the interaction between smoking and EBV antibody response on NPC risk.

In conclusion, our study may give new insight into the dramatically distinctive distribution of NPC and the still unknown tumorigenesis mechanisms from the perspective of EBV reactivation. We propose tailoring the NPC screening strategy according to individual host and environment factors. New approaches to inhibit EBV reactivation, such as smoking cessation or chemoprevention to decrease EBV reactivation, in high-risk populations may have potential in the primary prevention of EBV-associated diseases.

**Table 4**  
The interaction between smoking status and EBV seropositive rates for EBNA1-IgA and Zta-IgA among different risk areas.

Smoking status	Subjects in NPC non-endemic area			Subjects in NPC endemic area			AP <sup>b</sup>	P <sup>c</sup>	P <sup>d</sup>
	±	OR (95%CI) <sup>a</sup>	P	±	OR (95%CI) <sup>a</sup>	P			
<b>EBNA1-IgA<sup>e</sup></b>									
Never smoker	609/11	1.00 (reference)	/	429/66	9.53 (4.92–18.38)	<0.001			
≥ 20 pack-years	615/26	1.90 (0.92–3.91)	0.081	340/117	15.00 (7.90–28.46)	<0.001	0.31 (0.06–0.55)	0.024	0.645
<b>Zta-IgA<sup>f</sup></b>									
Never smoker	584/36	1.00 (reference)	/	443/54	2.25 (1.43–3.53)	<0.001			
≥20 pack-years	604/37	0.85 (0.52–1.37)	0.501	362/97	3.55 (2.32–5.41)	<0.001	0.41 (0.14–0.68)	0.003	0.045

<sup>a</sup> Adjusted for age (continuous variables), education (primary school or less, high school, university or more).  
<sup>b</sup> AP = attributable proportion due to interaction.  
<sup>c</sup> P values for the AP.  
<sup>d</sup> P values for multiplicative interaction analysis.  
<sup>e</sup> For EBNA1-IgA antibody, weak seropositive ones and seropositive ones were combined together to increase the statistic power since the limited positive sample size and represent the seronegative group vs. the combined group which consist of weak seropositive and seropositive status together with the reference cutoff defined by manufactures.  
<sup>f</sup> The seronegative group vs. the seropositive group with the reference cutoff defined by manufactures for Zta-IgA antibody.

## Conflicts of Interests

None.

## Author Contributions

WHJ conceived, designed the study and wrote the final draft. YQH participated in study design, performed data collection and analysis, drafted the manuscript. WQX, FHX, YFX, JBZ, HLY assisted the experiments. QSF, LZC, SMC and QL assisted participant recruitment and data collection. JIM assisted manuscript edition. YXZ participated in the study design. All authors read and approved the final manuscript.

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## Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.02.019>.

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