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ARTICLE

An Epidemiological and Molecular Study of the Relationship Between Smoking, Risk of Nasopharyngeal Carcinoma, and Epstein–Barr Virus Activation

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Background Elevated levels of antibodies against antigens in the Epstein–Barr virus (EBV) lytic phase are important predictive markers for nasopharyngeal carcinoma (NPC) risk. Several lifestyle factors, including smoking, have also been associated with NPC risk. We hypothesized that some specific lifestyle factors induce transformation of EBV from the latent to the lytic stage and contribute to NPC occurrence.

Methods We conducted a case–control study using data from male case patients (n = 1316) and control subjects (n = 1571) living in Guangdong Province, an area in China at high risk for NPC, to study potential NPC risk factors and EBV inducers. Two independent healthy male populations from a second high-risk area (n = 1657) and a low-risk area (n = 1961) were also included in the analysis of potential EBV inducers using logistic regression models. In vitro assays were performed to investigate the effect of cigarette smoke extract on EBV activation in two EBV-positive cell lines. All statistical tests were two-sided.

Results Smoking was associated with an increased risk of NPC among the Guangdong participants with 20–40 and 40 or more pack-years vs never smokers (OR = 1.52, 95% CI = 1.22 to 1.88 and OR = 1.76, 95% CI = 1.34 to 2.32, respectively; $P_{trend} < .001$). Smoking was the only factor linked to EBV seropositivity among the expanded control group and the independent low-risk population. In vitro experiments showed that cigarette smoke extract promoted EBV replication, induced the expression of the immediate-early transcriptional activators *Zta* and *Rta*, and increased transcriptional expression levels of *BFRF3* and *gp350* in the lytic phase.

Conclusion Smoking is not only associated with NPC risk in individuals from China but is also associated with EBV seropositivity in healthy males and is involved in EBV activation.

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Nasopharyngeal carcinoma (NPC) is rare in most populations around the world, but it is common in South China and Southeast Asia where the incidence can reach 20–50 cancers per 100 000 person-years (1–5). Epstein–Barr virus (EBV) is associated with the occurrence and development of NPC, and several specific serologic biomarkers have been identified and widely used for screening and early diagnosis of NPC in high-risk populations in some endemic areas such as South China (6–8), which include antibodies against antigens in the EBV lytic phase, ie, IgA antibodies against EBV capsid antigen (VCA-IgA) and Zta-IgA (9–14). A few long-term prospective studies have reported that elevated levels of antibodies against EBV antigens in serum precede the development of NPC and might have a causal role in the development of NPC, although there are other causal etiological factors that might play a role (9). Also, the molecular mechanism behind EBV seroreactitivity is largely unknown.

EBV establishes a life-long persistent infection in more than 90% of the population worldwide (15). The virus remains in a latent phase in healthy individuals carrying the virus with a low copy number of episomal virus maintained in resting memory B cells (16,17). The virus can be periodically reactivated throughout an individual's lifetime during periods of environmental stress. No specific natural inducer of EBV reactivation has been well defined although studies have shown that several different chemicals, such as phorbol-12-myristate-13-acetate (TPA) (18–20), sodium butyrate (19,21), and trichostatin A (22), have been found to induce the EBV lytic cycle. In addition, a few studies have searched for inducers

of EBV reactivation among environmental factors in human life. Two previous in vitro studies have observed that Cantonese-style salted fish (23) and Chinese herbs (24) may contain EBV-inducing substances, but these observations have not been made using epidemiological data.

After EBV is activated, it switches from a latent phase to a lytic phase. This switch is marked by the ability of serological tests to identify a series of antibodies against viral antigens expressed exclusively in the lytic phase. Previous studies have shown that elevated antibody titers against various EBV lytic gene products (25) and increasing viral DNA loads (26) have been detected in the serum of NPC patients, indicating that the lytic cycle and virus replication have been reactivated (19). Antibodies in the lytic stage of EBV are markedly elevated in NPC patients and high-risk individuals. However, the cause of this phenomenon has not yet been fully understood, and the etiological mechanism remains unclear. The switch from the latent phase to the lytic phase may be one of the crucial steps in the course of NPC development (27).

Previous epidemiological studies have documented that consumption of salted fish, consumption of other preserved foods, and smoking tobacco may be associated with elevated NPC risk (28). We hypothesized that specific factors among the confirmed or potential risk factors of NPC may act as environmental inducers to initiate the switch from the EBV latent stage to a lytic stage, therefore resulting in a series of elevated antibodies against viral antigens in the lytic phase.

To investigate the epidemiological relationship behind transformation of EBV from the latent to the lytic stage and NPC, we focused our study on eight suspected risk factors for NPC, including family history of NPC, smoking, and consumption of alcohol, tea, Chinese herbal tea, Cantonese slow-cooked soup, salted fish, and preserved vegetables. In vitro experiments in human EBV-positive cell lines were done to determine the molecular mechanism behind this transformation.

Materials and Methods

Study Populations

The study was reviewed and approved by the Human Ethics Approval Committee at Sun Yat-sen University Cancer Center. Written informed consent was obtained from each study subject. Characteristics of the study populations are summarized in Supplementary Table 1 (available online).

Guangdong Case–Control Study and 21-RCCP Population. A large-scale case–control study population has been previously described (29–31). Briefly, NPC case patients were identified from Sun Yat-sen University Cancer Center in Guangzhou of Guangdong province, China between October 1, 2005 and October 1, 2007. During the same period, healthy control subjects were recruited from physical examination centers in 21 regions in Guangdong Province (defined as 21 regions collected from the Cantonese population and abbreviated hereafter as [21-RCCP]) and matched to NPC case patients by geographical area, age (±5 years), and sex. All subjects were asked to complete an in-person interview. Demographic data including age, education, and family history of NPC in first-degree relatives were collected. Also, data on eight putative risk factors including family history of NPC, smoking, consumption of alcohol, tea, Chinese herbal tea, Cantonese slow-cooked soup, salted fish, and preserved vegetables were collected. The lifestyle questionnaire (Supplementary Materials, available online) was based on questionnaires issued by The University of Arizona Cancer Center (32) and Lifetime Drinking History issued by the European Monitoring Centre for Drugs and Drug Addiction (33). In detail, the dietary questionnaire was based on the Arizona Food Frequency Questionnaire (32); the tea questionnaire was based on the Arizona Tea Frequency Questionnaire (32); the smoking questionnaire was based on the Arizona Smoking Assessment Questionnaire (32). Alcohol consumption was measured using the Lifetime Drinking History (33) and Arizona Food Frequency Questionnaire (32). Regarding the intake of certain foods (salted fish, preserved vegetables, and slow-cooked soup) and drinks (tea and herbal tea), subjects were asked to choose from three intake frequency categories of less than monthly, monthly, and weekly or more.

For cigarette smoking, individuals who had smoked at least 100 cigarettes in their lifetime were defined as ever smokers, including current smokers and ex-smokers. Ex-smokers were defined as those subjects who had quit smoking more than 1 year before the interview. The cumulative amount of cigarette smoking was defined in pack-years by multiplying the number of packs of cigarettes smoked per day by the number of years of smoking. We investigated various cigarette smoking categories as follows: age at which the individual started smoking, the cumulative amount, and type of smoking (defined as filtered or nonfiltered cigarettes).

The consumption of alcohol was recorded as the average amount of all alcoholic beverages (including beer, wine, and liquor) that participants consumed during each time period in which regular drinking was consistent. A medium serving size (one drink) was defined as 12 ounces of beer, 5 ounces of wine, or 1.5 ounces of liquor (34).

A total of 1871 NPC patients and 2275 healthy control subjects were enrolled, and the response rates were 96% for the case patients and 66% for the control subjects. For the 21-RCCP study, 1316 male case patients ranging from age 14 to 80 years (mean age = 46.3 years) and 1571 male control subjects ranging from age 14 to 80 years (mean age = 46.9 years) were analyzed. Fifty of these case patients and 45 of the healthy control subjects were excluded from the analysis because of missing data.

Shihui and Yangquan Populations. To confirm the observations made in the 21-RCCP, we extended our study to two additional independent healthy populations. First, in Sihui City, Guangdong Province in a NPC endemic area (defined as Sihui population), participants were enrolled from September 1 to December 31, 2008. The subjects included in the Sihui population were healthy male residents from several administrative villages in Sihui City, and the educational levels of all study subjects were at the high school level or below. Initially, 1674 healthy males were enrolled, but 17 subjects were excluded for missing data. The response rate of the Sihui population was 72%, and 1657 healthy subjects ranging from age 18 to 67 years with a mean age of 45.5 years were included in the analysis.

Table 1. Multivariable analyses of Epstein–Barr virus (EBV), family history of nasopharyngeal cancer, and environmental factors in Guangdong men

Variable	No. of case patients	No. of control subjects	OR (95% CI)*	*P**
EBV VCA-IgA				
Negative	44	1296	1.00 (referent)	
Positive	1272	275	144 (103 to 202)	<.001
Family history of NPC				
No	1157	1517	1.00 (referent)	
Yes	146	54	3.50 (2.52 to 4.85)	<.001
Cigarette smoking, pack-years				
Never	357	519	1.00 (referent)	
<20	382	576	0.86 (0.71 to 1.05)	.13
<40	358	311	1.52 (1.22 to 1.88)	<.001
≥40	192	159	1.76 (1.34 to 2.32)	<.001
P_{trend}				<.001
Alcohol				
Nondrinker	615	627	1.00 (referent)	
≤1 drink per day	487	745	0.70 (0.59 to 0.83)	<.001
>1 drink per day	210	199	1.09 (0.87 to 1.37)	.47
P_{trend}				.31
Tea				
Less than monthly	351	257	1.00 (referent)	
Monthly	246	329	0.58 (0.46 to 0.73)	<.001
Weekly or more	697	982	0.55 (0.45 to 0.67)	<.001
P_{trend}				<.001
Herbal tea				
Less than monthly	312	293	1.00 (referent)	
Monthly	591	867	0.64 (0.53 to 0.78)	<.001
Weekly or more	398	394	0.89 (0.72 to 1.11)	.30
P_{trend}				.58
Slow-cooked soup				
Less than monthly	199	62	1.00 (referent)	
Monthly	162	154	0.33 (0.23 to 0.48)	<.001
Weekly or more	933	1338	0.24 (0.18 to 0.32)	<.001
P_{trend}				<.001
Salted fish				
Less than monthly	1046	1398	1.00 (referent)	
Monthly	150	80	2.50 (1.87 to 3.33)	<.001
Weekly or more	115	85	1.74 (1.29 to 2.35)	<.001
P_{trend}				<.001
Preserved vegetable				
Less than monthly	957	1364	1.00 (referent)	
Monthly	194	96	2.82 (2.17 to 3.67)	<.001
Weekly or more	163	102	2.22 (1.70 to 2.90)	<.001
P_{trend}				<.001

* Odds ratios (ORs) and *P* values were calculated using logistic regression analysis and adjusted for age (years, continuous variable) and education (high school or less, university or more). All tests were two-sided. CI = confidence interval; EBV VCA-IgA = IgA antibodies against Epstein–Barr virus capsid antigens.

Data from a second independent healthy population from Yangquan City, Shanxi Province in a NPC nonendemic area (defined as Yangquan population), were collected between March 1 and August 1, 2010. For the Yangquan population, the subjects were healthy male residents who attended the First General Hospital of Yangquan City for health examinations. Initially, 2016 healthy males were recruited, but 55 subjects were excluded from the study because of missing data. The response rate was 67%. Overall, data from 1961 healthy subjects ranging from age 16 to 87 years with a mean age of 47.0 years were analyzed.

All of the participants answered a structured questionnaire including demographic information and potential risk factors during a face-to-face interview. Three factors that are specific for Cantonese (21-RCCP and 1403 subjects of the Sihui population) were not included in the Yangquan population, including consumption of Cantonese herbal tea, slow-cooked soup, and salted fish. Only healthy males were included in the analysis because of the exceptionally low percentage of females who have reported their smoking history (less than 2% in the female population).

VCA-IgA Antibody Titers of EBV in Blood Specimens

For all three populations (the Guangdong, Shihui, and Yangquan populations), 5–10 mL of each subject's blood was collected. The VCA-IgA antibody titers of EBV were measured using a commercial kit (Zhongshan Bio-tech Co Ltd, Zhongshan City, China) based on standard immunoenzymatic techniques, which were described previously (35,36). In brief, the protocol steps are as follows: 1) B95-8 cell smears were prepared and aliquoted in the wells of slides to serve as antigen; 2) diluted sera were added to the wells; 3) the sera were incubated for 30 minutes at 37°C; 4) wells were washed with

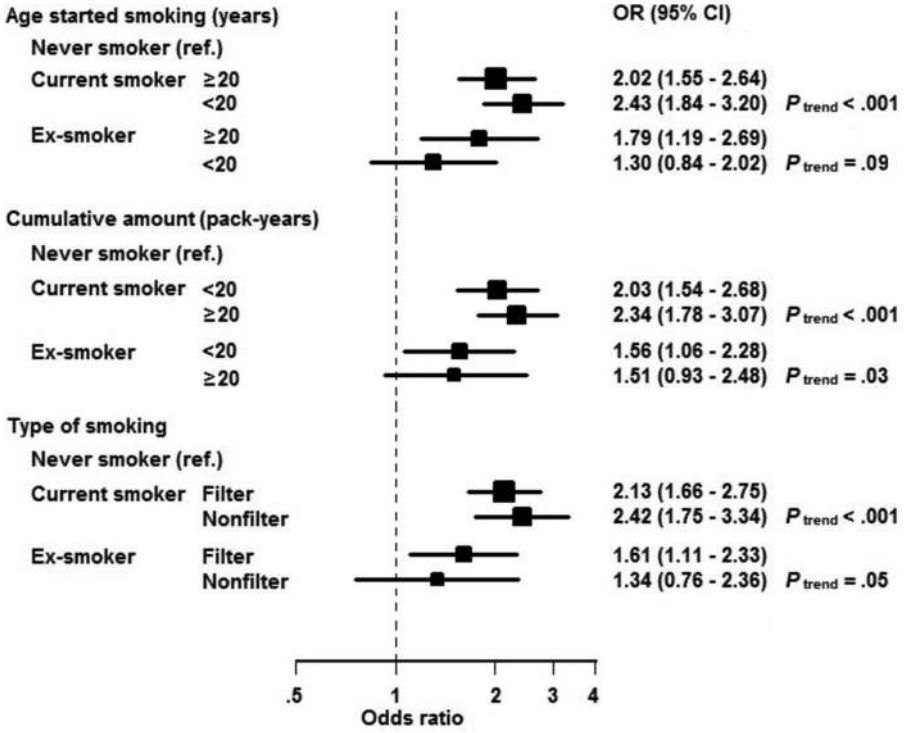

Figure 1. The relationship between cigarette smoking and EBV VCA-IgA antibody serostatus among current smokers and ex-smokers. Odds ratios (ORs) for the VCA-IgA seropositive vs seronegative current smokers and ex-smokers were calculated. Because of the small number of ex-smokers, we stratified smoking categories into two instead of three levels for age: started smoking (<20, ≥20 years) and cumulative amount (<20, ≥20 pack-years). Both groups were adjusted for age (years, continuous variable) and education (high school or less, university or more) using logistic regression analysis with never smokers as a reference (**ref.**), and the tests were two-sided. **Squares** = study-specific odds ratios; **horizontal lines** = study-specific confidence intervals (CIs); and **solid vertical line** = odds ratio of 1.0. EBV VCA-IgA = IgA antibodies against Epstein–Barr virus capsid antigens.

phosphate buffered saline three times; 5) peroxidase-conjugated antihuman IgA antibody (Zhongshan Bio-tech Co Ltd) was added, the wells were incubated at 37°C for 1 hour, and then washed three times with phosphate buffered saline; 6) the slides were flooded with 0.3 mg/mL aminoethylcarbazole solution (Sigma-Aldrich Co, St. Louis, MO) and 30% H_2O_2 (Guangzhou Chemical Reagent Factory, Guangzhou City, China) for 15 minutes. The slides were then examined under a light microscope (10×). Brown staining was considered positive. To minimize bias, samples were blinded to laboratory personnel. Serial dilutions of quality control sera (1:10, 1:20, 1:40, 1:80) were applied to each assay for the evaluation of intraset variability. To minimize experimental error, all tests were conducted in the same laboratory by the same technicians.

Serum Concentration of Total IgA

The serum concentration of total IgA was measured by immunoturbidimetry method using automatic analyzer 7600-020 (Hitachi Ltd, Tokyo, Japan) in the clinical laboratory of SYSUCC. The kits used in the assay were supplied by Sichuan Maker Biotechnology Co Ltd (Chengdu City, China).

Cell Culture

EBV-positive Akata, EBV-negative Akata (37), CNE2, and B95-8 cell lines were cultured as described in the Supplementary Methods (available online). The previous research reported that in vitro latently infected EBV-harboring cells could be reactivated to the lytic replication phase by treatment with TPA (20). The green fluorescent protein (GFP)-EBV-positive Akata cells start to produce EBV efficiently after cross-linking of cell surface immunoglobulin G (IgG) using anti-IgG antibodies (38,39). Treatment with 20 ng/mL TPA (stock dilution was prepared using ethanol) (Sigma-Aldrich) and 100 μg/mL rabbit anti-human IgG (Dako-Cytomation, Carpinteria, CA) served as positive controls for induction of EBV reactivation.

Preparation and Characterization of CSE

The CSE preparation process and determination of nicotine level in the CSE by high-performance liquid chromatography are summarized in the Supplementary Methods and Results and Supplementary Figure 1 (available online).

Flow Cytometry

Exponentially growing EBV-negative and EBV-positive Akata cell lines were treated with TPA (20 ng/mL), anti-IgG (100 μg/mL), CSE (0.5%–2%), or were untreated. The cells were washed twice with phosphate buffered saline at 24 or 48 hours posttreatment and were then evaluated by flow cytometry (FACS Diva Option, Becton Dickinson, Mountain View, CA) using signal wave length analysis.

Immunofluorescence Analysis

Immunofluorescence analysis was performed as a modification of the method described previously (40). EBV capsid antigen expression in the cell lines was evaluated by indirect immunofluorescence following sequential incubation with a serum mixture obtained

from ten NPC patients with high VCA-IgA titers, a polyclonal rabbit anti-human IgA (1:100) (Dako, Glostrup, Denmark), and a cyc3-conjugated anti-rabbit IgG (1:1000) (Jackson Laboratory, West-Grove, PA). Cellular expression of Zta or Rta was assessed using a monoclonal antibody specific for the Zta protein (1:40) (Dako) or anti-Rta (1:40) (Argene, Varilhes, France) and rhodamine-conjugated goat anti-mouse IgG (1:1000) (Jackson Laboratory). After three washes, the slides were stained with 0.5 μg/mL 4-,6-diamidino-2-phenylindole (1 mg/mL stock prepared in deionized water; Sigma-Aldrich) to visualize the nuclei and were examined using an Olympus confocal imaging system (Olympus FV1000) at 40× objective (oil immersion). Images were then analyzed via the Olympus FV 1000 software (Olympus, Fluoview, version 1.7, Tokyo, Japan). Experiments were performed in triplicate.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Cell lines treated with anti-IgG, TPA, or CSE or untreated cell lines were harvested at the indicated times. qRT-PCR was performed as described previously (41). Sequence data for the EBV genome were acquired from the GenBank sequence database (accession number V01555). The primers used for real-time RT-PCR to detect transcriptional levels of BZLF1, BRLF1, BFRF3, and gp350/220 are listed in Supplementary Table 2 (available online). Also, the primers used for qRT-PCR to detect the relative amount of EBV DNA are also shown in Supplementary Table 2 (available online). qRT-PCR and data collection were performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA). The data were analyzed using the RQ Manager Software (version 1.2, Applied Biosystems Inc) system.

Plasmid Construction, Transfection, and Luciferase Assay

The entire BRLF1 promoter sequence of the EBV genome from B95-8 was amplified with primers (5′-primer: 5′-GGCTCGAGA AAAAGGCCGGCTGACAT GGATTACTGG-3′; 3′-primer: 5′-GGGAGCTCTAAAGGCCCTGTCGTCGGGA GATAAAAGTG-3′) to generate a fragment from nucleotide 107144 to 106177 upstream of the starting codon ATG with XhoI and SacI sites at each end, respectively (42). The amplified PCR fragment was cloned into the promoterless pGL3-basic luciferase reporter plasmid (Promega, Madison, WI) to generate the pGL3-BRLF1-promoter. Genomic DNA from B95-8 cells was used as a template. Plasmids were prepared from *Escherichia coli* by CsCl–ethidium bromide centrifugation (43). Transfection of plasmids into CNE2 was performed by using the FuGENE HD transfection reagent (Roche, Mannheim, Germany) by following the manufacturer's protocol. Cells were treated with 20 ng/mL TPA or 2% CSE added to the culture medium 5 hours after transfection. The BRLF1 promoter luciferase assay was performed with the Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instructions.

Statistical Analyses

Throughout data processing, double entry of EpiData Entry software (EpiData Association, http://www.epidata.dk) was used. Logistic regression analysis was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) after adjusting for age (years, continuous variable) and education (high school or less, university or more). Linear trend tests for associations between exposure and EBV VCA-IgA antibodies were performed for continuous variables including smoking, alcohol, tea, herbal tea, salted fish, and preserved vegetables. The possible synergistic effect of smoking status and having lived in high/low-risk NPC areas was evaluated using the method proposed by Rothman (44). The synergism index (S) was calculated by the following equation: $S = (OR_{11} - 1)/(OR_{01} + OR_{10} - 2)$. STATA 10.0 (Stata Corp, College Station, TX) software and SAS 9.1 (SAS Institute, Cary, NC) were used.

The statistical power was calculated using software PASS 2008 (NCSS, LLC, Kaysville, UT) based on three parameters including α (0.05), relevant sample size, and effect size. For the analysis of the relationship between eight risk factors and NPC risk, all power values were greater than 0.999. Whereas for the analysis of the relationship between the factors and seropositivity of EBV VCA-IgA antibodies, power of greater than 0.999 was achieved for the factor of smoking. However, power ranged from 0.080 to 0.344 for other factors, suggesting that under the test level and sample sizes ($\alpha = 0.05$, n = 3228), we could not determine the statistical significance. All P values were calculated using two-sided tests, and P values less than .05 were considered to be statistically significant.

Results

Study of Risk Factors Related to NPC Risk in the Guangdong Case–Control Population

Individuals who reported a high cumulative amount of smoking (20–40 or ≥40 pack-years) had an increased risk of NPC (OR = 1.52, 95% CI = 1.22 to 1.88 and OR = 1.76, 95% CI = 1.34 to 2.32, respectively) compared with never smokers after adjustment for age and education ($P_{trend} < .001$). Only 4 of 1316 patients were diagnosed with differentiated NPC (World Health Organization type I); therefore, the histopathological type was not considered in the analysis.

The relationship between VCA-IgA antibody status and eight putative or potential risk factors with NPC risk was investigated. We observed that VCA-IgA positivity was statistically significantly associated with an increased risk of NPC (OR = 144, 95% CI = 103 to 202, $P < .001$) (Table 1). Consumption of salted fish and preserved vegetables (monthly, weekly or more) and a family history of NPC were also associated with an increased risk for NPC ($P < .001$), whereas consumption of tea or slow-cooked soup (monthly, weekly or more) were both associated with a statistically significantly reduced risk of NPC ($P < .001$). However, although consumption of herbal tea monthly or one or fewer alcoholic beverages per day was associated with a decreased risk of NPC ($P < .001$), increased levels of consumption of herbal tea or alcohol were not statistically significantly associated with NPC risk ($P_{trend} = .58$ and .31, respectively). These findings are consistent with previous reports of the relationship between smoking, consumption of alcohol, tea, Chinese herbal tea, Cantonese slow-cooked soup, salted fish, and preserved vegetables and NPC (29–31).

Relationship Between Risk Factors and EBV VCA-IgA Antibody Serostatus in High-Risk Areas for NPC

To identify potential risk factors for NPC that are associated with EBV, smoking, family history, consumption of alcohol, tea, herbal tea, slow-cooked soup, salted fish, and preserved vegetables were

Table 2. The dose–response relationship between smoking and Epstein–Barr virus VCA-IgA antibodies among two populations in China

Variable	Guangdong population*				Shanxi population				Pooled			
	No of VCA-IgA-positive men	No. of VCA-IgA-negative men	OR (95% CI)†	*P*†	No of VCA-IgA-positive men	No. of VCA-IgA-negative men	OR (95% CI)†	*P*†	No of VCA-IgA positive men	No. of VCA-IgA negative men	OR (95% CI) †	*P*†
Smoking												
Never smoker	95	749	1.00 (referent)		33	587	1.00 (referent)		128	1336	1.00 (referent)	
Ex-smoker	76	364	1.51 (1.09 to 2.11)	.02	13	174	1.35 (0.68 to 2.66)	.39	89	538	1.60 (1.19 to 2.15)	.002
Current smoker	432	1512	2.17 (1.70 to 2.78)	<.001	92	1062	1.64 (1.08 to 2.51)	.02	524	2574	2.01 (1.63 to 2.47)	<.001
P_{trend}				<.001				.02				<.001
Age started smoking, y												
Never smoker	95	749	1.00 (referent)		33	587	1.00 (referent)		128	1336	1.00 (referent)	
≥30	44	173	1.83 (1.22 to 2.73)	.003	3	94	0.58 (0.17 to 1.94)	.38	47	267	1.69 (1.17 to 2.43)	.005
20–29	232	887	1.98 (1.53 to 2.58)	<.001	45	563	1.47 (0.92 to 2.36)	.11	277	1450	1.90 (1.52 to 2.38)	<.001
<20	232	816	2.14 (1.64 to 2.79)	<.001	57	579	1.92 (1.22 to 3.04)	.005	289	1395	2.03 (1.62 to 2.55)	<.001
P_{trend}				<.001				.003				<.001
Cumulative amount, pack-years												
Never smoker	95	749	1.00 (referent)		33	587	1.00 (referent)		128	1336	1.00 (referent)	
<20	214	894	1.89 (1.45 to 2.45)	<.001	47	640	1.41 (0.88 to 2.27)	.15	261	1534	1.76 (1.41 to 2.21)	<.001
<40	185	688	2.04 (1.54 to 2.70)	<.001	33	404	1.50 (0.90 to 2.51)	.12	218	1092	1.94 (1.53 to 2.46)	<.001
≥40	108	286	2.74 (1.96 to 3.82)	<.001	24	180	2.52 (1.39 to 4.56)	.002	132	466	2.67 (2.01 to 3.55)	<.001
P_{trend}				<.001				.004				<.001
Type of smoking												
Never smoker	95	749	1.00 (referent)		33	587	1.00 (referent)		128	1336	1.00 (referent)	
Filtered cigarettes	365	1386	2.01 (1.57 to 2.58)	<.001	103	1209	1.61 (1.06 to 2.43)	.03	468	2595	1.82 (1.48 to 2.25)	<.001
Nonfiltered cigarettes	126	422	2.13 (1.57 to 2.90)	<.001	2	20	1.80 (0.40 to 8.17)	.45	128	442	2.65 (2.00 to 3.49)	<.001
P_{trend}				<.001				.03				<.001

* The two populations of men from 21 regions collected from the Cantonese population and Sihui population combined.

† Odds ratios (ORs) with 95% confidence intervals (CIs) and *P* values were calculated using logistic regression analysis and adjusted for age (years, continuous variable) and education (high school or less, university or more). All statistical tests were two-sided. VCA-IgA = IgA antibodies against Epstein–Barr virus capsid antigens.

compared between EBV VCA-IgA antibody seropositive and seronegative groups. A cutoff value of 1:10 was used to differentiate between seropositive and seronegative subjects, and it has been widely used in the screening of high-risk individuals in the endemic regions of Guangdong, China in other studies (35,36,45). Because we did not observe any heterogeneity between the 21-RCCP and the Sihui populations when we examined the effects of the factors on EBV serostatus ($P_{heterogeneity}$ = .25 to .97) (data not shown), we combined the subjects in the 21-RCCP and the Sihui population to maximize the statistical power. A total of 275 and 328 participants had a positive antibody titer (≥1:10), and a total of 1296 and 1329 participants had a negative antibody titer among the 21-RCCP and Shihui healthy populations, respectively (Supplementary Table 1, available online). We found that smoking was the only factor associated with seropositivity for the VCA-IgA antibody. Table 2 shows that individuals who started smoking at a younger age were associated with a higher risk of being seropositive for the VCA-IgA antibody when compared with never smokers (started smoking when age ≥ 30 years: OR = 1.83, 95% CI = 1.22 to 2.73; age 20–29 years: OR = 1.98, 95% CI = 1.53 to 2.58; age ≤ 20 years: OR = 2.14, 95% CI = 1.64 to 2.79; P_{trend} < .001). Moreover, the likelihood of being seropositive for VCA-IgA antibody increased with the cumulative amount of smoking (OR = 1.89, 95% CI = 1.45 to 2.45; OR = 2.04, 95% CI = 1.54 to 2.70; OR = 2.74, 95% CI = 1.96 to 3.82 for <20, 20–40, and ≥40 pack-years, respectively; P_{trend} < .001). With regard to the type of smoking, smoking filtered or nonfiltered cigarettes was associated with VCA-IgA antibody seropositivity (OR = 2.13, 95% CI = 1.57 to 2.90; OR = 2.01, 95% CI = 1.57 to 2.58, respectively).

Interestingly, the relationship between NPC risk and serum VCA-IgA antibody status among ex-smokers was weaker than that among current smokers (OR = 1.51, 95% CI = 1.09 to 2.11; OR = 2.17, 95% CI = 1.70 to 2.78, respectively; P_{trend} < .001), compared with the never smokers (Table 3). Strong associations between cigarette smoking and the VCA-IgA antibody serotype in current smokers were observed in all subgroups with odds ratios ranging from 2.02 to 2.43, and weaker associations were observed for ex-smokers with odds ratios ranging from 1.30 to 1.79 (Figure 1). The risk of being seropositive for the VCA-IgA antibody for ex-smokers was much lower than that for current smokers (adjusted OR = 0.69, 95% CI = 0.52 to 0.91, P = .008).

We also evaluated the associations of the remaining seven risk factors and EBV serotype but did not detect any statistically significant findings (Table 3). So far, among the eight factors that we focused on in our study, which were previously shown to be statistically significantly associated with NPC risk, smoking is the only factor associated with elevated titers of VCA-IgA antibody. In addition, associations between these eight factors and EBV serostatus were further adjusted for age, education, family history of NPC, and consumption of salted fish and preserved vegetables, and all the results were similar to the above results that included adjustment for age and education only (data not shown).

Cigarette Smoking and Seropositivity of VCA-IgA Antibodies in the Yangquan Population in a Low-Risk Area

All of the above findings prompted an investigation of whether or not the relationship was consistent between smoking habit and serostatus of the VCA-IgA antibody not only in populations in high-risk areas but also in low-risk areas for NPC. Thus, a third population in a low-risk area of Yangquan City in North China was investigated (Yangquan population). A total of 1961 subjects were recruited, including 138 subjects who were seropositive for VCA-IgA antibody, and the seropositive rate (7.0%) was much lower than that of the 21-RCCP (17.5%) and the Sihui population (19.8%). Nevertheless, a similar result that cigarette smoking was also related to the seropositivity of the VCA-IgA antibody in this area was observed, and it was also uniquely statistically significantly related to elevated titers of anti-VCA-IgA (Table 2).

However, the association between cigarette smoking and an increased risk of VCA-IgA antibody seropositivity in the Guangdong high-risk population (21-RCCP and Sihui population) was higher than that in the low-risk population (Yangquan population) among ever smokers in Guangdong and Yangquan (OR = 5.09, 95% CI = 3.47 to 7.48; OR = 1.60, 95% CI = 1.06 to 2.42, respectively), compared with never smokers among the Yangquan population (Table 4). The interaction effect (synergism "*S*" index) between the smoking status and regions of high-risk and low-risk areas for NPC on the VCA-IgA antibody seropositivity was 1.96 (95% CI = 1.30 to 2.97) when an additive model was used, but it was not statistically significant when a multiplicative model was used (P = .15).

Relationship of Cigarette Smoking and VCA-IgA Antibody Seropositivity Among the 21-RCCP, Sihui, and Yangquan Populations

To increase the power of detecting the association (46) between EBV serostatus and smoking, we jointly analyzed the data from the three study populations after finding no heterogeneity between the Guangdong (21-RCCP and Sihui population) high-risk populations and the low-risk Yangquan population ($P_{heterogeneity}$ = .15), except for one specific age group of those older than 55 years ($P_{heterogeneity}$ = .03). We observed a consistent trend indicating that as the amount of smoking increases, so does the likelihood of VCA-IgA antibody seropositivity among both the high-risk and low-risk populations (Table 2). The level of statistical significance of the results for each subgroup from the pooled analysis was increased (the corresponding P values decreased dramatically); for example, for the subgroup consisting of individuals whose cumulative amount of smoking was 40 pack-years or more, the P value decreased from 1×10^{-9} (in single analysis of Guangdong populations) to 1×10^{-12}, suggesting that the power was increased.

Relationship Between Smoking and Serum Concentration of Total IgA

We assessed the relationship between smoking and general IgA levels. Serum samples positive for the VCA-IgA antibody (n = 100, including 51 ever smokers and 49 never smokers) as well as serum samples negative for the antibody (n = 100, including 50 ever smokers and 50 never smokers) were identified. We did not observe any difference in the serum concentration of total IgA between seropositive (mean = 1.87 g/L, SD = 0.92 g/L) and seronegative (mean = 1.67 g/L, SD = 0.79 g/L) groups (P = .10). However, when the samples were divided into subgroups of ever smokers and never smokers, the serum concentration of total IgA in ever smokers (mean = 1.54 g/L, SD = 0.82 g/L) was lower than that in never

Table 3. The association between suspected risk factors for nasopharyngeal carcinoma and Epstein–Barr virus VCA-IgA antibody serostatus among healthy men in Guangdong*

Variable	VCA-IgA antibody serostatus*		OR (95% CI)	*P*
	No. of positive men	No. of negative men		
Age, y				
≤30	21	126	1.00 (referent)	
31–40	137	710	1.14 (0.69 to 1.87)	.61
41–50	198	882	1.30 (0.79 to 2.12)	.30
51–60	194	751	1.48 (0.91 to 2.42)	.12
≥61	53	156	2.03 (1.16 to 3.55)	.01
P_{trend}				<.001
Family history of NPC				
No	531	2331	1.00 (referent)	
Yes	22	84	1.15 (0.71 to 1.86)	.56
Smoking				
Never smoker	95	749	1.00 (referent)	
Ex-smoker	76	364	1.51 (1.09 to 2.11)	.02
Current smoker	432	1512	2.17 (1.70 to 2.78)	<.001
P_{trend}				<.001
Alcohol				
Nondrinker	248	1054	1.00 (referent)	
≤1 drink per day	212	1016	0.94 (0.76 to 1.15)	.53
>1 drink per day	93	351	1.12 (0.86 to 1.47)	.40
P_{trend}				.64
Tea				
Less than monthly	127	593	1.00 (referent)	
Monthly	108	472	1.13 (0.85 to 1.51)	.40
Weekly or more	315	1345	1.10 (0.87 to 1.38)	.44
P_{trend}				.50
Herbal tea				
Less than monthly	88	411	1.00 (referent)	
Monthly	311	1406	1.08 (0.83 to 1.41)	.56
Weekly or more	151	587	1.27 (0.94 to 1.70)	.12
P_{trend}				.10
Slow-cooked soup				
Less than monthly	166	700	1.00 (referent)	
Weekly or more	383	1702	0.96 (0.78 to 1.17)	.67
Salted fish				
Less than monthly	514	2204	1.00 (referent)	
Monthly	21	129	0.69 (0.43 to 1.11)	.12
Weekly or more	17	79	0.84 (0.49 to 1.43)	.51
P_{trend}				.19
Preserved vegetable				
Less than monthly	431	1930	1.00 (referent)	
Monthly	93	355	1.17 (0.91 to 1.50)	.23
Weekly or more	28	126	0.96 (0.63 to 1.47)	.86
P_{trend}				.58

* Odds ratios (ORs) and *P* values were calculated using logistic regression analysis and adjusted for age (years, continuous variable) and education (high school or less, university or more). All statistical tests were two-sided. CI = confidence interval; VCA-IgA = IgA antibodies against Epstein–Barr virus capsid antigens.

Table 4. An interaction analysis of smoking status and Epstein–Barr virus VCA-IgA antibody serostatus among high- and low-risk male populations*

Smoking status	Low-risk area (Yangquan population)			High-risk area (21-RCCP and Sihui population)		
	No of VCA-IgA-positive men	No. of VCA-IgA-negative men	OR (95%CI)	No of VCA-IgA-positive men	No. of VCA-IgA-negative men	OR (95%CI)
Never smoker	33	587	1.00 (referent)	95	749	2.49 (1.63 to 3.80)
Ever smoker	105	1236	1.60 (1.06 to 2.42)	508	1876	5.09 (3.47 to 7.48)
Interaction effect (synergism *S* index)†			1.96 (95% CI = 1.30 to 2.97)			

* Odds ratios (ORs) with 95% confidence intervals (CIs) and *P* values were calculated using logistic regression analysis and adjusted for age (years, continuous variable) and education (high school or less, university or more). All tests were two-sided, and *P* values less than .05 were considered to be statistically significant. 21- RCCP = 21-regions collected from the Cantonese population; VCA-IgA = IgA antibodies against Epstein–Barr virus capsid antigens.

† The synergism *S* index was calculated by equation $S = (OR_{11} - 1)/(OR_{01} + OR_{10} - 2)$.

smokers (mean = 2.01 g/L, SD = 0.84 g/L) ($P < .001$), suggesting that smoking is not associated with increasing IgA levels in general.

CSE and EBV Replication in EBV-Positive B-Cell Lines

The results obtained from the epidemiological study encouraged us to investigate whether the association of smoking exposure with elevated VCA-IgA antibodies was attributed to a direct reactivation of a switch from latent to lytic EBV. Thus, we performed in vitro experiments in cell lines to test this hypothesis.

To elucidate the effect of CSE on EBV replication, we tested whether or not CSE promotes EBV replication in Akata and B95-8 cells. Akata cells were first treated with CSE. As shown in Figure 2, A, the proportion of GFP-EBV-positive cells increased from 5.7% to 9.9%. Similarly, the intensity of GFP was enhanced in a dose-dependent manner following CSE treatment at both 24 and 48 hours ($P < .05$) (Figure 2, B–D). Moreover, an additive effect in the enhancement of fluorescent intensity was observed in cells treated with both CSE and TPA compared with cells treated with TPA alone. The increased expression of GFP was consistent with the enhanced EBV replication. The amount of EBV DNA in Akata cells was increased after treatment with anti-IgG, TPA, or CSE for 24 hours compared with untreated cells ($P < .05$) (Figure 2, E and F). Similar results were obtained in B95-8 cells (Figure 2, E). The relative number of EBV DNA copies increased as early as 4 hours after treatment with CSE and peaked after 12 hours; a 4-fold to 21-fold increase in the replication of EBV DNA was detected in Akata cells at different time points after CSE treatment (Figure 2, F). Taken together, these results suggest that CSE promotes EBV replication in Akata and B95-8 cells.

Expression of Lytic-Phase Late Gene Products of EBV After CSE Treatment

As the rate of replication of EBV DNA in Akata and B95-8 cell lines was increased after treatment with CSE, we tested if the expression of lytic-phase late genes, including the VCA p18 encoded BFRF3 and gp350, was induced in CSE-treated cells. As shown in Figure 3, A and B, the transcriptional levels of these genes in cells treated with anti-IgG, TPA, or CSE were elevated compared with untreated cells ($P < .05$). CSE induced a time-dependent increase in BFRF3 and gp350 expression in Akata cells. Furthermore, the relative transcriptional levels of these two genes increased 12 hours after induction by CSE and reached a peak at 36 hours ($P < .01$) (Figure 3, C and D). In addition, immunofluorescence staining showed an increased population of VCA-positive cells after 48 hours of CSE treatment in both Akata (1.70-fold induction; $P < .05$) and B95-8 (1.76-fold induction; $P < .01$) cells (Figure 3, E–G). Together, these data suggest that CSE induces the expression of late lytic gene products of EBV after CSE treatment.

Expression of EBV Transcription Factors After Treatment With CSE

Elevated mRNA levels of VCA and gp350, in addition to elevated protein levels of VCA, presumably reflect EBV reactivation in GFP-EBV-positive Akata and B95-8 cells after CSE treatment. Thus, we investigated if CSE induces the expression of BZLF1, which encodes Zta (BZLF1 transcription activator), and BRLF1, which encodes Rta (BRLF1 transcription activator). The Zta and Rta transcription factors play an important role in the switch from the latent cycle to the lytic cycle during EBV infection. The mRNA expression levels of BZLF1 and BRLF1 were statistically significantly enhanced in both cell lines after treatment for 24 hours with anti-IgG, TPA, or CSE. The induction of BZLF1 and BRLF1 expression by CSE treatment in GFP-EBV-positive Akata cells was 3.8-fold and 3.5-fold, respectively ($P < .01$), and the induction of both BZLF1 and BRLF1 in B95-8 cells was 4.5-fold ($P < .01$) (Figure 4, A and B). The expression of BZLF1 had the same trend, although there was no statistically significant increase at 4 hours ($P = .12$) (Figure 4, C). The increased BRLF1 mRNAs were observed as early as 4 hours after treatment ($P < .01$) and peaked at 12 hours ($P < .01$) compared with untreated cells (Figure 4, D). Also, immunofluorescence staining showed an increased population of Rta-positive cells and Zta-positive cells in both cell lines after 24 hours of CSE treatment (Figure 4, E–J).

Furthermore, we investigated the ability of CSE to activate EBV immediate-early promoters in CNE2 nasopharyngeal carcinoma cells. CSE treatment increased BRLF1 promoter activity by 2.87-fold, and TPA treatment also increased BRLF1 promoter activity by 2.5-fold (Figure 4, K). As expected, the control plasmid, pGl3-Basic, remained at background levels regardless of CSE or TPA treatment, and the background levels were statistically significantly lower than those exhibited by the pGL3-BRLF1-promoter ($P < .01$). Thus, these results suggested that CSE treatment enhances the expression of the EBV transcriptional factors, Zta and Rta, which may be responsible for the EBV latent-to-lytic switch.

Discussion

Cigarette smoking, a major risk factor for mortality in China (47), contributes to a variety of cancers, including lung cancer (48–50), hepatocellular carcinoma (51), colorectal cancer (52), head and neck cancer (53), and throat cancer (54). Cigarette smoke is a complex mixture of more than 4000 compounds, and many of these compounds, especially nitrosamine, are mutagenic or carcinogenic (57). Many studies have linked the exposure of tobacco smoking to NPC risk (8,55–66). In our study, we conducted a case–control study in males in high-risk areas of South China, and we observed a statistically significant association between cigarette smoking and the risk of NPC, which was consistent with the literature.

Cigarette smoking produces several reactive forms of agents, such as polycyclic aromatic hydrocarbons, aromatic amines, and N-nitrosamines, resulting in the formation of DNA adducts, which cause DNA damage (67,68). This mechanism may be important in NPC pathogenesis.

However, in NPC carcinogenesis, cigarette smoking may play an alternative role via induction of EBV reactivation. In this study, we verified that cigarette smoking and elevated VCA-IgA antibodies were both associated with NPC risk. Moreover, we observed consistently that cigarette smoking was related to seropositivity of the VCA-IgA antibody in three independent populations. In vitro assays provided direct evidence that CSE effectively induced EBV reactivation in both EBV-positive Akata and B95-8 cell lines. In our study, we further assessed the relationship between cigarette smoking and general IgA levels. The results suggest that cigarette smoking may be associated with a decreased serum concentration

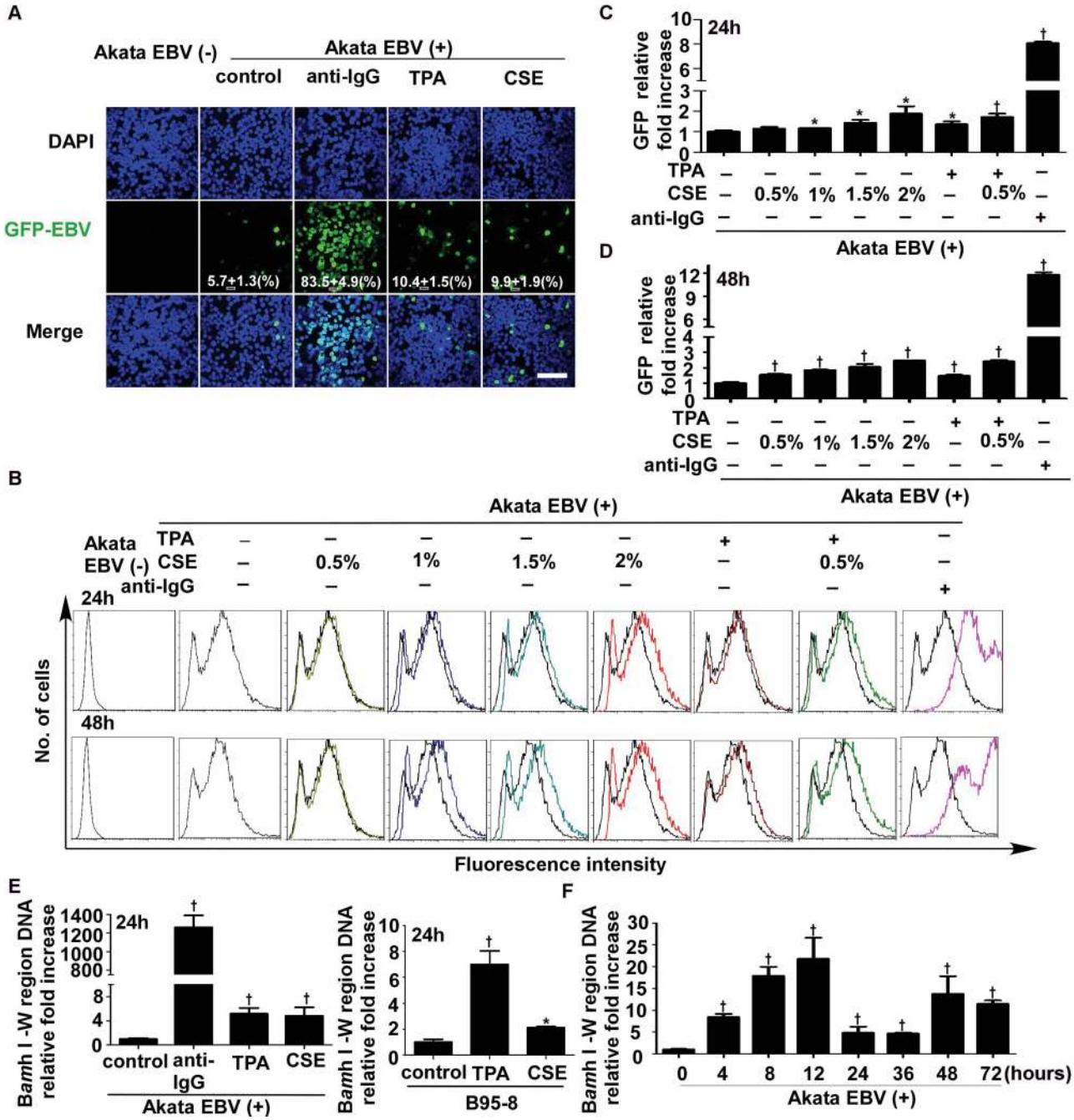

Figure 2. Effect of cigarette smoke extract (CSE) on Epstein–Barr virus (EBV) replication in EBV-positive B-cell lines. **A**) The proportion of GFP-EBV-positive cells was determined by immunofluorescence after EBV-positive and -negative AKATA cells were treated with 100 μg/mL anti-IgG, 20 ng/mL phorbol-12-myristate-13-acetate (TPA), or 2% CSE for 24 hours. The percentage of GFP-EBV-positive cells (**green**) was calculated relative to total cell populations (**blue**) in five random fields of view after treating the cells for 24 hours. Representative fields are shown. Scale bar = 100 μm. Data represent the mean and SD (n = 5). **B**) A flow histogram overlay of flow cytometric detection of green fluorescent protein (GFP)-EBV-positive cells is shown. The fluorescence intensities of EBV-positive and -negative AKATA cells treated with 0%, 0.5%, 1%, 1.5%, or 2% CSE, 20 ng/mL TPA, a combination of 0.5% CSE and 20 ng/mL TPA, or 100 μg/mL anti-IgG were compared with those of untreated cells for 24 or 48 hours. **Black lines** represent the untreated cells and **colored lines** represent treated cells. **C** and **D**) The relative fold increase of GFP expression among groups treated with 0%, 0.5%, 1%, 1.5%, or 2% CSE, 20 ng/mL TPA, a combination of 0.5% CSE and 20 ng/mL TPA, or 100 μg/mL anti-IgG for 24 or 48 hours is given. The relative fold increase of GFP expression was normalized to the amount for untreated GFP-EBV-positive Akata cells. **E** and **F**) The relative fold increase of EBV DNA in the BamHI-W region of GFP-EBV-positive Akata and B95-8 cell lines that were untreated or treated with 100 μg/mL anti-IgG, 20 ng/mL TPA, or 2% CSE after harvesting at the indicated times (0, 4, 8, 12, 24, 36, 48, and 72 hours) by real-time quantitative PCR are shown. β-globin served as the internal control. The data represent the mean and SD (**whisker bars**) from three independent experiments. Two-sided *P* values were calculated by Student *t* test. **P* < .05 compared with untreated cells. †*P* < .01 compared with untreated cells as the control.

of total IgA, which was consistent with previous reports (69,70). These findings indicate that smoking may increase seropositivity for the EBV VCA-IgA antibody, but not general IgA levels.

Few studies have indicated that cigarette smoking is associated with seropositivity of EBV. A 20-year follow-up study conducted in Taiwan found that long-term heavy cigarette smokers

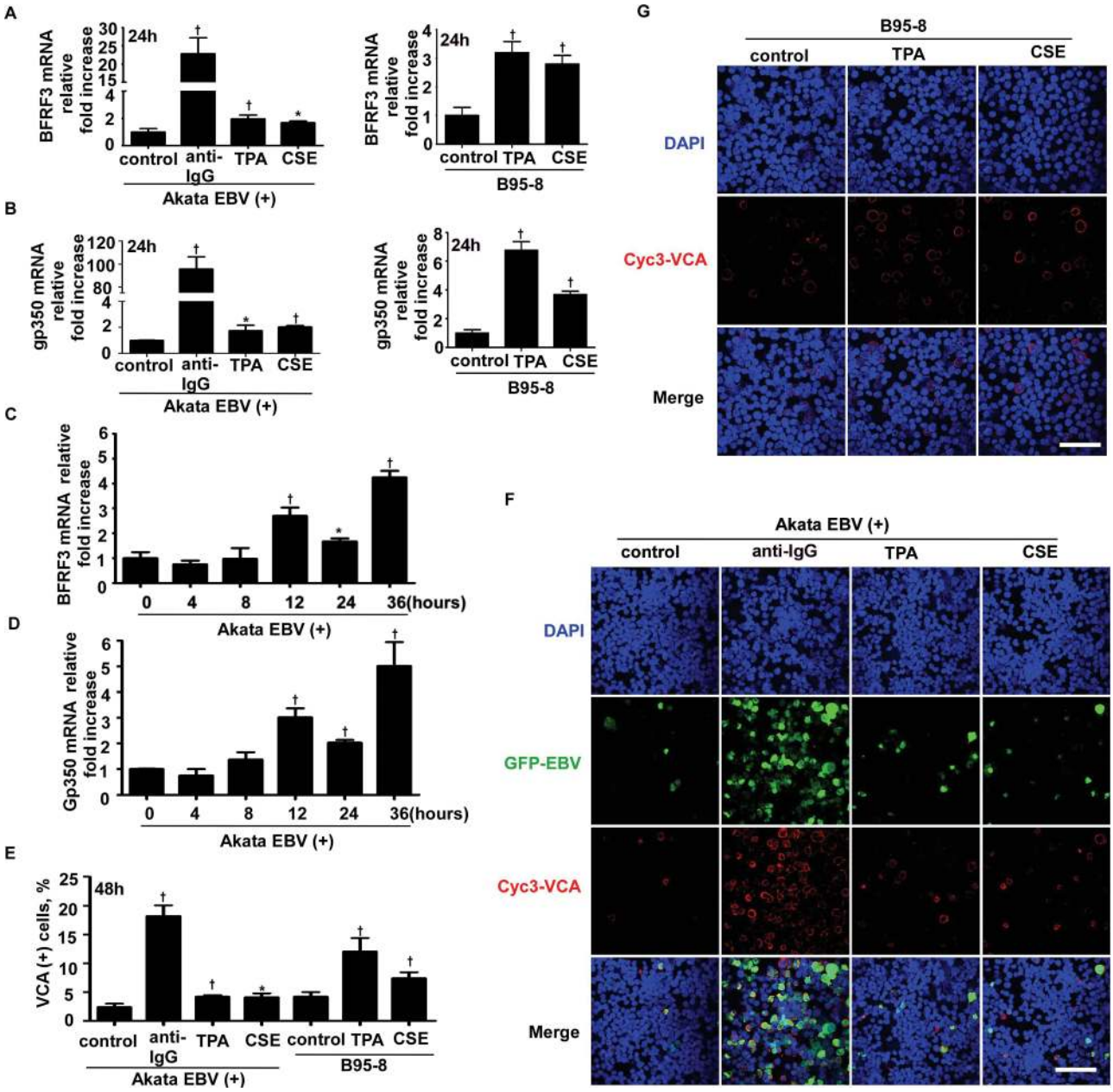

Figure 3. Expression of lytic-phase late gene products of Epstein–Barr virus (EBV) after treatment with cigarette smoke extract (CSE). (**A–D**) Expression of the viral lytic-phase late genes BFRF3 and gp350 was measured in green fluorescent protein (GFP)-EBV-positive Akata and B95-8 cell lines after treatment with 100 μg/mL anti-IgG, 20 ng/mL phorbol-12-myristate-13-acetate (TPA), or 2% CSE and harvested at 0, 4, 8, 12, 24, and 36 hours. RNA was extracted from the cells, and quantitative real-time polymerase chain reaction was done. The relative fold increase of transcripts was normalized to the amount of RNA harvested from untreated GFP-EBV-positive Akata cells. β-actin served as the internal control. Data represent the mean and SD (**whisker bars**) from three independent experiments performed in triplicate. **E**) Expression of virus capsid antigen protein (VCA) was detected by immunofluorescence after 2% CSE treatment for 48 hours. The percentage of cyc3-VCA-positive Akata EBV-positive and B95-8 cells shown in **F** and **G** (**red**) was calculated relative to the total cell number measured by 4-,6-diamidino-2-phenylindole (DAPI) staining (**blue**) in five random fields of view after treating the cells for 48 hours. Representative images are shown. Scale bar = 100 μm. Data represent the mean and SD (**whisker bars**) from three independent experiments. Two-sided *P* values were calculated by Student *t* test. **P* < .05 and †*P* < .01 compared with untreated cells as the control.

have a higher anti-EBV seropositivity rate than never smokers and short-term light smokers. However, the number of NPC case patients was small (n = 32) (8). In an analysis of 42 NPC patients in 2008, Abdulamir et al. (71) suggested that there may be an association between the serum levels of EBV IgA antibodies and smoking. In addition, in 2004, Chang et al. (72) reported that cigarette smoking is more common among EBV-positive Hodgkin lymphoma patients than among EBV-negative Hodgkin lymphoma patients. Although there has been a consistent trend showing a link between cigarette smoking and EBV-positivity in NPC or Hodgkin lymphoma patients, the sample size of these studies was usually small and could not demonstrate a statistically significant association between cigarette smoking and EBV because of inadequate statistical power. Moreover, these studies were conducted in NPC or Hodgkin lymphoma patients, and tumor burden is known to contribute to the reactivation of EBV (73). Therefore, it may be

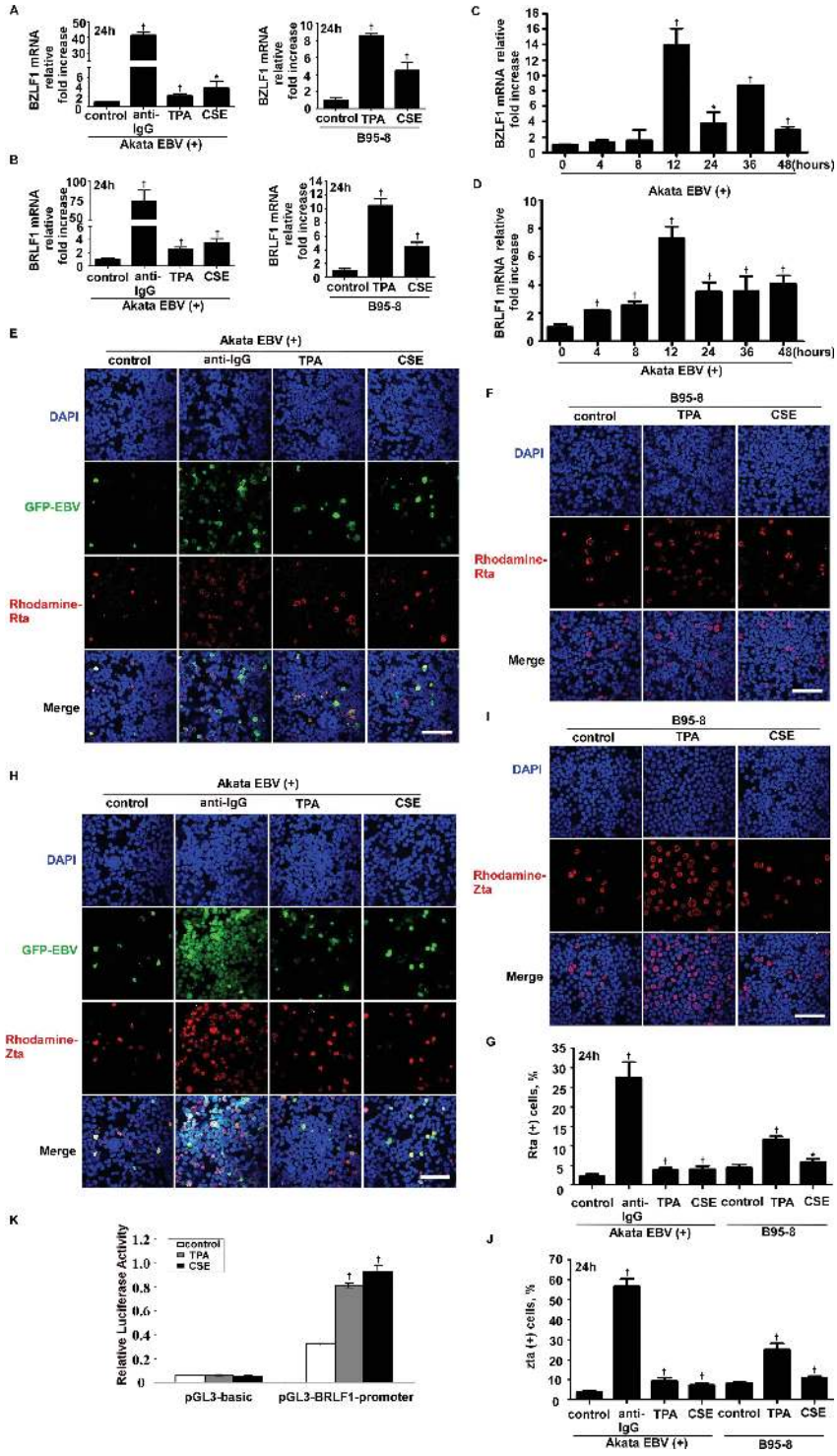

Figure 4. Expression of Epstein–Barr virus (EBV) transcriptional factors (Zta and Rta) in vitro after cell lines were treated with cigarette smoke extract (CSE). **A–D**) Expression of Zta and Rta was detected by quantitative real-time polymerase chain reactions after treatment with 2% CSE. EBV-positive Akata and B95-8 cell lines were treated or untreated with 100 μg/mL anti-IgG, 20 ng/mL phorbol-12-myristate-13-acetate (TPA), or CSE and harvested at 0, 4, 8, 12, 24, 36, and 48 hours, and RNA was extracted for quantitative real-time polymerase chain reactions. The relative fold increase of transcript expression was normalized to that of untreated Akata or B95-8 cells, respectively. β-actin was used as an internal control. Data represent the mean with SD (**whisker bars**) from three independent experiments. **E–J**) Expression of Rta and Zta protein was detected by immunofluorescence after treatment with 2% CSE for 24 hours. The percentage of Rta-positive cells or Zta-positive cells (**red**) was calculated relative to the total cell population (**blue**) in five random fields of view. Representative images are shown. Scale bar = 100 μm. Data represent the mean with SD (**whisker bars**) from three independent experiments. **K**) The EBV immediate-early BRLF1 gene promoter was activated by TPA or CSE. pGL3-BRLF1-promoter construct (0.5 μg) or pGl3-Basic (0.5 μg) was cotransfected with 0.01 μg of Renilla luciferase plasmid. Luciferase activity was monitored 24 hours after transfection. The data are represented as the ratio of firefly to Renilla luciferase activity. CNE2 cells, rather than EBV-negative Akata cells, were used in transfection experiments as these lymphoid cells exhibited low transfection efficiency. Data represent the mean with SD (**whisker bars**) from three independent experiments. Two-sided *P* values were calculated by Student t test. **P* < .05 and †*P* < .01 compared with the untreated cells as the control.

inappropriate to search for EBV-inducing agents in patients who are affected with EBV-associated tumors. Thus, healthy males were used in our study to avoid potential confounders.

Also, in vitro assays were used to investigate if EBV reactivation can be induced by CSE. Two B-cell lines, Akata and B95-8, with EBV latent infection were utilized. We demonstrated that EBV replication was promoted by CSE treatment and that the expression levels of lytic-phase gene products were enhanced, including VCA p18, gp350, and two key immediate early genes, BZLF1 and BRLF1, which stimulate a cascade of viral lytic gene expression (16,74,75).

It has been hypothesized that activated EBV might have a different impact on NPC tumorigenesis during different stages. In the early stage of tumorigenesis, the reactivation of EBV in B cells might facilitate epithelial cell infection with EBV. In vitro experiments suggested that producing massive viral particles of B cells and cell-to-cell contact might play a pivotal role for efficient infection of EBV (76,77). The frequent reactivation of EBV may not only increase the viral load in the individual but also change cytokine levels to effectively enhance EBV infection in nasopharyngeal epithelial cells (78). EBV reactivation in B cells could be triggered by cellular products of tumor cells (79,80). Conversely, EBV might act as a promoter for tumorigenesis in a feedback loop. EBV reactivation is associated with the elevations of levels of cytokines and growth factors, ie, interleukin-6 (81), interleukin-10 (81), transforming growth factor-β1 (82), and vascular endothelial growth factor (83), which could contribute to cell proliferation, immune system perturbation, and angiogenesis. Therefore, EBV reactivation in serum might establish cross-talk with tumor cells via modulating interactions between the infiltrating cells and the tumor cells in the microenvironment.

A previous study suggested that genetic variation may affect an individual's propensity for seropositive status of the VCA-IgA antibody (36). In our study, the seropositivity rate for the VCA-IgA antibody in ever smokers in a high-risk area was statistically significantly higher than that in a low-risk area when we examined the interaction effect by using an additive model. These results may be attributed to the specific genetic background of the subjects in the different areas. However, the effect did not reach statistical significance when a multiplicative model was used. Until now, no large-scale study has been conducted to evaluate genetic variants influencing VCA-IgA antibody titers.

To our knowledge, our study is the first attempt to systematically conduct a large-scale investigation aimed at characterizing the relationship between cigarette smoking and EBV reactivation as a potential novel mechanism of NPC etiology. Although cigarette smoking and the anti-EBV antibody have been reported to be related to NPC risk, the novelty of the current study is the establishment of a robust link between cigarette smoking and EBV activation. On the basis of our findings, cigarette cessation programs should be advocated for the primary prevention of NPC in China, especially in NPC endemic areas.

Our study is not without limitations. The individual contribution of smoking and EBV-positivity to the etiology of NPC was not investigated in this study. Future investigations are planned. Also, viruses other than EBV could also be activated by smoking, although none have been investigated. Here, we have provided evidence only for EBV. Studies of other viruses are being planned.

To summarize, we believe that searching for more environmental inducers specific to NPC high-risk areas has great value in NPC prevention. Future studies of the mechanisms by which cigarette smoking modulates the reactivation of EBV and further induces carcinogenesis of the nasopharynx are needed.

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Notes

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