

EPSTEIN-BARR VIRUS SEROREACTIVITY AMONG UNAFFECTED INDIVIDUALS WITHIN HIGH-RISK NASOPHARYNGEAL CARCINOMA FAMILIES IN TAIWAN

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Most adults have been infected with EBV. Many studies have indicated that antibodies against specific EBV antigens, particularly IgA antibodies, can be predictive or prognostic of EBV-associated malignancies, such as NPC. We hypothesized that healthy individuals from families with a history of multiple members affected with NPC (who therefore might be genetically susceptible to NPC themselves) might have an EBV antibody profile that is distinct from that seen in healthy individuals from the community at large. To explore this possibility and examine determinants of anti-EBV antibody levels in healthy, high-risk individuals, we evaluated data from 2 parallel studies of NPC in Taiwan, which included 1,229 healthy members of families in which 2 or more individuals were affected with NPC and 320 controls from the community at large. Blood collected from participants was tested for IgA antibodies against EBV VCA and EBNA-1 and for neutralizing antibodies against EBV DNase using standard assays. We observed evidence of familial aggregation of EBV seroreactivity among individuals from high-risk, multiplex NPC families. Anti-VCA IgA and anti-EBNA-1 IgA antibody seroprevalence in unaffected family members of NPC cases was 5–6 times higher than in members of the community ($p < 0.01$). This elevated seroprevalence among unaffected individuals from high-risk families was observed regardless of the relationship of the unaffected individual to the closest affected relative (siblings, parents, children or spouses). No sociodemographic or environmental factors examined were found to strongly and consistently correlate with elevated seroprevalence, but patterns emerged of increasing seroprevalence among older individuals and among females. Unaffected individuals from high-risk NPC families have elevated anti-EBV IgA antibody titers. The etiologic and clinical implications of this finding remain to be established.

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Key words: Epstein-Barr virus; nasopharyngeal carcinoma; susceptibility

EBV, a member of the herpes family of viruses, is known to infect the vast majority of adults worldwide, usually with lifelong persistence.¹ EBV typically remains latent in B cells, but for reasons that are not well understood, it periodically reactivates.² As a result of this intermittent reactivation, most individuals infected with EBV have detectable circulating levels of IgG antibodies against EBV antigens, most notably VCA and EBNA-1.

Most EBV infections are asymptomatic, but the virus can be associated with rare malignant transformations of lymphoid, epithelial or mesenchymal cells.³ Several malignancies are linked to EBV infection, most notably Burkitt's lymphoma and NPC.³ Individuals who develop EBV-associated malignancies present with specific patterns of anti-EBV antibodies that are rarely seen in the

general population.⁴ These patterns vary by tumor type but typically involve the detectability of IgA antibodies against VCA and EBNA-1, which are rarely seen among normal individuals after convalescence from EBV infection.^{2,5–7}

Numerous studies have shown both diagnostic and prognostic utility of EBV serology for EBV-associated malignancies.^{6,8–11} For example, many studies have shown that anti-VCA IgA, DNase or EBNA-1^{5,6,12–14} are found routinely in NPC and can be used to assist in its diagnosis. Interestingly, some studies have shown that normal individuals with serologic patterns similar to those associated with malignancies are at elevated risk of subsequent malignancy (*i.e.*, EBV serology is predictive of future disease occurrence). Studies in China and Taiwan have observed, for example,

Abbreviations: CI, confidence interval; EBNA-1, Epstein-Barr nuclear antigen-1; EBV, Epstein-Barr virus; GMT, geometric mean titer; NPC, nasopharyngeal carcinoma; OD, optical density; OR, odds ratio; VCA, viral capsid antigen.

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that individuals with detectable levels of IgA against EBV VCA and DNase are at a greatly elevated risk of developing NPC in subsequent years.^{4,15}

Although EBV is the strongest risk factor for NPC, other exogenous factors have been associated with this disease, including smoking, diet and occupational exposures.^{16–26} Family history of NPC and host susceptibility factors such as HLA, cytochrome P-450 2E1 and DNA repair gene (*XRCC1* and *hOGG1*) polymorphisms have also been associated with disease risk.^{27–32} Genetic susceptibility loci linked to NPC development have been identified on chromosomes 4 and 14 (Diehl *et al.*, unpublished)³³ Thus, both environmental and genetic factors have been proposed to be involved in NPC pathogenesis.

Based on the accumulated evidence, we reasoned that individuals without NPC from families with a history of multiple members affected with NPC (*i.e.*, high-risk families) might have a distinct antibody pattern compared to individuals in the general community. This distinct pattern could have important clinical implications if individuals from high-risk families with elevated titers are shown to have elevated risk of occult prevalent or incident NPC. To explore this idea, we combined data on antibody levels from 2 parallel studies of NPC in Taiwan conducted collaboratively by the National Taiwan University and the United States National Institutes of Health. One was a case-control study using controls from the community and the other, a study of families with multiple individuals affected with NPC. We evaluated whether antibodies against specific EBV markers were more frequently observed in unaffected individuals from high-risk NPC families (*i.e.*, multiplex families) than among community controls as well as the determinants of detectability of these antibodies.

MATERIAL AND METHODS

Study population

Data from 2 studies of NPC conducted in Taiwan were utilized for comparison of anti-EBV antibody titers across different groups (*i.e.*, relatives of NPC-affected individuals and individuals from the community at large). One was a case-control study and the second, a study of families with 2 or more individuals affected with NPC. The 2 studies were conducted by the same collaborating institutions and used similar data-collection instruments and testing methods. Human subject review committees in Taiwan and the United States approved both projects. All participants provided informed consent.

Case-control study. The methods used in case ascertainment and control selection have been described in detail.³⁰ Briefly, 2 hospitals in Taipei, Taiwan, referred incident cases of NPC among men and women <75 years of age diagnosed between 15 July 1991 and 31 December 1994. Cases were required to be residents of Taipei or the surrounding county. Three hundred seventy-eight histologically confirmed cases were identified. The National Household Registration System was used to select one population control per case from the same district or township as the cases. Controls were matched to cases by gender and age in 5-year age groups. Three hundred seventy-four controls were identified.

Family study. 14,471 individuals diagnosed with NPC between 1980 and 1999 were identified through an extensive review of records from the nationwide tumor registry in Taiwan, supplemented by information from listings obtained from 10 tertiary-care hospitals throughout the island and prospective identification at selected outpatient clinics that treat NPC. These individuals were contacted by mail or phone and/or directly at participating clinics and asked about their family history of NPC. Information was successfully obtained from 45% of individuals, from which 475 potentially eligible multiplex families were identified. We further restricted our study to multiplex families where 2 or more of the affected individuals were first-, second- or third-degree adult relatives and available for sampling or could have their genotype inferred by sampling of a spouse and adult offspring. If a family

had only a parent-child combination of affected subjects, it was excluded since this pedigree structure provides little information for linkage studies. Based on these additional criteria, a total of 246 eligible families were identified.

Both NPC-affected and unaffected members of these multiplex families were eligible for study. All affected individuals were eligible, as were all parents and up to 5 unaffected siblings. When an affected family member was deceased or otherwise unavailable for sampling, the spouse and up to 3 adult offspring were also recruited, to allow us to infer the genotype of the NPC case. Finally, when affected individuals were more distant relatives, such as cousins, we recruited their shared second-degree relatives, such as unaffected aunts and uncles, to genetically connect the NPC cases.

To date, 5,009 (819 affected and 4,190 unaffected) individuals have been identified as eligible for the study. For the 1,349 individuals (26.9%) determined to be deceased, family members were contacted as a proxy. Of identified individuals, 3,166 (63.2%) had been contacted as of this analysis. Of the 3,166, 167 (5.3%) refused participation, and recruitment is pending for 828 (26.1%). Consent and questionnaire data were obtained for 2,171 (68.6%, 468 affected and 1,703 unaffected).

Data and specimen collection

Case-control study. Personal interviews collected information about sociodemographic characteristics and other exogenous and lifestyle factors hypothesized to be associated with NPC. Participants were also asked to consent to the collection of approximately 30 ml blood. A total of 369 cases (98%) and 320 controls (86%) gave informed consent and completed both interview and blood collection.

Family study. An extensive family history questionnaire was administered to each NPC-affected individual within a family. For deceased cases, proxy interviews were conducted with a close family member (usually a sibling). This questionnaire obtained complete pedigree information and information on age, cancer status (for NPC and other cancers) and vital status for all family members. Risk factor interviews collected information about sociodemographic characteristics and other exogenous and lifestyle factors hypothesized to be associated with NPC. The risk factor questionnaire was administered to all eligible individuals, irrespective of NPC status. The questionnaire used in our family study was an abbreviated version of the questionnaire used in our case-control study described above. Decisions on how to reduce the length of the questionnaire were made based on results of the case-control study and information obtained from the literature. Participants were also asked to consent to the collection of approximately 30 ml blood for genotyping and EBV antibody testing. As of the date of this analysis, blood has been collected from 1,930 subjects (88.9%, 278 affected and 1,652 unaffected). Results from EBV testing were available on a total of 191 affected individuals (68.7%) and 1,229 unaffected family members (74.4%).

Each unaffected family member was assigned a relationship to the affected family members. Since every unaffected family member had at least 2 affected individuals within the family, relationship status was assigned based on the closest affected relative. For example, unaffected individuals having a sibling and a cousin affected with NPC were assigned a relationship status of "sibling." First-degree relatives and spouses were prioritized from highest to lowest as follows: sibling, parent, child and spouse. Therefore, a relationship of "sibling" was assigned to individuals with both a sibling and any other first-degree relative affected. To determine whether unaffected individuals had extensive daily contact with an affected individual within the family, families were contacted and asked about living arrangements. This information was available on 1,077 of the 1,229 unaffected individuals (87.6%) in our analysis. Unaffected individuals were classified as living together with an affected individual if they resided in the same household as an affected individual at the time of sampling.

EBV testing

Serum obtained from blood drawn from each individual was tested for antibody titers to several EBV antigens associated with NPC. Anti-VCA IgA was tested by the indirect immunofluorescence assay.⁵ VCA results were positive when anti-VCA IgA antibody was detected at a 1:10 or greater dilution of serum. Anti-DNase antibody levels were evaluated *via* an enzyme neutralization assay.³⁴ One unit DNase activity was defined as the amount of enzyme required to convert 1 μ g of dsDNA into acid-soluble material in 10 min at 37°C. A result was considered positive for anti-DNase antibody when 2 units or more of DNase activity were neutralized by 1 ml serum. Anti-EBNA-1 IgA antibodies were detected by ELISA.³⁵ A positive result for anti-EBNA-1 IgA was defined as OD₄₀₅ > 0.20. The positivity cut-offs described above for the various assays were chosen based on data from previous studies that demonstrated their sensitivity and specificity in distinguishing NPC cases from unaffected individuals.^{4,12,35}

Testing of specimens obtained from individuals enrolled in our case-control and family studies occurred in different years. To assure that differences in antibody profile noted between these 2 groups were not explained by systematic differences in results obtained at different time points, a subset of specimens ($n = 288$ for anti-VCA and anti-DNase evaluation, $n = 263$ for anti-EBNA1 evaluation) originally tested at different times (one-third tested as part of our case-control study before 1996; one-third tested in the early years of our family study, between 1996 and late 1998; and one-third tested in the most recent years of our family study) were selected and retested to assess the reproducibility of testing results. Results of this retesting effort indicate very good reliability for the anti-EBNA1 assays (agreement = 87.8%, $\kappa = 0.73$) and anti-DNase (agreement = 86.1%, $\kappa = 0.71$) and modest reproducibility for the anti-VCA assay (agreement = 74.3%, $\kappa = 0.41$). When the anti-VCA results were examined more closely, agreement between the original and retesting results was higher for specimens from the case-control study (agreement = 85.4%, $\kappa = 0.61$) than for specimens from the early or late phases of our family study (agreement = 67.7% and 69.8%, $\kappa = 0.29$ and 0.38 for the early and late phases of the family study, respectively). Given that modest reliability was observed for the anti-VCA IgA assay used, we included the anti-VCA IgA results in our analysis. This was done for 2 reasons. First, the anti-VCA IgA results are consistent with those seen for anti-EBNA-1 IgA, which had very good assay reliability. Second, anti-VCA IgA is arguably the most accepted EBV antibody marker of NPC.

Analysis

EBV antibody status among the various groups of unaffected individuals was compared using Pearson's χ^2 test. Both overall positivity (positive *vs.* negative) and distribution of titers among those positive (*i.e.*, high *vs.* medium *vs.* low) were examined. These comparisons were conducted separately for each of the 3 EBV markers evaluated. Comparisons were also performed using a measure of EBV seroreactivity that combined information across the 3 EBV markers (*i.e.*, positive for any of the 3 markers *vs.* negative for all).

To evaluate possible sociodemographic and exogenous determinants of EBV seroreactivity in unaffected individuals from our family study, EBV antibody status was initially compared by the various factors of interest using Pearson's χ^2 test. Factors examined included age group, gender, ethnicity, educational level attained, smoking status, betel quid use and household contact. These comparisons were conducted separately for each of the 3 EBV markers evaluated. Logistic regression analyses conditioned on family were also performed to evaluate determinants of antibody positivity while controlling for possible confounding factors.

RESULTS

Seropositivity to anti-VCA IgA was observed among 77.1% of cases from our case-control study; 44.9% of those positive had a positive response at dilutions $\geq 1:160$. Similarly, 83.6% of cases from our case-control study were positive for DNase neutralizing antibodies (proportion of those positive with ≥ 4 units of neutralization activity: 92.8%) and 78.5% positive for anti-EBNA-1 IgA (proportion of those positive with OD₄₀₅ readings ≥ 0.37 : 64.3%). These results confirm the high rate of seroreactivity to these markers among NPC cases. In the family study, seroreactivity among affected individuals for the 3 markers of interest was as follows: 67.5% positive for anti-VCA IgA, 73.3% for DNase neutralizing antibodies, 52.9% for anti-EBNA-1 IgA. The lower rates of seroreactivity observed among affected individuals in our family study compared to our cases in the case-control study are due to the fact that a majority of the affected individuals within our family study were recruited after treatment. It has been observed that EBV seroreactivity declines following successful treatment.^{36,37} Since our primary objective was to evaluate whether seroreactivity to EBV differs between unaffected individuals from high-risk multiplex families and individuals from the community, the NPC cases described above will not be included in the analyses that follow.

When community controls from our case-control study and unaffected members from our family study were compared, they differed significantly with respect to age distribution ($p < 0.01$ for age categories; 8.2% *vs.* 18.6%, respectively, were ≤ 30 years old), gender (69.6% and 46.9% male, respectively; $p < 0.01$) and smoking (52.5% *vs.* 67.8% never-smokers, respectively; $p < 0.01$). These 2 groups did not differ significantly with respect to ethnicity ($p = 0.27$), educational attainment ($p = 0.09$) or betel quid chewing ($p = 0.12$). The differences observed in age and gender distribution were due to the fact that community controls in our case-control study were matched to NPC cases with respect to these 2 factors, whereas no such matching was applied in our family study. Since NPC cases tend to occur predominantly in males and after the age of 30, the distribution among controls in our case-control study was biased accordingly.

The frequency of positivity for antibodies against each of the 3 EBV antigens evaluated was examined in community controls from our case-control study and unaffected family members from our family study (Table I). In addition, among seropositive individuals, the distribution of responses was compared (percentage seropositive with high titers and GMT among positives also shown in Table I). The frequency of positive antibody tests among unaffected family members was significantly different from that for controls from the case-control study, with unaffected family members having a consistently higher seropositivity rate for IgA antibodies against VCA (6.0 times higher), EBNA-1 (5.3 times higher) and when all 3 markers were combined (2.6 times higher). Also, unaffected family members were more likely than controls from the case-control study to test positive for more than one marker (11.3% compared to 3.5%, $p < 0.001$). When individuals positive for each of the markers were examined, the distribution of antibody levels between the 2 groups was not significantly different for any of the 3 antigens. Unaffected family members were classified according to their relationship to the NPC cases in the family: parents, children, siblings and spouses (as defined in Material and Methods). Few differences in overall positivity or in the distribution among positives were observed. Spouses, who might be expected to differ from genetically related family members in immune profile, had levels similar to those seen for other family groups.

To analyze the elevated antibody levels observed among unaffected individuals from our high-risk families, we next evaluated sociodemographic and other environmental factors as possible determinants of EBV seroprofile in this group of individuals. Results for each of the 3 EBV antibodies examined are presented in Table II. Overall results across all 3 EBV antibodies are also

TABLE I—DISTRIBUTION OF EBV SEROPOSITIVITY AMONG COMMUNITY CONTROLS AND UNAFFECTED INDIVIDUALS FROM HIGH-RISK FAMILIES

Group	Number ¹	Number (%) Positive	<i>p</i> value	Number (%) of positive individuals with high titers ²	GMT
Anti-VCA IgA					
Community controls	319	15 (4.7)		1 (6.7)	1:23
Unaffected individuals from high-risk families	1,229	349 (28.4)	<0.01	97 (27.8)	1:43
Unaffected individuals from high-risk families by relationship to affected individuals ³					
Siblings	569	149 (26.2)		43 (28.9)	1:46
Parents	431	125 (29.0)	0.29	28 (22.4)	1:37
Children	96	34 (35.4)		11 (32.4)	1:42
Spouses	125	36 (28.8)		12 (33.3)	1:45
DNase neutralizing activity					
Community controls	320	35 (10.9)		17 (48.6)	3.8
Unaffected individuals from high-risk families	1,229	145 (11.8)	0.67	58 (40.0)	3.6
Unaffected individuals from high-risk families by relationship to affected individuals ¹					
Siblings	569	83 (14.6)		33 (39.8)	3.7
Parents	431	34 (7.9)	0.01	11 (32.4)	3.2
Children	96	13 (13.5)		8 (61.5)	4.3
Spouses	125	13 (10.4)		6 (46.2)	3.7
Anti-EBNA1 IgA					
Community controls	261	10 (3.8)		4 (40.0)	0.32
Unaffected individuals from high-risk families	1,229	247 (20.1)	<0.01	81 (34.0)	0.34
Unaffected individuals from high-risk families by relationship to affected individuals ³					
Siblings	569	121 (21.3)		39 (32.2)	0.33
Parents	431	81 (18.8)	0.70	27 (33.3)	0.34
Children	96	19 (19.8)		8 (42.1)	0.33
Spouses	125	22 (17.6)		6 (27.3)	0.33
Any of the 3 markers					
Community controls	267	49 (18.4)		N/A	N/A
Unaffected individuals from high-risk families	1,229	589 (47.9)	<0.01		
Unaffected individuals from high-risk families by relationship to affected individuals ³					
Siblings	569	281 (49.4)			
Parents	431	194 (45.0)	0.27		
Children	96	52 (54.2)			
Spouses	125	56 (44.8)			

¹One community control missing anti-VCA IgA data; 59 individuals missing anti-EBNA-1 IgA data. ²High titer defined as $\geq 1:160$ dilution for anti-VCA IgA, ≥ 4 units for DNase neutralizing activity and $OD_{405} \geq 0.37$ for anti-EBNA1 IgA. None of the differences observed between community controls and unaffected individuals from high-risk families was significant at the $p = 0.05$ level. ³Eight individuals whose relationship to affected individuals was other than sibling, parent, child or spouse excluded.

presented. For VCA IgA antibodies, a marginally significant elevation in positivity was observed among females ($p = 0.05$). For DNase neutralizing antibodies, significant elevations in positivity were observed at older ages ($p = <0.01$) and among females ($p = 0.02$). In addition, elevated seroprevalence to DNase neutralizing antibodies was observed among family members who did not reside in the same household as an NPC case ($p = 0.01$). When anti-EBNA-1 IgA antibodies were examined, the only significant finding was an elevation in seroprevalence among former smokers ($p = 0.04$). In analyses that combined all 3 EBV antibody markers, only the elevation in antibody positivity among older individuals was statistically significant ($p < 0.01$). No significant differences were noted for any of the antibody markers examined for ethnicity, education and betel nut use.

In fully adjusted logistic regression models that included age, gender, ethnicity, education, smoking, betel quid use and household contact, largely similar patterns to the univariate results presented in Table II were observed. For anti-VCA IgA, the modest elevation in seropositivity seen among females persisted but was no longer statistically significant (OR = 1.2 comparing females to males, 95% CI 0.81–1.7). For DNase neutralizing antibodies, the significant increase in seropositivity observed at older ages persisted (OR = 2.9 for >60 years compared to ≤ 30 years, 95% CI 1.3–3.7) and the elevated seropositivity observed among females persisted but was no longer statistically significant (OR = 1.5, 95% CI 0.91–2.6). The effect observed for household contact also persisted in adjusted models but was no longer statistically significant (OR = 2.1 for individuals living in different

compared to the same household as an NPC case, 95% CI 0.96–4.6). Finally, a significant effect was observed for betel nut use (OR = 3.0 for former users compared to never users, 95% CI 1.0–8.9; OR = 2.2 for current users compared to never users, 95% CI 0.92–5.4). For anti-EBNA-1 IgA, the elevation in seropositivity observed among former smokers was attenuated and no longer statistically significant (OR = 1.2 comparing former to never smokers, 95% CI 0.60–2.4). For the analysis that examined positivity to any of the 3 EBV markers examined, the elevation in seropositivity observed at older ages persisted (OR = 1.8 for >60 years compared to ≤ 30 years, 95% CI 1.1–3.0).

Among those who tested positive for one of our EBV markers, an additional analysis was undertaken. The percentage of individuals positive for the marker of interest with antibody levels in the highest tertile were examined by the demographic and descriptive variables in Table II (data not shown). Four significant differences were noted. A higher proportion with high IgA antibody levels against VCA was observed among individuals of non-Fujianese ethnicity (34.6% among non-Fujianese compared to 24.2% among Fujianese, $p = 0.04$), a higher proportion of individuals with high DNase neutralizing antibody levels was observed among older individuals (55.6% for individuals >60 compared to 14.3% for individuals ≤ 30 , $p = 0.04$), a higher proportion of individuals with high DNase neutralizing antibody levels was observed among individuals with lower educational attainment (52.8% for those with a junior high school or less education, 35.9% for those with a high school education and 15.6% for those with some technical school and/or university education; $p < 0.01$) and a higher pro-

TABLE II – DISTRIBUTION OF EBV SEROPOSITIVITY BY SOCIODEMOGRAPHIC AND OTHER ENVIRONMENTAL FACTORS AMONG UNAFFECTED INDIVIDUALS FROM HIGH-RISK FAMILIES FOR VARIOUS EBV MARKERS

	Number ¹	Anti-VCA IgA		DNase neutralizing antibodies		Anti-EBNA-1 IgA		Overall ²	
		% positive	<i>p</i> value	% positive	<i>p</i> value	% positive	<i>p</i> value	% positive	<i>p</i> value
Age (years)									
0–30	222	24.3		6.3		18.5		41.4	
31–40	261	28.4		12.6		16.1		43.3	
41–50	283	25.8	0.13	9.5	<0.01	20.9	0.19	46.3	<0.01
51–60	202	28.2		14.9		22.8		54.0	
> 60	228	34.7		15.8		24.1		56.6	
Gender									
Male	576	25.7		9.6		20.7		45.5	
Female	653	30.8	0.05	13.8	0.02	19.6	0.67	50.1	0.11
Ethnicity									
Fujian	838	28.2		12.4		20.4		48.2	
Other	361	29.6	0.60	10.8	0.43	19.9	0.88	48.5	0.93
Education									
Jr. high or less	535	31.4		13.5		21.5		51.4	
High school	363	27.0	0.15	10.7	0.34	19.6	0.62	46.6	0.14
Tech school/university	301	25.6		10.6		18.9		44.9	
Smoking status									
Current	302	26.5		11.9		19.2		43.7	
Former	84	25.0	0.42	4.8	0.10	31.0	0.04	51.2	0.18
Never	813	29.8		12.7		19.6		49.7	
Betel									
Current	67	22.4		19.4		17.9		44.8	
Former	48	35.4	0.31	14.6	0.12	31.3	0.14	54.2	0.61
Never	1,084	28.7		11.4		19.9		48.3	
Household contact									
Same	173	27.2		6.4		16.7		42.2	
Different	904	28.5	0.71	13.0	0.01	21.0	0.22	49.5	0.08

¹Thirty-Three individuals have missing age information; 30 individuals have missing ethnicity, education, smoking and betel quid information. Household contact information was available for 1,077 individuals, as described in Material and Methods. ²Overall positivity defined as positive for any of the 3 EBV markers evaluated.

portion of individuals with high IgA antibody levels against EBNA-1 was observed among females (39.1% compared to 26.1% among males, $p = 0.03$).

DISCUSSION

Results of our study provide clear evidence for the presence of elevated EBV seroreactivity among unaffected individuals from high-risk, multiplex NPC families. We examined the presence and levels of antibodies against 3 EBV antigens closely associated with NPC and only rarely detected among nondiseased individuals: anti-VCA IgA, DNase neutralizing antibodies and anti-EBNA-1 IgA. For 2 of these 3 markers (anti-VCA IgA and anti-EBNA-1 IgA), higher rates of positivity were noted among unaffected individuals from high-risk families compared to individuals from the general community. Overall, positivity to one or more of the 3 antibodies evaluated was observed for approximately half of unaffected individuals from high-risk families compared to 18% of controls from the general population ($p \leq 0.01$). The fact that significant elevations in seroprevalence were noted for both markers that specifically measure levels of IgA antibodies (anti-VCA IgA and anti-EBNA-1 IgA) but not for the marker that measures combined levels of antibodies (DNase neutralizing antibodies, including IgA and IgG antibodies) suggests that elevations in antibodies against EBV among unaffected individuals from high-risk NPC families are restricted to elevations in IgA antibodies.

The elevations observed in our study can be explained by genetic predisposing factors, shared environmental factors or a combination of both. Our observation that elevation in seropositivity was evident for spouses of affected individuals (who are genetically unrelated to the affected cases) suggests that shared environmental factors are likely important determinants of seroreactivity. However, a genetic component to the observed familial aggregation cannot be ruled out based on our data. For example,

the elevations in EBV seropositivity observed among the genetically unrelated spouses of our NPC cases are consistent with a recessive mode of transmission for a disease gene(s) that unaffected family members often carry.

Although additional studies are required before the significance of our findings can be fully understood, it is tempting to speculate on the possible clinical implications of the observed elevations in EBV seropositivity among unaffected individuals from high-risk NPC families. Studies have demonstrated that individuals who are seropositive to anti-EBV VCA IgA and DNase neutralizing antibodies are at increased risk of both prevalent and incident NPC.^{4,15} If it is shown that seropositive individuals from high-risk multiplex families are at a similarly increased risk of NPC, one might envision the use of such serologic tests to identify members of high-risk NPC families predisposed to NPC who might benefit from more extensive clinical evaluation.

In our study, we evaluated sociodemographic and environmental factors associated with seropositivity to EBV among unaffected individuals from NPC families. No strong and consistent correlations of seropositivity were noted across all 3 markers examined. However, some evidence was seen for a correlation between older age and female gender and elevated seropositivity rates. It has previously been shown that anti-EBV antibody titers increase with age,³⁸ presumably because the cumulative probability of exposure to EBV increases with time. Similarly, previous evidence supports the notion that antibody responses in females tend to be more robust than in males,^{39–41} possibly due to hormonal influences on immunologic responses.

We did not observe higher seroprevalence among unaffected individuals who lived in the same household as an NPC case. Indeed, for DNase neutralizing antibodies, the opposite was observed. One possible explanation for the lack of association between living in the same household as an NPC case and being seropositive might be that we obtained information on residential

status at the time of blood sampling, while household sharing in earlier years (*e.g.*, during childhood, when most agree initial infection with EBV takes place) might be a more important predictor of seroreactivity patterns to EBV antigens. Also, exogenous sources of EBV (*e.g.*, re-exposure through contact with EBV-shedding family members who develop NPC) might be a less important predictor of seroreactivity against EBV than other poorly understood factors that influence frequency of EBV reactivation of endogenous virus in individuals initially infected during childhood.

The limitations of our study include the following. First, differences were noted between our community controls and unaffected family members with respect to age, gender and cigarette smoking. These differences might theoretically explain the differences in seroprevalence observed in the 2 groups. However, the differences observed between the 2 study groups with respect to age, gender and smoking were relatively small, while the differences in seropositivity were striking; it is therefore unlikely that these minor differences between community controls and unaffected family members account for our findings. Furthermore, unaffected family

members were likely to be younger and to smoke less than community controls, and this would tend to bias our findings toward the null rather than away from it. Second, while our reproducibility study confirmed the reliability of the DNase neutralizing antibody and anti-EBNA-1 IgA assays, only modest reproducibility was observed for the anti-VCA IgA assay. The fact that our observations with respect to anti-VCA IgA were similar to those for anti-EBNA-1 suggest that misclassification due to assay variability cannot explain our findings.

In this analysis, we confirmed the presence of elevated EBV IgA seroreactivity among unaffected individuals from high-risk NPC families. Future evaluation of whether these individuals with elevated levels of EBV antibodies are at increased risk of prevalent or incident NPC will help to determine the clinical utility of EBV serologic testing among healthy individuals from families at high risk of NPC.

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