Two Epstein-Barr Virus–Related Serologic Antibody Tests in Nasopharyngeal Carcinoma Screening: Results From the Initial Phase of a Cluster Randomized Controlled Trial in Southern China

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A nasopharyngeal carcinoma (NPC) mass screening trial using a combination of immunoglobulin A antibodies to Epstein-Barr virus capsid antigen and nuclear antigen-1 by enzyme-linked immunosorbent assay in addition to indirect mirror examination in the nasopharynx and/or lymphatic palpation (IMLP) was conducted in southern China. Cantonese aged 30–59 years residing in 2 cities randomly selected by cluster sampling, Sihui and Zhongshan, were invited to participate in this screening from May 2008 through May 2010. Participants were offered fiberoptic endoscopy examination and/or pathologic biopsy if their serologic tests reached our predefined level of high risk or if results from the physical examination indicated possible cancer (i.e., were IMLP positive). A total of 28,688 individuals were voluntarily screened in the initial round. The overall NPC detection rate was 0.14% (41/28,688) with an early diagnosis rate of 68.3% (28/41) during the first year of follow-up. Thirty-eight of 41 cases (92.7%) were detected among the high-risk group, and 7 of 41 cases (17.1%) were detected among the IMLP-positive group. The 2 Epstein-Barr virus serologic tests by enzyme-linked immunosorbent assay could be a feasible alternative for NPC screening in endemic areas. Further follow-up is needed to examine whether screening has an effect on decreasing mortality from NPC in these areas.

Abbreviations: CI, confidential interval; EA/IgA, immunoglobulin A antibodies to Epstein-Barr virus early antigen; EBNA1/IgA, immunoglobulin A antibodies to Epstein-Barr virus nuclear antigen-1; EBNA1/IgG, immunoglobulin G antibodies to Epstein-Barr virus nuclear antigen-1; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A; IgG, immunoglobulin G; IMLP, indirect mirror examination in the nasopharynx and/or lymphatic palpation; NPC, nasopharyngeal carcinoma; SYSUCC, Sun Yat-sen University Cancer Center; VCA/IgA, immunoglobulin A antibodies to Epstein-Barr virus capsid antigen.

Editor’s note: An invited commentary on this article appears on page 251, and the authors’ response appears on page 254.

Nasopharyngeal carcinoma (NPC) has remained a major cause of death, with a persistently high incidence in southern China and some other parts of the world for over a century (1–3). Despite previous efforts, the high susceptibility of southern Chinese to develop NPC is still a mystery (4). Evidence accumulated so far indicates potential roles of genetic susceptibility, Epstein-Barr virus (EBV) infection, and other environmental factors in the pathogenesis of NPC (5, 6). NPC tends to present at an advanced stage at
diagnosis because the primary anatomic site of the tumor is located in a silent area and its early symptoms are inconspicuous (7). Long-term survival differs substantially between patients with advanced- and early stage NPC, with 4-year survival rates for patients with stage I–IV disease 96.7%, 94.1%, 82.6%, and 67.1%, respectively (8). It thus highlights the need to improve the early diagnosis rate of NPC through screening.

The long-term impact of screening on NPC-specific mortality is unknown. Since the 1970s, several pilot efforts have been launched to conduct NPC mass screening in a few high-risk counties in southern China (9–13) by measuring 2 traditional seromarkers, immunoglobulin A (IgA) antibodies to EBV capsid antigen (VCA/IgA) and EBV early antigen (EA/IgA), using immunofluorescence assay. Although testing for these 2 seromarkers as a screening method did increase the early detection rate of NPC (10, 13), such limitations as the relatively low positive predictive value, lack of a standardized method, high intraobserver variation, and time-consuming protocols make it less applicable in large-scale population screenings (14).

Recently, several enzyme-linked immunosorbent assay (ELISA) kits for EBV-related antibodies have been developed that offer better performance in NPC detection with also relatively lower cost (15, 16). Our previous results showed that the combination of tests for IgA antibodies to EBV VCA/IgA and EBV nuclear antigen-1 (EBNA1/IgA) by ELISA outperformed the traditional NPC screening scheme regarding both sensitivity and specificity (17).

Sihui and Zhongshan cities are located along the Pearl River in Guangdong province, China, and are well known for a high incidence (20/100,000 person-years) of NPC (2, 18). During the last 20 years, NPC incidence rates have remained high in both cities, with incidence stable in Sihui while slightly increasing in Zhongshan (2, 18, 19). Thus, a cluster randomized screening trial with combined VCA/IgA and EBNA1/IgA measurement by ELISA as the primary screening method was launched in year 2008 in these 2 cities to examine the influence of the new screening strategy on NPC-specific mortality. The initial screening round was completed in year 2010. In this report, we aimed to describe the demographics and other characteristics of the screened population, the fiberoptic endoscopy rate in the initial round of screening, the NPC detection rates in different risk groups defined by EBV antibody levels and indirect mirror examination in the nasopharynx or lymphatic palpation, and the characteristics of NPC cases diagnosed during the first year of follow-up.

**MATERIALS AND METHODS**

**Study population**

Sixteen candidate towns with a population of 70,000–120,000 in Sihui and Zhongshan were selected as the screening areas and randomly assigned to either the screening or control arm (Figure 1). All eligible residents in the 8 screening towns (7 in Sihui city and 1 in Zhongshan city) were invited to participate in the screening between May 2008 and May 2010. Written informed consent was obtained at the time of recruitment. Inclusion criteria included 1) being aged 30–59 years; 2) being Cantonese; 3) having no prevalent NPC; 4) having an Eastern Cooperative Oncology Group score of 0–2; and 5) having a good physical or psychological condition and consciousness. Exclusion criteria included 1) having severe cardiovascular, liver, or kidney disease and 2) having prevalent NPC.

In order to maximize the participation rate, members of the target populations were contacted by their village doctors who introduced the screening program with information leaflets containing information on NPC in general (such as possible causes of NPC, early symptoms, diagnosis, treatment, and prognosis), the advantages and potential side effects of the screening method, and the follow-up strategies in case of a positive test result. The screening was also promoted by local television stations in the screening towns. This mass screening study (NCT00941538 ClinicalTrials.gov) was approved by the Ethics Review Committee of the Sun Yat-sen University Cancer Center (SYSUCC, YP2009051).

**Serologic tests and definition of different populations**

The participants were invited to donate 6 mL of blood collected in 2 tubes, 1 with potassium-ethylenediaminetetraacetic acid and 1 without. Within less than 8 hours, all the blood samples were processed in local hospital laboratories. Samples were separated into sera, plasma, and buffy coat and stored at −20°C. In Sihui, all samples were transported to SYSUCC within 1 week after collection, where the serologic tests were performed. In Zhongshan, all the serologic tests were performed in the laboratory of the local town hospital.

Serum samples were used for anti-EBV antibody testing. The commercial kits for testing VCA/IgA (EUROIMMUN AG, Lübeck, Germany) (20) and EBNA1/IgA (Zhongshan Bio-Tech Company, Zhongshan, China) (21) were selected as the optimal combination from a case-control study. In brief, 6 seromarkers (VCA/IgA, EA/IgA, EBNA1/IgA, EBNA1/immunoglobulin G (IgG), immunoglobulin A antibodies to BZLF1 transcription activator protein (Zta/IgA), and immunoglobulin G antibodies to BRLF1 transcription activator protein (Rta/IgG)) were measured by ELISA. Serum samples were collected from 45 patients with early stage NPC, 52 patients with late-stage NPC, 99 healthy controls in Sihui city, and 80 patients with non-NPC cancers from SYSUCC. A logistic regression model was used to identify an optimal biomarker panel to discriminate NPC from controls. The combination of 2 seromarkers (VCA/IgA and EBNA1/IgA), with a sensitivity of 92.8%, a specificity of 91.6%, and an area under the receiver operating characteristic curve of 0.97, was identified, and a prediction formula was developed: Logit = −3.934 + 2.203 × VCA/IgA + 4.797 × EBNA1/IgA.

Of note, 2 different ELISA kits for EBNA1/IgA were available, the first with a sample incubation time of 60 minutes and the second with an incubation time of 30 minutes. We also evaluated the diagnostic performance for the EBNA1/IgA with a 30-minute incubation time in a 2-stage study (17). A similar diagnostic performance for the
A combination of VCA/IgA and EBNA1/IgA was achieved with a sensitivity of 95.3%, a specificity of 94.1%, and an area under the curve of 0.97. Because the EBNA1/IgA ELISA kit with a 60-minute incubation time was the only product approved by the Chinese Food and Drug Administration for marketing when the mass NPC screening was initiated, the 60-minute kit was used throughout the whole study period.

The NPC incidence rate in the screening target population is relatively low (about 50/100,000 person-years) (2, 18, 22). Thus, it is important that the false positive rate, which equals 1 – specificity, should be small enough to avoid unnecessary fiberoptic endoscopy/biopsies and psychological stress for the NPC screening participants. On the other hand, the true positive rate (equal to sensitivity) should be acceptable (23). We used 2 minimally acceptable false positive rates of 3% and 7% as the high-risk and medium-risk cutoff values (with the corresponding logistic regression $P = 0.98$ and 0.65, respectively); the corresponding true positive rates (sensitivities) were 58.8% and 78.8%.
respectively. If the baseline serologic results fulfilled the definition of high risk, the participants were referred for diagnostic examinations, and different screening intervals were assigned to the high-risk, medium-risk, and low-risk groups.

Quality control for the measurement of VCA/IgA and EBNA1/IgA was performed in accordance with the protocols suggested by the manufacturers. Additionally, as a supplement, we randomly retested 1% of the samples \( (n=281) \) for mass screening samples in the year 2011 (about 2 years after the baseline storage) to evaluate the accuracy of these 2 serarkers. The proportions of overall agreement for VCA/IgA and EBNA1/IgA were 0.88 (95% confidence interval (CI): 0.85, 0.92) and 0.89 (95% CI: 0.86, 0.93), respectively, when the cutoff was set according to the manufacturer’s protocol. The proportions of agreement among the original positive tests for VCA/IgA and EBNA1/IgA were 0.71 (95% CI: 0.55, 0.87) and 0.49 (95% CI: 0.35, 0.62), respectively. The kappa values for VCA/IgA and EBNA1/IgA were 0.51 (95% CI: 0.36, 0.65) and 0.58 (95% CI: 0.44, 0.71), respectively. Using the triage logistic formula in this study, we found that the proportion of overall agreement for the logistic formula (high risk vs. medium risk and low risk) was 0.90 (95% CI: 0.87, 0.94) when the cutoff for the logistic regression \( P \) was set as 0.98. The proportion of agreement among the original high-risk group was 0.79 (95% CI: 0.65, 0.93) with a kappa value of 0.61 (95% CI: 0.48, 0.74).

Preliminary physical examination

Indirect mirror examination in the nasopharynx and/or lymphatic palpation (IMLP) was performed in the Sihui Cancer Institute or the People’s Hospital in Zhongshan. Participants with a positive result to IMLP were informed and invited for further fiberoptic endoscopy examination. The criteria for a positive result to IMLP included 1) lymphatic palpation showing abnormal enlargement of the lymph nodes in the upper neck of 0.5 cm × 0.5 cm and/or 2) an elevated, nodular, rough, or ulcerative nasopharyngeal surface found in the indirect mirror examination in the nasopharynx that led to a conclusion of suspicious NPC. An unsatisfactory result was defined if the nasopharynx could not be observed clearly by indirect mirror or with missing data.

Diagnostic tests—fiberoptic endoscopy and biopsy

Within 6 months of the baseline tests, participants categorized as the high-risk group and/or with an IMLP-positive result were invited for fiberoptic endoscopy performed by the local otorhinolaryngologists at the Sihui Cancer Institute or the People’s Hospital in Zhongshan. Nasopharyngeal biopsies were also performed if suspicious lesions were observed in the endoscopy. Histopathologically diagnosed patients were immediately given advice for treatment.

NPC was classified according to the World Health Organization classification (24), and tumor stage was defined according to the 2008 Staging System of China (25).

Screening interval and follow-up

Screening tests were repeatedly conducted according to the screening protocol. In brief, the high-risk and medium-risk groups were reexamined annually, and the low-risk group every 4 years, after the initial round of screening. Repeated blood samples were collected accordingly.

NPC cases were identified by the research team among the high-risk group and, as a complement, were identified through linkage to the cancer registries in these 2 cities annually. NPC diagnoses from the medium-risk and low-risk groups were mainly ascertained through the cancer registries. When an NPC record was found in the cancer registries, medical records were reviewed to verify the diagnosis and collect detailed data (i.e., a pathological result, imaging record, and tumor stage). The Sihui and Zhongshan cancer registries were established in 1978 and 1970 on the basis of a 3-level network, called the Three Ranks Cancer Prevention and Control Network. The network covers all the areas of Sihui and Zhongshan, comprising the cancer research institutes in both cities, the regional hospitals of each town or community, and local general practitioners (community health service stations). The percentage of death-certificate-only cases of NPC was 5% during 1987–2007, and 90.3% of NPCs were diagnosed pathologically in Sihui (2). The percentage of death certificate only was 0.03%, and 96.3% of NPCs were diagnosed pathologically in Zhongshan during 1970–2007 (18).

Information on death was obtained from the causes of death registers and the rosters of village (community) committees annually, and information on migration was obtained from the local public security bureau and the rosters of village (community) committee every 3 years.

Statistical analysis

Possible baseline differences among participants in the 2 cities were compared by chi-squared tests. The proportions of the high-risk, medium-risk, and low-risk participants were also compared between the 2 cities. The rates of fiberoptic endoscopy examination and biopsy were compared among the 3 risk groups.

All statistical tests were 2 sided. \( P < 0.05 \) was considered to be statistically significant.

RESULTS

Demographics of screened participants

A total of 28,688 residents voluntarily participated in the screening (Figure 1), representing about 21% of the total population aged 30–59 years in the screening towns. Participants were all Han Chinese and more likely females. Age groups were well represented (Table 1). On average, 2.1% of the participants had at least one first degree relative with NPC.

Screening results

Overall, 3.0% (862/28,688) of the participants were classified as the high-risk group, 7.6% (2,184/28,688) as the medium-risk group, and 89.4% (25,642/28,688) as the...
low-risk group on the basis of the combination of serologic results (Table 2). Seventy-eight participants (0.3%) were classified as IMLP positive, 98.0% (28,122/28,688) as IMLP negative, and 1.7% (488/28,688) as having unsatisfactory results. The sex distribution of the high-risk group was similar between Sihui and Zhongshan, that is, more males than females.

Diagnostic evaluation of subjects with suspicious results

A total of 932 participants had suspicious results from the initial serologic tests (i.e., high-risk group) and/or IMLP examination (i.e., IMLP positive) (Table 3). Among these high-risk participants, 71.0% (661/932) underwent further diagnostic evaluation by fiberoptic endoscopy, and 13.3% of them (88/663) underwent nasopharyngeal biopsy within 1 year of screening. The high-risk group had a higher fiberoptic endoscopy rate (76.1%; 656/862) compared with the IMLP-positive group (15.4%; 12/78). The biopsy rate was similar between these 2 groups: 10.1% (87/862) among the high-risk group and 10.3% (8/78) among the IMLP-positive group.

Forty-one NPC cases were detected during the first year of follow-up after the initial screening, yielding an overall
diagnosis rate of 0.14% (Table 3). NPC diagnosis rates were 0.008% (2/25,642), 0.05% (1/2,184), and 4.4% (38/862) in the low-risk, medium-risk, and high-risk groups, respectively. The diagnosis rates were 9.0% (7/78), 0.11% (32/28,122), and 0.41% (2/488) in the IMLP-positive, -negative, and unsatisfactory results groups, respectively. Within each serologic risk group, participants with IMLP-positive results had a higher NPC diagnosis rate than those with IMLP-negative results. For example, participants who were serologically at high risk and IMLP negative had a diagnosis rate of 3.6% (30/841), while participants who were serologically at high risk and IMLP positive had a diagnosis rate of 75.0% (6/8). There was only 1 case (2.5%) who was IMLP positive but not classified as high risk by serology, leading to a diagnosis rate of 1.4% (1/70).

Clinical characteristics of NPC patients

Among the 41 NPC cases, 38 (92.7%) were detected among the high-risk group and 7 (17.1%) among the IMLP-positive group (Table 4). Twenty-nine (75.6%) cases had no symptom at the time of screening, and 38 cases were diagnosed by our screening staff in the participating hospitals. Twenty-eight (68.2%) patients were diagnosed at stages I and II, and all patients were diagnosed with World Health Organization histopathology type III.

DISCUSSION

In the present study, we report results from the initial round and first year of follow-up of an NPC screening trial in 2 high-risk areas in southern China. By use of the new risk-group classification strategy that includes a combination of VCA/IgA and EBNA1/IgA ELISA tests, the proportion of high-risk individuals was narrowed down to 3.0% (only 3 of 41 NPCs were missed), which is lower than the proportions reported in previous mass screening trials using traditional VCA/IgA and EA/IgA by immunofluorescence assay in southern China. For example, Zeng et al. (9) and Zong et al. (13) previously reported the proportions of high-risk populations in Wuzhou and Zhongshan cities as 5.3% and 6.7%, respectively. A reduced false positive rate could avoid unnecessary cost in the following diagnostic procedures and the potential psychological stress perceived by individuals incorrectly classified as high risk.

An NPC diagnosis rate of 4.4% in the high-risk group is higher than those reported previously from Wuzhou city (1.9%), Cangwu county (2.5%), and Zhongshan city (1.5%) (9, 10, 13). Because the prevalence of NPC in our study (0.14%) is similar to those reported in earlier studies from Sihui and Zhongshan (2, 18, 19), the high diagnosis rate is likely due to the better diagnostic value of the new strategy.

The majority of NPC cases in this screening trial were diagnosed at early stages without noticeable clinical symptoms.
symptoms. In an earlier mass screening in the 1980s, Zeng et al. (9, 10) reported that the early diagnosis rate was 100% in Wuzhou city and 58.2% in Cangwu county. One previous report in year 1992 from Zhongshan showed that about 80.5% of cases were diagnosed at early stage in the initial screening round (13). The different early diagnosis rates between previous studies and ours could be partially explained by the poor resolution of x-ray imaging technology in tumor stage classification in the 1980s. To the best of our knowledge, the present study is the first reporting an early diagnosis rate in NPC mass screening after the introduction of the new NPC staging classification system in China incorporating magnetic resonance imaging technology.

Our study also aimed to shed light on the potential of IMLP examination in NPC screening. According to our findings, we believe that the IMLP examination does not contribute sufficient information during NPC screening. Over 90% of the NPC patients were successfully detected by serologic examination, whereas only 17% of the patients were identified by IMLP examination. The fiberoptic endoscopy rate was much higher in the high-risk group compared with the IMLP-positive group (76.1% vs. 15.4%), indicating that the result of serologic tests for the EBV antibody is more convincing than the result of IMLP in this population. Furthermore, the substantial difference in the IMLP-positive rates between Sihui and Zhongshan (0.37% vs. 0.20%; \( P < 0.001 \)) suggested a large interinvestigator variation of the IMLP examination, making it a less objective assessment.

In the present study, we had different follow-up strategies for individuals classified into different risk groups. Closer follow-up is conducted for individuals with positive screening results; the screening intervals for the high-risk, medium-risk, and low-risk groups are 1, 1, and 4 years, respectively. Previous studies (22, 26, 27) showed that individuals with consistently high serologic titers of EBV antibodies had a higher risk of NPC. Therefore, we decided that participants with positive screening results in the initial round should be evaluated more closely. The different cutoff values for high- and medium-risk groups were not based on a formal analysis but rather on an informal consensus among study investigators. Optimized performance criteria should be set by cost-benefit analysis for NPC screening with enough follow-up time.

Although the NPC screening trial in southern China has a history of more than 30 years (3, 28), no controlled results have been reported so far. Most studies utilized hospital or clinic-based data and are subject to different methodological limitations. To estimate whether the screening approach increases the early diagnosis rate of NPC, we analyzed the characteristics of NPC cases in the unscreened population in Sihui during the same period. A total of 239 NPC cases were found in the unscreened populations in both the screening towns and the control towns between May 2008 and December 2010. Among 237 cases with data on clinical stage in the cancer registries, 89 cases were from the screening towns, and 148 cases were from the control towns. Among these cases, 36.0% (32/89) in the screening towns and 25.7% (38/148) in the control towns were at early stages. Therefore, we believe that screening significantly improves the early diagnosis rate, since 68.3% of the cases were diagnosed at early stages in the screened population. However, prospective randomized trials are needed to provide sound evidence for the

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**Table 4.** Clinical Characteristics of Nasopharyngeal Carcinoma Patients Detected at Initial Round and During 1 Year of Follow-up by Combination of Serologic Tests and IMLP in Sihui and Zhongshan Cities, Guangdong Province, China, 2008–2010

<table>
<thead>
<tr>
<th>Screening Method</th>
<th>No. of NPCs</th>
<th>Symptom</th>
<th>Clinical Stage*</th>
<th>Histopathologic Typeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Serologic examination</td>
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<tr>
<td>High risk</td>
<td>38</td>
<td>9</td>
<td>29</td>
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<tr>
<td>Medium risk</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
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<tr>
<td>Low risk</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<td>IMLPc</td>
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<td>+</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>1</td>
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<tr>
<td>–</td>
<td>32</td>
<td>1</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>Unsatisfactory</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: IMLP, indirect mirror examination in the nasopharynx and/or lymphatic palpation; NPC, nasopharyngeal carcinoma; TNM, tumor-node-metastasis.

* Clinical stage was defined according to the 2008 staging system in China (25): Stage I, T1 with N0 and M0; Stage II, T1 with N1 or T2 with N0/N1 and M0; Stage III, T1/T2 with N2 or T3 with N0–N2 and M0; and Stage IV, T1–T3 with N3 or T4 with N0/NX and M0 or MX.

b Histopathologic type was defined according to the World Health Organization classification: Type I, keratinizing squamous cell carcinoma; Type II, differentiated nonkeratinizing carcinoma; and Type III, undifferentiated nonkeratinizing carcinoma (24).

c+, suspicious for cancer; –, not suspicious for cancer; unsatisfactory, although with normal lymphatic results, the nasopharynx couldn’t be observed clearly by indirect mirror or because of missing data.
potential effect of screening in reducing NPC-specific mortality (29).

There are some limitations to the present study. First of all, some factors influencing the parameters in the logistic regression formula may limit its application to other populations. Second, not all subjects received further diagnostic tests after a positive screening result. Even for the high-risk group, the fiberoptic endoscopy rate was only 76.1%. Therefore, a few cases of NPC might have been missed. Third, no further diagnostic evaluations were conducted for any of the participants in the medium-risk and low-risk groups, and an adjustment for the verification bias is needed while estimating the real diagnostic value of these 2 assays. In order to evaluate the influence of the verification bias, we conducted a follow-up evaluation in the screened population through linkages to the cancer registries in Sihui and Zhongshan. During 1 extra year of follow-up, another 8 NPC cases were found, including 6 cases from the high-risk group, 1 case from the medium-risk group, and 1 case from the low-risk group, showing that the NPC diagnosis rate is low anyway in the medium-risk and low-risk groups. We therefore believe that verification bias should have limited influence on our study results. Another limitation is the generalizability of our results because the screening covered only about 20% of the target population. The relatively low participation rate may partially be due to the dramatically developing economy in China, making people migrate around for temporary jobs. A lack of external validity, however, does not interfere with the internal validity of the present study.

In conclusion, to our knowledge, this is the only ongoing NPC mass screening study that uses the new biomarkers and test method in southern China. EBV VCA/IgA and EBNA1/IgA tests by ELISA may be a feasible alternative to the conventional immunofluorescence assay for NPC screening in the endemic areas. Further follow-up is needed to determine whether the screening program has an impact on decreased mortality from NPC in these areas.

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