Multiparametric Detection of Antibodies against Different EBV Antigens to Predict Risk for Nasopharyngeal Carcinoma in a High-Risk Population of China

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Abstract

In this study, we aimed to use the combined detection of multiple antibodies against Epstein–Barr virus (EBV) antigens to develop a model for screening and diagnosis of nasopharyngeal carcinoma (NPC). Samples of 300 nasopharyngeal carcinoma patients and 494 controls, including 294 healthy subjects (HC), 99 non-nasopharyngeal carcinoma cancer patients (NNPC), and 101 patients with benign nasopharyngeal lesions (BNL), were incubated with the EUROLINE Anti-EBV Profile 2, and band intensities were used to establish a risk prediction model. The nasopharyngeal carcinoma risk probability analysis based on the panel of VCA P125 IgA, EBNA-1 IgA, EA-D IgA, EBNA-I IgG, EAD IgG, and VCA P19 IgG displayed the best performance. When using 26.1% as the cutoff point in ROC analysis, the AUC value and sensitivity/speciﬁcity were 0.957 and 90.7%/88.1%, respectively. The diagnostic speciﬁcity and sensitivity of the EUROLINE Anti-EBV Proﬁle 2 assay for both nasopharyngeal carcinoma and early-stage nasopharyngeal carcinoma were higher than that of mono-antibody detection by immune-enzymatic assay and real-time PCR (EBV DNA). In the VCA-IgA–negative group, 82.6% of nasopharyngeal carcinoma patients showed high probability for nasopharyngeal carcinoma, and the negative predictive value was 97.1%. In the VCA-IgA–positive group, 73.3% of healthy subjects showed low probability. The positive predictive value reached 98.2% in this group. The nasopharyngeal carcinoma risk probability value determined by the EUROLINE Anti-EBV Proﬁle 2 might be a suitable tool for nasopharyngeal carcinoma screening.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common squamous cell cancers of the head and neck in Southern China. Besides dietary and genetic inﬂuences, Epstein–Barr virus (EBV) infection has been proven to be an important factor for nasopharyngeal carcinoma onset. EBV is a member of the herpesvirus family and leads to a persistent infection in over 90% of adults (1, 2). After EBV infection of human nasopharyngeal epithelial cells, different EBV antigens are expressed at different stages of infection. Nasopharyngeal carcinoma tumor cells show a distinct gene-expression pattern (3) and are accompanied by aberrant antibody responses to various EBV proteins and antigen complexes (4).

In general, EBV healthy carriers show a limited diversity of IgG antibodies directed toward EBNA-1, VCA-p18, VCA-p40, gp125, p160, and immediate early protein BZLF1, whereas nasopharyngeal carcinoma patients show abnormal elevated IgA reactivity to EBNA-1 and VCA-p18 (5, 6). Many studies have indicated a close relationship between EBV infection and nasopharyngeal carcinoma, as revealed by elevated IgG and IgA responses to EBV viral capsidantigen (VCA), early antigen (EA), and Epstein–Barr nuclearr antigen (EBNA) complexes (4, 7). Fachirol was first to illustrate increases in IgA and IgG antibody diversity in nasopharyngeal carcinoma using immunoblot technology (8).

The VCA complex is composed of more than 30 different proteins. Several of these proteins have been considered as antigenic markers. The major VCA-p18 (VCA p19 = p18, BFRF3) protein has a unique immunodominant and virus-specific antigenic domain in its C-terminus. This domain of VCA-p18 contains several small peptide regions which can be targets for VCA-IgM, IgG, and IgA antibodies (6). The VCA-p40 (BdRF1) protein forms the highly repetitive nuclear capsid scaffold and is reactive with IgG and IgA antibodies in nasopharyngeal carcinoma patients. The VCA-p23 (p22 = p23, BLKRF2) and the VCA-p143 (BNRF1) proteins are located between the virion capsid and envelope.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerpreventionresearch.aacrjournals.org/).

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These two proteins require a largely intact polypeptide sequence to be recognized by human antibodies, suggesting involvement of conformational epitopes. VCA-p160 (BcLF1) contains only a few small linear antigenic epitopes and was first identified as major VCA marker (9). gp125/110 (BAlF4) and gp350/220 (BLF1) are additional immunodominant determinants of the VCA complex. They function as a target for cytolytic antibody responses capable of killing the target cell (10).

EBNA is a multiprotein complex composed of six proteins (EBNA 1–6). EBNA-1 is a DNA-binding protein that binds to the specific site in viral nucleic acid sequences and mediates the viral replication cycle (11).

EBV infects B cells and induces B-cell immortalization. Antibodies against EBNA-1 are present only after the disintegration of B cells (12, 13). Thus, anti-EBNA-1 antibodies can be used as an indicator for late infection with EBV (14). Furthermore, EBNA-1 is the only viral protein expressed in all EBV-associated tumors and in the latent period (15).

For nasopharyngeal carcinoma diagnosis, VCA-IgA and EBNA-1 IgA detection by ELISA has been the most commonly used marker for nasopharyngeal carcinoma screening. However, the sensitivity and specificity were not satisfactory (16–18). Hsu’s research found that the sensitivity and specificity of VCA-IgA by ELISA in Chinese patients were 91.9% and 83.8%, respectively, compared with EBNA-1 IgA with sensitivity and specificity values of 80.0% and 80.0%, respectively (19). Fachiroh reported that the sensitivity/specificity values were 86.2/92.0% (EBNA-1 IgA) and 84.1/90.3% (VCA-p18 IgA) for single-peptide assays and 95.1/90.6% for the combined VCA plus EBNA-1 IgA ELISA. This may be due to the diversity of antigens. Detection of a single EBV antibody does not provide the sensitivity and specificity needed for screening or early detection.

The antibody diversity analysis of Fachiroh revealed that the EBV antigen diversity of IgG and IgA varies considerably between individual patients with nasopharyngeal carcinoma and seems to be driven by different independent B-cell triggering events (5). Paramita and colleagues used EBV RecombLine-based immuno blot assay in screening and diagnosis of nasopharyngeal carcinoma in populations with high EBV prevalence for the first time. EAd-p47/54 and -p138 IgG show combined overall sensitivity and specificity for nasopharyngeal carcinoma diagnosis of 92.6% and 98.3%, respectively. However, the study was performed with a small number of samples, and results were semiquantitative. Detection of a single EBV antibody does not provide the sensitivity and specificity needed for screening or early detection.

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Immunofluorescence assays (IFA) are still widely used, providing a summary result of responses to multiple individual EBV proteins and epitopes (5, 20). But this method is restrained by technical difficulties and subjective result interpretation, and is not suitable for high-throughput testing.

Cell-free EBV DNA (EBV DNA) has been shown to be an independent prognostic marker. Besides its prognostic value, it is positively correlated with nasopharyngeal carcinoma tumor burden and has shown capacity to monitor the therapeutic effect (21–23).

A commercial anti-EBV antibody profiling test system which detects different antibody isotypes against different EBV proteins in parallel could further improve the ability to discriminate nasopharyngeal carcinoma patients from normal subjects. Few studies have explored the use of antibody isotype profiling against multiple EBV proteins in nasopharyngeal carcinoma profiling.

Serological tests for VCA-IgA and EA-IgA antibodies were performed with an IFA supplied by the Shanghai Institute of Biological Products. EIA slides were prepared from the B95 cell line for VCA and the Raji cell line for EA. Plasma samples were screened at a dilution of 1:10, followed by 2-fold serial dilutions. The antibody titer was the reciprocal of the highest dilution clearly showing a brown color within 15% of the cells. Levels of VCA-IgA and EA-IgA were determined by titration, with the cutoff values set at 1:40 for VCA-IgA and 1:10 for EA-IgA (25).
Real-time quantitative PCR (EBV DNA)

Real-time quantitative PCR of EBV DNA from plasma was carried out at SunYat-sen University Cancer Center (26). The real-time quantitative PCR system was developed for EBV DNA detection toward the BamHI-W region. The system consisted of the amplification primers W-44F (5'-AGT CTC TGC CTC CAG GCA-3') and W-119R (5'-ACA GAG GCCTGTCACC G-3') and the dual-labeled fluorescent probe W-67T (5'-[FAM] CACITGCTGTA-AAGTCCAGGCTCC [TAMRA]-3'). Sequence data for the EBV genome were obtained from the GenBank sequence database (26).

EUROLINE Anti-EBV Profile 2

Antibodies of class/isotype IgG or IgA against EA-D, p22, EBNA-1, VCA p19, and VCA gp125 were detected in serum using the EUROLINE Anti-EBV Profile 2 test kit (EUROIMMUN AG). The detection was performed according to the manufacturer's instructions. Briefly, serum was incubated with the strip coated with VCA gp125 (BALF4), VCA p19 (P19 = p18 = VP26; BFRF3), p22 (P22 = p23; BLRF2), EBNA-1, and EA-D for 30 minutes at room temperature. After a washing step, alkaline phosphatase-labeled anti-human IgG (or IgA) was added to the strip for a further 30-minute incubation. Finally, the substrate was added for the color reaction. The incubated and dried membrane chips attached to the support matrix were analyzed by means of digital scanning using a Canon LiDE series scanner. The intensity of each band on the EUROLINE test strip was evaluated by the EUROLineScan program. P(Yi

Statistical analysis

The 300 nasopharyngeal carcinoma patients and the 494 controls were randomly assigned to a training set (164 nasopharyngeal carcinoma samples vs. 228 normal controls) and a validation set (136 nasopharyngeal carcinoma samples vs. 266 normal controls). The data of the training set were used to establish a regression function for a risk prediction model for nasopharyngeal carcinoma which was further verified by the validation set. The resulting regression function was used to calculate the probability of nasopharyngeal carcinoma for each study subject.

Results

Characteristics of study population

In total, 794 adult patients were studied. Nasopharyngeal carcinoma patients were grouped into early (n = 66) and late (n = 234) nasopharyngeal carcinoma cases. Non-nasopharyngeal carcinoma cancer patients included patients with breast cancer (n = 11), cervical cancer (n = 11), colon cancer (n = 1), endometrial cancer (n = 1), esophageal cancer (n = 10), liver cancer (n = 8), hypopharyngeal cancer (n = 1), laryngopharyngeal carcinoma (n = 1), lung cancer (n = 13), NK/T-cell lymphoma (n = 1), ovarian cancer (n = 10), prostate cancer (n = 1), stomach cancer (n = 10), thyroid cancer (n = 17), and tongue cancer (n = 3). The age and gender of the patients are summarized in Supplementary Table S1.

EUROLINE Anti-EBV Profile 2

Antibodies against EA-D, p22, EBNA-1, VCA p19, and VCA gp125 of isotypes IgG and IgA were evaluated by measuring the intensity values of the following bands (IgG for EA-D, p22, EBNA-1, VCA gp125) were significantly higher in patients with nasopharyngeal carcinoma compared with those in all controls (Supplementary Fig. S1A, all P < 0.001; Supplementary Table S2). The ROC curves for individual bands that discriminate the cancer groups from all controls are shown in Supplementary Fig. S1B. The AUC values ranged from 0.473 (VCA p19 IgG) to 0.913 (EBNA-1 IgA). EBNA-1 IgA had the largest AUC. The cutoff value and test performance of different indexes were provided and compared. The Anti-EBV EUROLINE had the best AUC (0.951, P < 0.001; Supplementary Table S2).

The distribution of the probability for nasopharyngeal carcinoma (%) according to age and gender

The individual distribution of nasopharyngeal carcinoma probability is shown in Fig. 1A. The probability for nasopharyngeal carcinoma (%) was greater than 80% in more than 80% of patients, whereas more than 80% of healthy people had a probability of less than 20%.

There were no remarkable differences in P values (P > 0.05) between males and females in the different groups (Fig. 1B).

Assessment of the probability for nasopharyngeal carcinoma risk in study population

There was no significant difference in nasopharyngeal carcinoma probability between the training and the validation cohorts,
with mean probabilities of nasopharyngeal carcinoma subjects in the training and validation cohorts of 81.2% (95% CI, 76.7%–85.7%) and 80.9% (95% CI, 76.1%–85.66%), respectively. In the training cohort or in the validation cohort, as shown in Fig. 2, a statistically significant difference in P values was found between the nasopharyngeal carcinoma group and the other control groups (P < 0.001), whereas there was no significant difference between non-nasopharyngeal carcinoma, BNL, and HC. The mean probability values for non-nasopharyngeal carcinoma, BNL, and HC in the training cohort were 13.3% (95% CI, 8.9%–17.7%), 17.8% (95% CI, 10.8%–24.8%), and 12.4% (95% CI, 9.7%–15.1%), respectively. The values for non-nasopharyngeal carcinoma, BNL, and HC in the validation cohort were 25.3% (95% CI, 16.3%–34.3%), 13.2% (95% CI, 16.3%–18.6%), and 13.9% (95% CI, 11.4%–16.3%), respectively (Fig. 2).

Diagnostic performance of the prediction model for nasopharyngeal carcinoma

The quality of the prediction model for identifying nasopharyngeal carcinoma correctly is analyzed by using ROC curves in Fig. 3. The risk model produced similar AUC values for the training and validation cohorts, resulting in AUCs of 0.950 (95% CI, 0.928–0.973) and 0.953 (95% CI, 0.933–0.973), respectively.
The ROC curves were also used to compare the diagnostic capability of the risk model using the complete data set including the controls with or without the non-nasopharyngeal carcinoma and BNL groups. ROC analysis revealed comparable AUCs if the control set was used with (AUC, 0.951; 95% CI, 0.936–0.966) or without non-nasopharyngeal carcinoma and BNL (AUC, 0.957; 95% CI, 0.942–0.972).

When using 26.1% as the cutoff point, the sensitivity and specificity of the EUROLINE were 90.7% and 88.1%, respectively (Table 1). However, using the same cutoff for the detection of nasopharyngeal carcinoma patients versus controls without non-nasopharyngeal carcinoma and BNL group (D). The sensitivity and specificity were evaluated by using 26.1% as the cutoff point.

Table 1. Comparison of sensitivities and specificities of markers by IEA and RT-PCR and Western blot strip

<table>
<thead>
<tr>
<th>VCA</th>
<th>EA</th>
<th>VCA and EA</th>
<th>DNA</th>
<th>Anti-EBV EUROLINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1:20</td>
<td>&gt;1:10</td>
<td>&gt;1:100</td>
<td>&gt;26.1%</td>
<td>26.1%</td>
</tr>
<tr>
<td>Sensitivities</td>
<td>81.9</td>
<td>67.3</td>
<td>68.6</td>
<td>69.5</td>
</tr>
<tr>
<td>Specificities</td>
<td>82.4</td>
<td>68.8</td>
<td>68.3</td>
<td>75.2</td>
</tr>
</tbody>
</table>

The optimum combination of VCA and EA detected by IEA. The cutoff values were VCA ≥ 1:20 and EA ≥ 1:10.

Sensitivity was evaluated based on all nasopharyngeal carcinoma patients (n = 300).

Sensitivity was evaluated based on early-stage (I and II) nasopharyngeal carcinoma patients (n = 66).

Sensitivity was evaluated based on T1-T2 stage nasopharyngeal carcinoma patients (n = 94).

Sensitivity was evaluated based on T3-T4 stage nasopharyngeal carcinoma patients (n = 206).

Specificities were evaluated based on healthy controls. Controls with benign nasopharyngeal lesions and non-nasopharyngeal carcinoma cancer patients were not included, to more closely simulate a mass screening.

Specificities were evaluated based on all controls. Controls with benign nasopharyngeal lesions and non-nasopharyngeal carcinoma cancer patients and healthy controls were included.

Figure 3.
ROC curve analysis of individual probability for nasopharyngeal carcinoma. ROC curves of probability for nasopharyngeal carcinoma with nasopharyngeal carcinoma versus controls in the training set (A) and the validation set (B) and all the cases (C), and for nasopharyngeal carcinoma patients versus controls without non-nasopharyngeal carcinoma and BNL group (D). The sensitivity and specificity were evaluated by using 26.1% as the cutoff point.
The nasopharyngeal carcinoma diagnostic performance of other methods

The sensitivity and specificity of different methods are summarized in Table 1.

With a cutoff value of 1:20, the sensitivity of VCA-IgA detected by IEA was 81.9% for all nasopharyngeal carcinoma cases and 71.6% for early-stage nasopharyngeal carcinoma cases, with specificities of 95.9% (HC) and 97.0% (all controls). The sensitivity of EA IgA IEA was lower compared with VCA IgA IEA, with comparable specificities of 67.3% and 92.9% (HC) and 95.1% (all controls), respectively. The combined test results for VCA IgA and EA IgA IEAs reached a sensitivity of 67.3% for nasopharyngeal carcinoma and 53.7% for early-stage nasopharyngeal carcinoma with a specificity of 98.3% (HC) and 98.4% (all controls).

Real-time quantitative PCR of viral DNA burden showed a sensitivity of 75.5% for nasopharyngeal carcinoma cases and 47.8% for early-stage nasopharyngeal carcinoma cases.

Comparison of the performance between EUROLINE, IEA, and real-time PCR reveals that the best comprehensive diagnostic performance is provided by the EUROLINE with a sensitivity and specificity of 90.7% and 88.1%, respectively. For the diagnosis of early-stage (I and II and T1-2) nasopharyngeal carcinoma, the sensitivity of the EUROLINE is significantly higher than that of other methods, except for VCA-IgA IEA in stages I and II (Supplementary Table S3).

To compare the performance of the risk model with the VCA-IgA test, the total cases were divided into two groups according to the qualitative IEA test result of VCA-IgA. In the VCA-IgA-negative group, there were 69 nasopharyngeal carcinoma patients and 479 control cases, where by the negative predictive value (NPV) of VCA-IgA was 87.4%. In this group, 82.6% of nasopharyngeal carcinoma patients were found to have a high probability of nasopharyngeal carcinoma, and the NPV was 97.1%. In the VCA-IgA-positive group, there were 231 nasopharyngeal carcinoma patients and 15 control cases, and the positive predictive value (PPV) of VCA-IgA was 93.9%. In this group, 73.3% of control subjects were found to have a low probability. The PPV reached 98.2% in this group. When the VCA-IgA-negative and VCA-IgA-positive groups included only nasopharyngeal carcinoma patients and healthy subjects, 83.3% of healthy subjects in the VCA-IgA-positive group were found to have a low probability of nasopharyngeal carcinoma. The PPV determined in the VCA-IgA-positive group using the risk model was as high as 99.1%, whereas the NPV in the VCA-IgA-negative group was about 95% (Table 2).

The probability for nasopharyngeal carcinoma (%) values from EUROLINE model and clinical stage of nasopharyngeal carcinoma

The probability for nasopharyngeal carcinoma (%) values for patients at different clinical stages of nasopharyngeal carcinoma was compared. The median of the probability for nasopharyngeal carcinoma (%) value in patients with stage III and IV nasopharyngeal carcinoma was higher than the median in patients with early stages of nasopharyngeal carcinoma (stages I and II). A significant overall difference was found (P < 0.001). When investigating the tumor-node-metastasis (TNM) classifications, no significant differences were found for the probability for nasopharyngeal carcinoma (%) levels among T classes or between M0 and M1 classes (Fig. 4). However, patients with and without tumor spread to the lymph nodes showed different probability for nasopharyngeal carcinoma (%) levels in this study. The absence of tumor cells from the regional lymph nodes significantly lowered the probability for nasopharyngeal carcinoma (%) value in nasopharyngeal carcinoma patients (Fig. 4). However, the degree of spread to the lymph nodes had no influence on the probability for nasopharyngeal carcinoma (%) value levels.

Discussion

Previous studies revealed considerable heterogeneity in antibody responses to individual proteins in nasopharyngeal carcinoma patients, which suggested that single antibody detection is insufficient to meet the needs of nasopharyngeal carcinoma diagnosis and combined detection is necessary (27, 28).

Paramita used the Mikrogen EBV Recomb Line assay with special emphasis to detect IgG and IgA antibodies in nasopharyngeal carcinoma, non-nasopharyngeal carcinoma cancer patients, and healthy regional controls as well as infectious mononucleosis patients. They also found out that IgG and IgA signals against the individual EBV markers were significantly lowered the probability for nasopharyngeal carcinoma (%) value in nasopharyngeal carcinoma patients (Fig. 4). However, the degree of spread to the lymph nodes had no influence on the probability for nasopharyngeal carcinoma (%) value levels.

The EUROLINE anti-EBV profile assay provides a versatile, well-standardized, and automated approach for simultaneous assessment of IgG and IgA responses to the individual
immunodominant EBNA-1, VCA, and EA-D markers. In this study, by means of logistic regressions, we produced different models using different combinations of the potential Anti-EBV EUROLINE biomarkers and found that a restricted panel composed of VCAgp125 IgA, EBNA-1 IgA, EA-D IgA, EBNA-1 IgG, EA-D IgG, and VCAp19 IgG comprised the potential markers for nasopharyngeal carcinoma screening. With an AUC of 0.95, our risk prediction model with a selected antibody panel showed a suitable capability to that from other studies for nasopharyngeal carcinoma screening. Pengal used six tumor-associated antigens as a biomarker panel for the detection of nasopharyngeal carcinoma (AUC = 0.855; ref. 30). Cao and colleagues used a model testing for VCA-IgA, which performed well for nasopharyngeal carcinoma prediction in the third year (AUC = 0.807; ref. 31).

Coghill and colleagues evaluated the ability of anti-EBV-IgA antibodies to detect the incidence of nasopharyngeal carcinoma among high-risk Taiwanese individuals. The result showed that EBNA-1 IgA had the best performance with sensitivity and specificity of 92.3% and 80%, respectively (32).

As presented here, existing EBV-IgA antibodies are also shown to be a useful diagnostic biomarker of nasopharyngeal carcinoma, but they have a high false-negative rate and unsatisfactory sensitivity and specificity, which is in agreement with a study by Ji and colleagues (33). In addition, a screening program designed to detect disease and lower mortality needs highly sensitive screening tools that will target individuals at high disease risk for early detection, enabling effective treatment in a population at very high risk of developing nasopharyngeal carcinoma. The specificity is also important to define, as a good screening test in the absence of a sensitive diagnostic work-up does not have high utility. Sensitivity and specificity are often conflicting. So the number of screen positives sent to receive a test for each real nasopharyngeal carcinoma case detected also needs to be considered. The Anti-EBV EUROLINE panel with sensitivity and specificity of 90.7% and 88.1%, respectively, maybe more suitable for the nasopharyngeal carcinoma high-risk population of China.

The false-positive rate and false-negative rate of serological tests are important factors affecting the accuracy of nasopharyngeal carcinoma diagnosis. High false-negative rates will prevent the timely diagnosis of nasopharyngeal carcinoma patients in endemic areas, particularly the symptomless, early-stage patients. High false-positive rates will cause unnecessary worry and excessive examination. However, the large number of seropositive subjects who require close follow-up is still a big burden. Nasopharyngeal endoscopy combined with biopsy of suspicious lesions is currently the gold standard of clinical nasopharyngeal carcinoma detection. But as endoscopy presentations may be subtle or obscured in early nasopharyngeal carcinoma lesions, it is sometimes difficult to diagnose nasopharyngeal carcinoma by endoscopy. To make a definite nasopharyngeal carcinoma diagnosis and avoid misdiagnosis of subclinical cancers, it is not uncommon to perform multiple biopsies for some patients. Additional MRI scanning may be helpful to depict subclinical cancers missed at endoscopy (34). However, these techniques are either invasive or expensive and, most importantly, cannot be performed by general physicians; thus, they are not suitable for mass screening programs, especially in rural areas where only limited health care facilities are available (18). The analysis of the diagnostic performance of the risk model in VCA-IgA-negative and –positive groups showed that 82.6% of VCA-IgA-negative patients with nasopharyngeal carcinoma in our study had positive Anti-EBV EUROLINE panel results (with cutoff value of 26.13%); whereas...
83.3% of VCA-IgA–positive healthy people had negative Anti-EBV EUROLINE panel results. These data indicated that most of the false-positive and false-negative results obtained by monoparameter (VCA-IgA) assay could be corrected by our prediction model with the Anti-EBV EUROLINE panel. The Anti-EBV EUROLINE panel had a high PPV in VCA-IgA–positive people and a high NPV in VCA-IgA–negative individuals. These results suggest that the Anti-EBV EUROLINE panel is suitable supplement to the VCA-IgA assay for nasopharyngeal carcinoma screening.

We further investigated if the Anti-EBV EUROLINE panel will enhance early detection of nasopharyngeal carcinoma. The Anti-EBV EUROLINE panel displayed a sensitivity of 77.9% for early-stage nasopharyngeal carcinoma (stages I and II) samples in the present study which is higher than values published for EBV DNA test and four-gene methylation panel (sensitivity 51.2% and 64.6%) as reported in the literature by Yang and colleagues (35). Peng and colleagues used an anti-antibody panel for screening early nasopharyngeal carcinoma with a sensitivity ranging from 61.1% to 70% (30). Thus, the Anti-EBV EUROLINE panel complements the detection of early nasopharyngeal carcinoma.

In addition to the limited funds, EBV DNA detection was not carried out in a large number of the control group, but previous studies suggest that the specificity of EBV DNA is higher than 98% [23, 36, 37]. The EUROLINE Anti-EBV Profile 2 and the risk probability analysis for nasopharyngeal carcinoma proved to have better performance than mono-antibody assay and to be minimally traumatic, sensitive, simple, low-cost, and easy to perform. These attributes together make our method extraordinarily amenable to widespread use in nasopharyngeal carcinoma screening programs. More importantly, we provide a novel parameter for probability of nasopharyngeal carcinoma (p) for the result display. Euroline is an alternative commercial test system, comparable with the Mikrogen product evaluated by Paramita and colleagues. We expect that our panel maybe be used as a novel noninvasive approach to screen for nasopharyngeal carcinoma in regions with high incidence rates.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: W. Meyer, Y.-H. Peng, W. Liu
Development of methodology: S. Chen, T. Scheper, W. Meyer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wang, J. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Meyer
Writing, review, and/or revision of the manuscript: H. Chen, J. Li, W. Meyer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Lu, L. Li, J. Fu
Study supervision: T. Scheper, Y.-H. Peng

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