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 VCAgp125 IgA, EBNA-1 IgA, EA-D IgA, EBNA-1 IgG, EAD IgG, and VCAp19 IgG displayed the best performance. When using 26.1% as the cutoff point in ROC analysis, the AUC value and sensitivity/specificity were 0.951 and 90.7%/86.2%, respectively, in nasopharyngeal carcinoma and all controls. In nasopharyngeal carcinoma and controls without the non-nasopharyngeal carcinoma and BNL groups, 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. Cancer Prev Res; 10(9): 1–9. ©2017 AACR.

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 invasion 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 B2LF1, 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 nuclear antigen (EBNA) complexes (4, 7). Fachiroh was first to illustrate increases in IgA and IgG antibody diversity in nasopharyngeal carcinoma using immunoblot technique (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, BLRF2) and the VCA-p143 (BNRF1) proteins are located between the virion capsid and envelope.
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 (BLLF1) 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 immunoblot 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 (scored by + or −; ref. 20). The assay based on immunoblot still needs to be improved in accuracy and simplicity, and panels also need to be optimized.

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 detection. Some studies have focused on the use of individual recombinant EBV proteins, but only the IgA isotype has been analyzed. The study by Hsu and colleagues demonstrated 98% sensitivity and 82% specificity in nasopharyngeal carcinoma detection using the combination of anti-EA and anti–EBNA-1 IgA (19). In a small pilot study, we showed that improvement of nasopharyngeal carcinoma diagnosis can be achieved by detecting EBV (EA-D p45–IgG) using Western blot based on a whole EBV extract (24).

Here, we present the analysis of an alternative commercial anti-EBV antibody profile comprising IgG and IgA isotypes against the target antigens VCA gp125, VCA p19, EBNA-1, p22, and EA-D. The capability of this antibody profile and the resulting risk prediction model to detect nasopharyngeal carcinoma was compared with the established immune-enzymatic assay (IEA) tests for VCA-IgA and EA-IgA as well as real-time quantitative PCR of EBV DNA.

Materials and Methods

Ethics statement

This study was approved by the Institutional Review Board of Sun Yat-sen University Cancer Center. All patients provided written-informed consent for the collection and publication of their medical information at the first visit to our center, which was filed in their medical records, and the ethics committees approved this consent procedure. The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number as RDDA2017000161.

Study population

In total, 794 adult patients were enrolled between 2014 and 2015 in SunYat-sen University Cancer Center. The population included a group of nasopharyngeal carcinoma patients as well as a control group. The nasopharyngeal carcinoma group comprised 300 newly diagnosed patients enrolled prior to any treatment (radiotherapy or chemotherapy). The control group included 3 subgroups: healthy subjects (HC, n = 294), non-nasopharyngeal carcinoma cancer patients (NNPC, n = 99), and subjects with benign nasopharyngeal lesions (BNL, n = 101).

Nasopharyngeal carcinoma diagnosis was histologically confirmed by biopsy and further tests, including head and neck MRI and chest X-rays for all participants. The stages of nasopharyngeal carcinoma progression were classified according to the 2009 Union for International Cancer Control classification. Patients were further grouped into early and late nasopharyngeal carcinoma cases accordingly.

IEA

Serological tests for VCA-IgA and EA-IgA antibodies were performed with an IEA 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-41F (5'-AGT CTC TGC CTC CAG GCA-3') and W-119R (5'-ACA GAG GCGCTGTCCG CACG G-3') and the dual-labeled fluorescent probe W-67T (5'- [FAM] CACGTGCTGTA-AAGTCCAGCCT GCC[TAMRA]-3'). Sequence data for the EBV genome were obtained from the GenBank sequence data base (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 (EUROIMMUN AG). The following evaluation principle is applied: The membrane chip is localized by image recognition using the software. Absolute gray values are measured by the software and normalized by comparing them with the background signal. The intensity (or grayscale level) over the entire chip is defined, and the band positions and band intensities are measured.

Modeling for nasopharyngeal carcinoma risk

Using the training cohort, a regression function was established as a prediction model for nasopharyngeal carcinoma risk. This model consisted of the band intensities of VCA gp125 IgA, EBNA-1 IgG, and EA-D IgG on the EUROLINE line blot as determined by the EUROLineScan program. P(Yi = 1) = 1/(1 + Exp(-(1.886-0.042*VCA gp125_ A-0.101*EBNA-1_ A-0.059*EA-D_ A-0.016*EBNA-1_ G-0.041*EAD_ G+0.023*VCA p19_ G)).

In the resulting regression, function P is the probability for nasopharyngeal carcinoma, VCA gp125_ A is the intensity value of VCA gp125 IgA, EBNA-1_ A is the intensity value of EBNA-1 IgA, VCA p19_ G is the intensity value of VCA p19 IgG, EA-D_ A is the intensity value of EA-D IgA, EBNA-1_ G is the intensity value of EBNA-1 IgG, and EA-D_ G is the intensity value of EA-D IgG on the EUROLINE strip.

Based on a cutoff level of $P \geq 50\%$, 87.8\% of the subjects in the training cohort could be classified correctly. Note that 88.3\% of the validation cohort and 88\% of the total population could be classified correctly.

Statistical analysis

The 308 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.

An ROC curve was constructed by plotting sensitivity versus 1-specificity, and the AUC was analyzed according to Hanley and McNeil. The cutoff value was identified from the respective ROC. All statistical tests were two-sided, and a $P$ value $<0.05$ was considered statistically significant. Values are given with 95\% confidence interval (95\% CI) if applicable. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 19.0 (IBM Corp.).

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 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 Supplemental 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 staining intensity of each band. We compared the intensity value of each band between nasopharyngeal carcinoma subjects and all controls (HC, NNPC, and BNL; Supplementary Fig. S1A). The intensity values of the following bands (IgG for EA-D, p22, EBNA-1, and VCA gp125; IgA for EA-D, p22, EBNA-1, VCA p19, and VCA gp125) were significantly higher in patients with nasopharyngeal carcinoma compared with those in all controls (Supplementary Fig. S1A, all $P < 0.005$). 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 IgG). EBNA-1 IgG 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.

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**Figure 1.**

A, Distribution of nasopharyngeal carcinoma (NPC) probability in 300 NPC cases and 294 HC. B, Distribution of probability for NPC in different gender groups. [Control includes non-nasopharyngeal carcinoma (NNPC), benign nasopharyngeal lesions (BNL), healthy subjects (HC)].

**Figure 2.**

Probability for nasopharyngeal carcinoma (NPC) of individual patients and controls (non-nasopharyngeal carcinoma (NNPC), benign nasopharyngeal lesions (BNL), healthy subjects (HC)). A, Scatter plot of probability for NPC from NPC sera ($n = 164$) and NNPC sera ($n = 58$), BNL sera ($n = 51$), and HC sera ($n = 120$) in training set. B, Scatter plot of probability for NPC from NPC sera ($n = 136$) and NNPC sera ($n = 41$), BNL sera ($n = 50$), and HC sera ($n = 174$) in validation set. C, Scatter plot of probability for NPC from all NPC sera ($n = 300$) and NNPC sera ($n = 99$), BNL sera ($n = 101$), and HC sera ($n = 294$). Black horizontal lines are means. (Statistically significant difference was found between the NPC and other control groups; no significant difference between NNPC and BNL and HC in training set or validation set).
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, 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></th>
<th>VCA &gt;1:20</th>
<th>EA &gt;1:10</th>
<th>VCA and EA*</th>
<th>DNA &gt;1:1000</th>
<th>Anti-EBV EUROLINE</th>
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</thead>
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<td>68.6</td>
<td>69.5</td>
<td>90.7</td>
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<td>53.7</td>
<td>53.7</td>
<td>41.8</td>
<td>76.1</td>
</tr>
<tr>
<td>Sensitivitiesc</td>
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<td>52.1</td>
<td>52.1</td>
<td>45.7</td>
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</tr>
<tr>
<td>Sensitivitiesd</td>
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<td>68.8</td>
<td>68.3</td>
<td>73.2</td>
<td>94.2</td>
</tr>
<tr>
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<td>92.9</td>
<td>98.3</td>
<td>73.2</td>
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<tr>
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<td>97.0</td>
<td>95.1</td>
<td>98.4</td>
<td>83.2</td>
<td>86.2</td>
</tr>
</tbody>
</table>

aThe optimum combination of VCA and EA detected by IEA. The cutoff values were VCA ≥ 1:20 and EA ≥ 1:10.
bSensitivity was evaluated based on all nasopharyngeal carcinoma patients (n = 300).
cSensitivity was evaluated based on early-stage (I and II) nasopharyngeal carcinoma patients (n = 66).
dSensitivity was evaluated based on T1-2 stage nasopharyngeal carcinoma patients (n = 94).
eSensitivity was evaluated based on T3-4 stage nasopharyngeal carcinoma patients (n = 206).
fSpecificities 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.
gSpecificities were evaluated based on all controls. Controls with benign nasopharyngeal lesions and non-nasopharyngeal carcinoma cancer patients and healthy controls were included.
early-stage nasopharyngeal carcinoma leads to a sensitivity of 76.1% (Table 1). The sensitivity of the EUROLINE was different for different tumor sizes. It had the highest sensitivity of all methods. The sensitivity of the EUROLINE in T1-2 and T3-4 stages was 84.0% and 93.6%, respectively.

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 stronger in nasopharyngeal carcinoma patients, but with lower serum levels and reduced antigen diversity (Supplementary Fig. S1).

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 Li 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
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 autoantibody 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.

Indeed, in 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 screen for nasopharyngeal carcinoma in regions with high incidence rates.

In conclusion, the risk probability analysis using the multiparametric detection of antibodies against EBV among high-risk individuals may reduce the number of subjects needed to be closely followed up and could serve as part of an nasopharyngeal carcinoma screening program in high-risk populations. 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, I. 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, I. Li, J. Fu
Study supervision: T. Scheper, Y.-H. Peng

Acknowledgments
All the author thanks Jana Langner for doing the statistics and Guihua Sun for assistance in specimen collection.

Grant Support
The study (W. Liu and H. Chen) was supported by grants from the National Natural Science Foundation of China (no. 81472008).

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Received February 2, 2017; revised April 20, 2017; accepted July 21, 2017; published OnlineFirst July 28, 2017.

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Cancer Prevention Research

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