

RESEARCH ARTICLE

EA-D p45-IgG as a Potential Biomarker for Nasopharyngeal Carcinoma Diagnosis

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Abstract

Aim: To identify new biomarkers for NPC diagnosis with an anti-EBV Western blot test kit. **Methods:** Serum samples from 64 NPC patients and healthy subjects with four specific VCA-IgA/EA-IgA profiles were tested with an anti-EBV Western blot test kit from EUROIMMUN AG. Proteins were quantified with scores of intensity visually assigned to the protein bands. The markers which showed statistical differences between the NPC and non-NPC subjects were further evaluated in another 32 NPC patients and 32 controls in comparison with established biomarkers including VCA-IgA, EA-IgA, EBV-related protein IgG, and EBV DNA. **Results:** Among the markers screened, EA-D p45-IgG showed a statistically significant difference ($p < 0.05$) between NPC and non-NPC subjects with VCA-IgA positivity. In 32 VCA-IgA positive NPC patients and 32 control subjects, the diagnostic accuracy of EA-D p45-IgG was 78.1% with a positive predictive value of 77.8% and a negative predictive value of 78.6%. In the verification experiment, the specificity and sensitivity of EA-D p45-IgG were 75.0% and 90.6%, respectively. **Conclusions:** EA-D p45-IgG might be a potential biomarker for NPC diagnosis, especially among VCA-IgA positive subjects.

Keywords: EBV - NPC - Western blot - viral capsid antigen - early antigen - EBV nuclear antigen - diagnosis

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Introduction

Nasopharyngeal carcinoma (NPC) is a rare tumor in most countries. However, it is very common in southern regions of China, especially in Guangdong Province, with the prevalence of 10 to 30 per 100 000 population (Su et al., 2002; Yau et al., 2006; Cao et al., 2011; Wei et al., 2011). Diagnosis in the early stage is not easy because of its anatomical location and mild early symptoms (Seong et al., 2000; Lin et al., 2003). Since treatments at late stages are often less effective (Yau et al., 2006) early diagnosis of NPC is critical for achieving good treatment results (Ji et al., 2011). One important feature of NPC is that it is associated with Epstein-Barr virus (EBV) infection. The majority of NPC patients have a variety of EBV antigens, and anti-EBV antibody serological testing has become an important tool for NPC diagnosis (Henle et al., 1970; Henle et al., 1973; Ho et al., 1976; Lynn et al., 1985; Brooks et al., 1992; Cochet et al., 1993; Ng et al., 2006; Ai et al., 2012). Traditional assays of anti-EBV antibodies have been very useful in clinical diagnosis of NPC, however, high false-positive rates may occur in endemic areas (Chang et al., 2008).

VCA-IgA and EA-IgA antibody tests are most

commonly used in clinical diagnosis and screening of NPC (Henle et al., 1973; De et al., 1988; Zong et al., 1992). However, the lack of specificity of the VCA-IgA assay and sensitivity of the EA-IgA assay limits their effectiveness in NPC diagnosis. For example, while more than 90% of NPC patients are VCA-IgA positive (Cao et al., 2006) 90% of people who are VCA-IgA positive do not have NPC (Zhang et al., 2002). VCA-IgA positive patients often need further examinations such as the CT/MR review which is quite costly. Thus the suboptimal performance of the traditional biomarker assays affect the accuracy and cost of NPC diagnosis.

EBV DNA is considered to be a state-of-the-art quantitative blood biomarker in current NPC research. However, it has been reported that EBV DNA load is independent of serological parameters and does not reflect the number of intact tumor cells (Sheen et al., 1998; Lin et al., 2001). In addition, EBV DNA load quantified is expensive. Therefore, the EBV DNA load as a primary screening assay for the diagnosis of NPC is limited (Stevens et al., 2005).

In our study, we detected the EBV-related proteins with a western blot test kit, determined the scores of each test strip according to the color depth (Paramita et

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Table 1. Scores of Some Representative Samples

Sample	p22	p27	p33	p40,41,42	p43	p45	p65	p79	p93	p125
DP-NPC 16	1	4	0	3	3	2	2	4	1	1
DP-NPC 15	0	4	1	2	2	3	2	2	0	1
DP-NPC 14	1	4	1	3	3	1	1	4	1	2
SP-NPC 16	2	3	0	2	2	0	1	0	0	0
SP-NPC 15	1	3	0	0	0	1	1	0	1	0
SP-NPC 14	1	3	0	2	2	3	1	0	0	0
SP-NON-NPC 16	0	0	0	1	0	2	0	0	1	0
SP-NON-NPC 15	0	1	0	1	0	0	0	1	1	0
SP-NON-NPC 14	0	0	0	2	1	1	0	0	1	0
DN-NOR 16	0	0	0	0	0	0	1	0	0	0
DN-NOR 15	3	0	0	1	0	0	1	0	0	0
DN-NOR 14	0	3	0	1	1	1	0	1	0	0

VCA-p22, VCA-p33, VCA-p65, VCA-p125, VCA-p42, VCA-p41, and VCA-p40 as VCA makers, EA-R p93, EA-D p45, and EA-D p43 as EA markers, and p79 as EBNA1 marker and p27 for further antigens

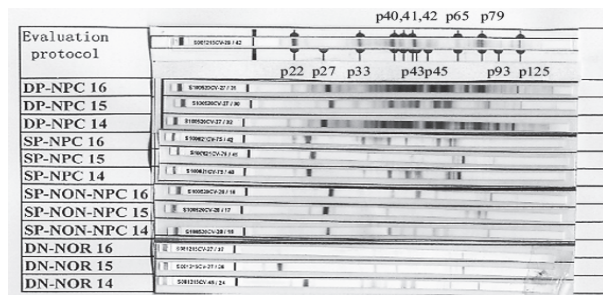


Figure 1. The Results of Some Samples Detected by Anti-EBV Western Blot (IgG) Test Assay Evaluation Protocol: A membrane strip of the same lot was incubated with a positive reference serum in every test kit. Each protein band was visually assigned a score of 0, 1, 2, 3, or 4 according to their intensity

al., 2008), and compared scores of the VCA-IgG, EA-IgG, and EBNA1-IgG antibodies in four subject groups with specific VCA-IgA/EA-IgA profiles. The results demonstrate that EA-D p45-IgG is a potential biomarker for NPC diagnosis.

Materials and Methods

Specimen Collection

Sera samples were collected from 64 NPC patients and healthy subjects (HS) with four specific VCA-IgA /EA-IgA profiles (16 NPC patients with VCA-IgA positive and EA-IgA positive, 16 NPC patients with VCA-IgA positive and EA-IgA negative, 16 HS with VCA-IgA positive and EA-IgA negative, and 16 HS with VCA-IgA negative and EA-IgA negative). In addition, sera and plasma samples were collected from 32 patients with newly diagnosed NPC, eight subjects with other tumors, and 24 healthy subjects to verify the screened markers. The ratio of males to females in each group was 3:1, with ages from 23 to 76, at an average of 38. Among the 32 newly diagnosed NPC patients, there were 26 cases of undifferentiated non-keratinizing carcinoma and six cases of differentiated non-keratinizing carcinoma. In accordance with the clinical nasopharyngeal '92 Fuzhou staging, the patients were classified as one case of stage I, two cases of stage II, 26 cases of stage III, and three cases of stage IV.

NPC Patients were diagnosed at SunYat-sen University Cancer Center from October 2010 to October 2011.

The patient samples were taken after the diagnosis was confirmed but before treatment was received. All diagnoses of NPC had been confirmed by biopsy, and the patients had undergone routine check-ups including head and neck MRIs and chest x-rays. The healthy subjects had undergone routine health examination. Healthy subjects with VCA-IgA positive did not develop NPC during the 3 years after the VCA-IgA test.

Venous blood was collected and the sera samples were separated by centrifugation. The samples were stored at -80 °C for later testing.

Immunoenzymatic Assays

Serological tests for VCA-IgA and EA-IgA antibodies were taken with IEA (Yi et al., 1980) method supplied by Shanghai Institute of Biological Products. IEA were prepared from B95 cell line for VCA, and Raji cell line for EA. Plasma were screened at a dilution of 1:10, and followed by two-fold serial dilutions. The antibody titer was the reciprocal of the highest dilution clearly showing brown color within 15% of the cells. Levels of VCA IgA and EA-IgA were determined by titration, with the cut-off values setting at 1:40 for VCA-IgA and 1:10 for EA-IgA.

Real-Time Quantitative PCR

Real-time quantitative PCR of EBV DNA was carried out at SunYat-sen University Cancer Center (Lo et al., 1999). 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 GGCCTGTCCACC G-3') and the dual-labeled fluorescent probe W-67T (5'-[FAM] CACTGTCTGTAAAGTCCAGCCTCC [TAMRA]-3'). Sequence data for the EBV genome were obtained from the GenBank sequence data base.

Anti-EBV Western Blot (IgG) Test Assay

EBV-IgG was measured using anti-EBV western blot (IgG) test kit from EUROIMMUN AG, Lübeck, Germany (lot numbers of s100520cv-27, s100520cv-28, and s100621cv-75). The EUROIMMUN anti-EBV western blot test strip is an EBV whole extract including VCA-p22, VCA-p33, VCA-p65, VCA-p125, VCA-p42, VCA-p41, and VCA-p40 as VCA makers, EA-R p93,

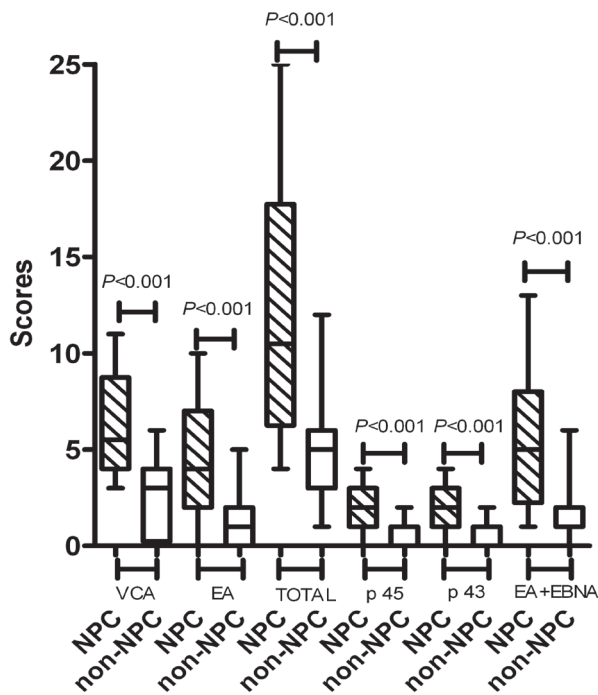


Figure 2. Scores of the Six Screened Markers in NPC Patients and Non-NPC Subjects by Anti-EBV Western Blot (IgG) Test Assays

EA-D p45, and EA-D p43 as EA markers, p79 as EBNA1 marker, and p27 for further antigens. The intensity of the antigen bands correlates with the antibody titer. The results were presented as scores of 0, 1, 2, 3, and 4, respectively, according to the intensity of each band in comparison with the evaluation protocol (Figure 1 and Table 1). The study was carried out by three individuals. One person was responsible for collecting specimens and performing assays, and the other two people assigned the scores of each strip test under blind conditions.

Calculation and Statistical Analysis

All statistical analyses were performed using SPSS software version 19.0 (Chicago, IL, USA). Sensitivity = the positive cases of NPC group/ the total cases of NPC group × 100%. Specificity = the negative cases of healthy group/ the total cases of healthy group × 100%. Receiver operating characteristic (ROC) curves were plotted by sensitivity versus (1 - specificity). Comparisons between groups were made using one-way analysis of variance (ANOVA) with least significant difference t-test for post hoc analysis. All statistical tests were two-sided, and a *p*-value of <0.05 was considered to be statistically significant.

Results

We first aimed to investigate the potential of several EBV-related proteins as biomarkers of NPC. We collected sera samples from 64 NPC patients and healthy subjects based on the results of VCA-IgA and EA-IgA detected by traditional IEA. After assigning scores to specimen strips, we calculated and analyzed the scores of each band. To identify proteins that are differentially present in NPC sera, we compared the scores of the protein bands in the sera of

Table 2. Scores of the Six Markers (IgG) in the Four Study Groups

Index	DP-NPC (\bar{x} ±SD)	SP-NPC (\bar{x} ±SD)	SP-NON-NPC (\bar{x} ±SD)	DN-NOR (\bar{x} ±SD)
Total	16.31±5.88	8.13±3.77	5.94±2.32	3.65±1.59
EA	6.44±2.83	2.69±2.06	1.19±1.33	2.88±2.90
EA+EBNA	7.75±3.44	3.63±2.22	2.00±1.37	1.88±1.20
VCA	7.88±2.25	4.44±1.63	3.94±1.18	1.06±1.34
p45	2.88±1.26	1.31±0.95	0.38±0.62	0.31±0.60
p43	2.81±1.33	1.31±1.08	0.69±0.70	1.33±1.33

Data are presented as mean ± SD

Table 3. The Differential Diagnosis of EA-D p45-IgG and EA-IgG + EBNA1-IgG in 32 NPC Patients (n=16) and Healthy Subjects (n=16) with VCA-IgA Single Positive

Index	Accuracy(%)	Positive predictive value(%)	Negative predictive value(%)
ED-D p45-IgG	78.1(25/32)	77.8(14/18)	78.6(11/14)
EA-IgG+EBNA1-IgG	65.6(21/32)	72.7(8/11)	61.9(13/21)

NPC patients with that of healthy subjects (non-NPC). A panel of markers was selected based on the analysis of the screening data in which the scores of these markers were higher in the NPC group than that in the healthy subjects. Six markers (EVB-IgG, VCA-IgG, EA-D p43, EA-IgG, EA-IgG + EBNA1-IgG, and EA-D p45-IgG) were found to show statistically significant differences (*p* < 0.001) between the NPC group and non-NPC group (Figure 2). It suggests that these six markers may be useful in differential diagnosis of NPC. In order to assess the potential of these markers in NPC diagnosis among the VCA-IgA single positive subjects, we performed statistical analyses of the four groups of samples, namely, the sera of VCA-IgA and EA-IgA double positive NPC patients (DP-NPC), VCA-IgA single positive NPC patients (SP-NPC), VCA-IgA single positive non-NPC patients (SP-NON-NPC), and VCA-IgA and EA-IgA double negative healthy subjects (DN-NOR), using one-way ANOVA and LSDt-test.

The results showed that the mean scores of total, EA + EBNA and p45 gradually declined in the four groups (Table 2). Except VCA-IgG, the other five markers were not statistically different between the SP-NON-NPC and DN-NOR group. EA-IgG + EBNA1-IgG and EA-D p45-IgG showed statistically significant differences (*p* < 0.05) between the SP-NPC group and SP-NON-NPC group, and the other markers were not statistically different (Figure 3). The EA-IgG + EBNA1-IgG and EA-D p45-IgG were significantly different between any two groups (DP-NPC, SP-NPC and SP-NON-NPC).

In 32 cases of NPC and healthy subjects with VCA-IgA single positive, the accuracy, positive predictive value and negative predictive value of EA-D p45-IgG were 78.1%, 77.8%, and 78.6%, respectively (Table 3).

We then assessed the six screened markers in the sera and plasma samples from another 32 patients with newly diagnosed NPC, eight patients with other tumors, and 24 healthy subjects. We tested VCA-IgA, EA-IgA and EBV-related protein IgG in sera and EBV DNA in

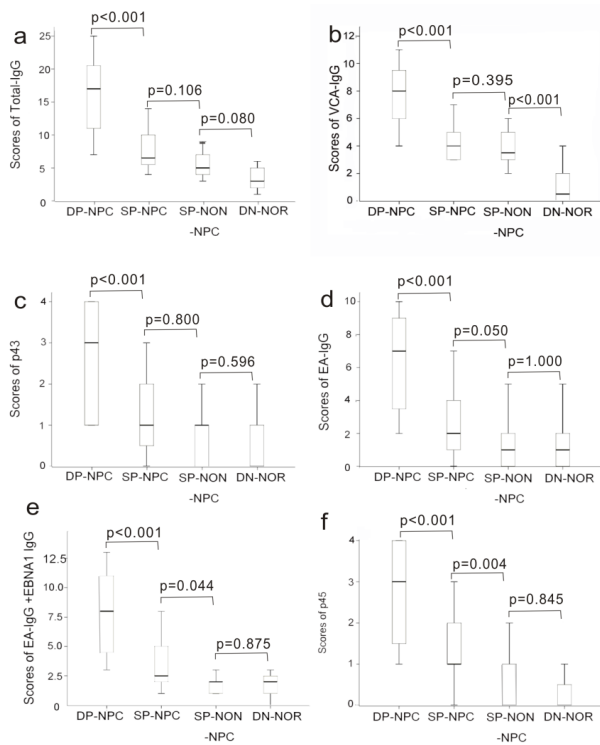


Figure 3. Scores of the Six Markers in the Four Study Groups (a) total EVB-IgG, (b) VCA-IgG, (c) EA-D p43, (d) EA-IgG, (e) EA-IgG + EBNA1-IgG, (f) EA-D p45-IgG

Table 4. Diagnostic Values of the Six Markers in Comparison with That of VCA-IgA, EA-IgA, and EBV DNA

Index	Cut-off value	Sensitivity %	Specificity %
Total-IgG	6	87.5(28/32)	71.9(23/32)
EA-IgG	2	81.3(26/32)	68.8(22/32)
EA+EBNA-IgG	3	75.0(24/32)	78.1(25/32)
VCA-IgG	4	84.4(27/32)	71.9(23/32)
p45-IgG	1	53.0(17/32)	87.5(28/32)
p43-IgG	1	90.6(29/32)	75.0(24/32)
VCA-IgA	1:40	90.6(29/32)	87.5(28/32)
EA-IgA	1:10	75.0(24/32)	93.8(30/32)
EBV-DNA	1000	71.9(23/32)	93.8(30/32)

VCA-p22, VCA-p33, VCA-p65, VCA-p125, VCA-p42, VCA-p41, and VCA-p40 as VCA markers, EA-R p93, EA-D p45, and EA-D p43 as EA markers, p79 as EBNA1 marker, and p27 for further antigens

plasma. Sensitivity and specificity are popular parameters for assessing diagnostic values of biomarkers. Sensitivity is the probability of a positive test among patients with disease, while specificity is the probability of a negative test among subjects without disease. We evaluated the sensitivity and specificity of the six screened markers for NPC diagnosis by defining the cut-off values through ROC analysis (Figure 4). The areas under the curve (AUC) of EVB-IgG, VCA-IgG, EA-D p43-IgG, EA-IgG, EA-IgG + EBNA1-IgG, and EA-D p45-IgG were 0.892, 0.886, 0.800, 0.839, 0.858, and 0.876, respectively. Accordingly, the cut-off values, sensitivity, and specificity of total IgG, EA-IgG, EA + EBNA-IgG, VCA-IgG, p43-IgG, and p45-IgG for NPC diagnosis in the confirmation study were

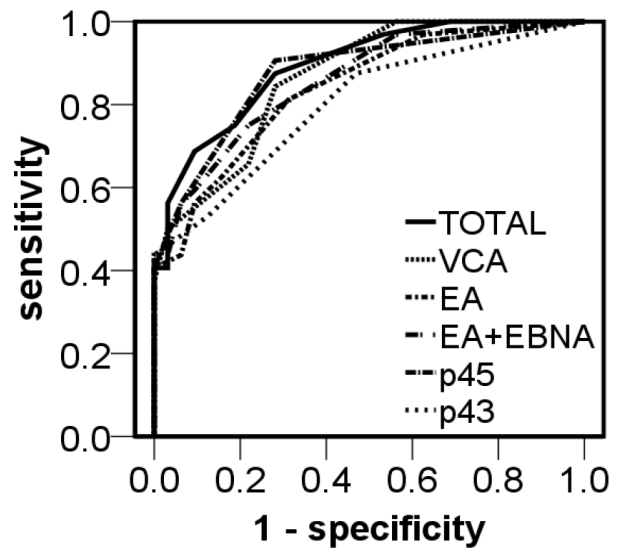


Figure 4. Receiver Operating Characteristic (ROC) Analysis of the Six Markers. The areas under the curve (AUC) of EVB-IgG, VCA-IgG, EA-D p43, EA-IgG, EA-IgG + EBNA1-IgG, and EA-D p45-IgG were 0.892, 0.886, 0.800, 0.839, 0.858, and 0.876, respectively

determined to be >5, 87.5 %, 71.9%; > 1, 81.3%, 68.8%; >2, 75.0%, 78.1%; >3, 84.4 %, 71.9 %; >0, 53.0%, 87.5%; and >0, 90.6%, 75.0%, respectively (Table 4).

EBV DNA load, VCA-IgA, and EA-IgA are widely known as the biomarkers of NPC. For this reason, EBV DNA load in plasma and VCA-IgA and EA-IgA in sera were analyzed to enable a comparison with the six screened markers in diagnosis of NPC. The sensitivity and specificity of EBV DNA load, VCA-IgA, and EA-IgA were 71.9% and 93.8%, 90.6% and 87.5%, and 75.0% and 93.8 %, respectively (Table 4).

Discussion

EB viruses can proliferate in the lymphocytes and make the cells transform, and can long term subculture. The virus-infected cells have the genome of EBV and can produce various EBV-related antigens, including EBV nuclear antigen (EBNA), early antigen (EA), membrane antigen (MA), viral capsid antigen (VCA), and lymphocyte membrane antigen (LYDMA). Except LYDMA, the EBNA, MA, VCA, and EA of NPC patients can all induce IgG and IgA antibody production. After the human body is infected, EA-IgG and VCA-IgG will peak in 5 weeks and EBNA1-IgG in 5 months. At present, the high false positive rate of the screening biomarker and low sensitivity of the diagnosis biomarker make accurate early diagnosis of NPC difficult (Tsang et al., 2004).

This study is the first to use anti-EBV western blot test kit to measure the sera levels of EBV-associated protein IgG antibodies in an effort to identify new biomarkers for the detection and prognosis of NPC. Although anti-EBV western blot test kit has been used to investigate infectious mononucleosis disease (IM), its application in the screening of EBV-associated proteins for NPC diagnosis has never been reported. Previous studies had reported some markers for NPC, but no one was unable to go beyond the role of nasopharyngeal cancer screening

until now.

EBV-related serological testing has been used for NPC diagnosis. Buisson et al. (1999) used recombinant proteins (VCA p23, EA p138, EA p54, and EBNA-1 p72) to compare with an immunofluorescence assay using 291 sera samples. The results showed that the accuracy was 94.5% for detecting primary EBV infection with an immunoglobulin G anti-VCA p23 band correlating with reactivation. While several EBV-related antibodies have been established as biomarkers for NPC diagnosis, the diagnostic potential of EBV proteins has not been fully explored. Only a small number of EBA proteins have been studied and the diagnostic fragments have not been identified. In this study, we aimed to identify additional EBV-related protein as new potential biomarkers for NPC diagnosis. We screened sera samples from NPC patients and healthy subjects with specific VCA-IgA/EA-IgA profiles using the anti-EBV western blot test kit from EUROIMMUN AG. Statistically significant differences were observed in the scores of six markers (EBV-IgG, VCA-IgG, EA-D p43-IgG, EA-IgG, EA-IgG + EBNA1-IgG, and EA-D p45-IgG) between NPC patients and healthy subjects. These were selected as potential NPC biomarkers and further analyzed. EA-IgG + EBNA1-IgG and EA-D p45-IgG scores were statistically different ($p < 0.05$) between NPC patients and healthy subjects with VCA-IgA single positive. We demonstrated for the first time that EA-D p45-IgG can be potentially used for NPC diagnosis among VCA-IgA single positive subjects. The EBV-induced early antigens (EA) are among several antigen complexes that have been identified in EBV-infected cells. The EA complex is composed of diffuse (EA-D) and restricted (EA-R) components. The activity of EA-D is suppressed during latent infection. BMRF1, the gene that encodes EA-D, is closely associated with the gene encoding EBV DNA polymerase, and EA-D is essential for the activity of this polymerase. EA-D forms a complex with EBV DNA polymerase which may play a role in viral replication.

In summary, in this study of 32 VCA-IgA positive NPC patients and 32 healthy subjects, the diagnostic accuracy of EA-D p45-IgG was 78.1% with a positive predictive value of 77.8% and a negative predictive value of 78.6%.

To compare the diagnostic value of the six screened markers with the established markers, we tested VCA-IgA, EA-IgA, EBV-related protein IgG, and EBV DNA in parallel with the six screened markers in another study involving 32 patients with newly diagnosed NPC, eight patients with other tumors, and 24 healthy subjects. EA-D p45-IgG demonstrated good diagnostic performance with the AUC, specificity, and sensitivity of 0.876, 71.8%, and 90.6%, respectively, with a cutoff value of 1. The performance of EA + EBNA IgG was less satisfying with the AUC, specificity, and sensitivity of 0.858, 71.9%, and 84.4%, respectively, with a cutoff value of 3. The performance of both EA-D p45-IgG and EA + EBNA IgG was not as good as that of the traditional markers of VCA-IgA, EA-IgA, and EBV DNA in our study (Luo et al., 2009), but EA-D p45-IgG did show value in NPC diagnosis among subjects with VCA-IgA single positive. Therefore, EA-D p45-IgG might be used as a biomarker

secondary to VCA-IgA for preliminary NPC screening. It will greatly reduce the false positive rate of VCA-IgA.

In this study we investigated a significant number of EBV-related antibodies as potential NPC biomarkers among NPC patients and healthy subjects with diverse VCA-IgA/EA-IgA profiles. Reagents and experimental procedures were handled with high standards through the entire study. The specificity and sensitivity of VCA-IgA, EA-IgA, and EBV DNA observed were consistent with that previously reported (Luo et al., 2009; Han et al., 2012), validating the quality of our study. Our preliminary results identified EA-D p45-IgG as a promising new NPC biomarker. Future evaluation of these markers (and in combination with other markers, particularly IgA antibody) for NPC diagnosis among high-risk individuals is warranted.

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