RESEARCH ARTICLE

Diagnostic Significance of Combined Detection of Epstein-Barr Virus Antibodies, VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA for Nasopharyngeal Carcinoma

Yong-Lin Cai[1&], Jun Li[1&], Ai-Ying Lu[1], Yu-Ming Zheng[1], Wei-Ming Zhong[2], Wei Wang[3], Jian-Quan Gao[2], Hong Zeng[1], Ji-Ru Cheng[1], Min-Zhong Tang[1,4*]

Abstract

The objective of this study was to investigate the diagnostic significance of EBV antibody combined detection for nasopharyngeal carcinoma (NPC) in a high incidence region of southern China. Two hundred and eleven untreated NPC patients, 203 non-NPC ENT patients, and 210 healthy controls were recruited for the study. The titers of VCA/IgA and EA/IgA were assessed by immunoenzyme assay, and the levels of Rta/IgG and EBNA1/IgA were determined by enzyme-linked immunosorbent assay. The levels of VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA demonstrated no association with gender or age ($p>0.05$). The receiver operating characteristic curve and the area under the curve were used to evaluate the diagnostic value. The sensitivity of VCA/IgA (98.1%) and the specificity of EA/IgA (98.5%) were the highest. When a logistic regression model was used to combine the results from multiple antibodies to increase the accuracy, the combination of VCA/IgA+Rta/IgG, whose area under the curve was 0.99, had the highest diagnostic efficiency, and its sensitivity, specificity and Youden index were 94.8%, 98.0% and 0.93 respectively. The data suggest that the combination of VCA/IgA+Rta/IgG may be most suitable for NPC serodiagnosis.

Keywords: Nasopharyngeal carcinoma - epstein-barr virus - antibody - tumor marker - serodiagnosis

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Introduction

As a member of the herpesviridae, Epstein-Barr virus (EBV) infected more than 90% of the human population (Bocian et al., 2011). It has been well accepted that EBV infection is strong association with nasopharyngeal carcinoma (NPC) (Brennan, 2006; Han et al., 2012). The World Health Organization (WHO) classified NPC into two types, namely, the keratinizing squamous cell carcinoma and non-keratinizing carcinoma in 1992. The latter, which is by far the most dominant type in endemic area of southern China, is an undifferentiated or poorly differentiated carcinoma, in which the EBV infection exhibits type II latency pattern. EBV latency-associated gene expression is consistently detected in NPC tumor cells and is regarded therefore as one of the factors in the oncogenesis (Raab-Traub, 2002; Burgos, 2005). Although viral expression in NPC cells is largely restricted to EBV nuclear antigen-1 (EBNA1) and latent membrane protein-1 (LMP1), and LMP2A (Burgos, 2005; Hariwiyanto et al., 2010), transcription of a large variety of viral genes including those associated with EBV replication cycle was detected (Zhang et al., 1998; Xue et al., 2000; Walling et al., 2001; Chen et al., 2013). In situ studies identified lytic EBV antigens in nonmalignant epithelial cells (Tao et al., 1995) and infiltrating lymphoid cells (Wen et al., 1997). It is a special feature of NPC that the patients sustain high levels of a broad spectrum of EBV antibodies. The presence of antibodies against both latent and replication (lytic) antigens of EBV suggests that both latent and lytic EBV infections are present in NPC patients.

The presence of immunoglobulin A (IgA) to EBV capsid antigens (VCA) and early antigens (EA) may be biomarkers for EBV reactivation and serves as predictive markers for NPC in Chinese populations(Deng et al., 1995; Chien et al., 2001). Upon EBV reactivation, two key immediate early lytic genes, BZLF1 and BRLF1,

*[1]Wuzhou Health System Key Laboratory for Nasopharyngeal Carcinoma Etiology and Molecular Mechanism, [2]Department of Oncology, Wuzhou Red Cross Hospital,Wuzhou, Guangxi Province, [3]Research Center of Clinical Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province, [4]College of Life Science and Bio-Engineering, Beijing University of Technology, Beijing, China [&]Equal contributors *For correspondence: gxtom2000@gmail.com.*

encoding Zta (BZLF1 transcription activator) and Rta (BRLF1 transcription activator) respectively, are transcribed, and consequently activate several downstream viral promoters and lead to an ordered cascade of viral gene expression (Liu et al., 2003). Rta was reported to disrupt viral latency in an epithelial cell-specific fashion (Ragoczy et al., 1998). Feng et al. (2000) reported that EBV remains in the latent state in blood of both NPC patients and healthy individuals; and in NPC patients, viral reactivation occurs in local tumor tissues with a specific expression of BRLF1, which subsequently results in induction of a strong humoral immune response to its protein product. The specific expression of EBV BRLF1 mRNA and especially its protein product, Rta, could be regarded as a tumor antigen for NPC, and may be useful as a serologic parameter for the screening and diagnosis of patients with NPC (Feng et al., 2001; Ren et al., 2006). The latent antigens such as EBNA1 probably derive from most of NPC cells in which EBV latency was present, and part of tumor tissues where there is a very active EBV replication may produce the lytic antigens of EBV. Moreover, the infiltrating lymphocytes in NPC may contribute significantly to the elevation of antibodies to the lytic antigens of EBV as well (Niedobitek, 2000). Therefore, the diversity of EBV antigens in NPC could provide the conditions, which are conducive for a vigorous antibody response, to the virus, and this would give rise to a general and sustained elevation of serum levels of EBV antibodies.

At present, no single biomarker for cancer is considered adequately sensitive and specific for cancer screening. It is expected that the results of multiple markers will need to be combined in order to yield adequately accurate classification. In this article, we aimed to investigate the value of combined detection of EBV antibodies including VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA in evaluating the clinicopathologic features and diagnosis for NPC in a high incidence region of southern China.

Materials and Methods

Patient selection

NPC cases and controls were recruited from Wuzhou Red Cross Hospital during November 2006 to November 2008. Two hundred and eleven newly diagnosed NPC patients (mean age 48±10 years) were confirmed histologically, and those main type histological accounting for 94.8% (200/211) was non-keratinizing carcinoma. Clinical stages were categorized by the project of Fuzhou 1992' Staging, 4 cases were at stage I, 25 at stage II, 74 at stage III, 96 at stage IVa and 12 at stage IVb.

Two subgroups of control were recruited: patient controls and healthy controls. The patient control group included 203 non-NPC ear-nose-throat (ENT) patients (mean age 42±14 years) with complaints, such as epistaxis, tinnitus, vertigo, otitis media, sinusitis, vasomoto rhinitis, but who were histologically negative for NPC. Two hundred and ten healthy participants (mean age 40±10 years) were recruited for the healthy control group. Informed consent was obtained for all the individuals. Institutional Review Boards at Wuzhou Red Cross Hospital approved the study protocol and informed consent.

Serological detection

Serum from peripheral blood samples were obtained by venipuncture at study enrollment and stored at-70°C until use. Serological testing for VCA/IgA and EA/IgA was done by immunoenzymatic assay. B95-8 cells were used for the detection of VCA/IgA antibodies, and Raji cells were used for EA/IgA test. Sera at 1:10 to 1:640 dilutions were added to cells in separate wells and the slides were incubated at 37°C for 30min at a humid atmosphere and washed 3 times with PBS. Horseradish peroxidase labeled antihuman IgA antibody at the appropriate dilution was added to the slides, and the slides were further incubated at 37°C for 30 min, washed 3 times with phosphate buffered saline (PBS) and immersed into diaminobenzene solution with H_2O_2 for 10 min. Positive and negative controls were included in each experiment.

The kits of EBV Rta/IgG (Tarcine BioMed, Beijing) and EBNA1/IgA (Zhongshan Bio-tech, Zhongshan) were done by enzyme-linked immnunosorbent assay (ELISA). The tests were performed according to manufacturer's instructions. Briefly, 0.1ml of 1:10 (1:20 for EBNA1/IgA) diluted duplicate serum specimen were added to the microplate wells. After incubation at 37°C for 30 min., the wells were washed 5 times with PBS and then reacted with 0.1 ml of sheep radish peroxidase conjugated rabbit anti-human IgA for 30 min (20 min for EBNA1/IgA) at 37°C. After washing 5 times with PBS, TMB solution was added to the well and incubated for 10 min at 37°C. The color development was stopped by the addition of 0.1 ml (0.05 ml for EBNA1/IgA) of 2M H_2SO_4. The level of antibodies present in the serum specimens is indicated by the absorbance (A) value measured at 450nm. A strong positive control serum and a weakly reactive normal control serum were concurrently tested in each test run. Interassay variations were minimized by expressing antibody levels in relative A (rA) values calculated as ratios of mean A values of test samples to that concurrently determined for a positive reference serum (Cheng et al., 2002).

Statistical analysis

Data are presented as *Median (Quartile lower-Quartile upper)*. The titers of VCA/IgA and EA/IgA, and rA value of Rta/IgG and EBNA1/IgA were compared among multiple groups using *Kruskal-Wallis H* rank test, or between two groups using *Mann-Whitney U* rank test. The binary unconditional logistic regression models were established with the method of Forward Likelihood-Ratio for various combined determinations of antibodies, respectively. Using the predicted probability as the analyzed variable, the receiver operating characteristic (ROC) curve was generated to evaluate the diagnostic values of different combined determinations including sensitivity, specificity and Youden index, and the area under the ROC curve (AUC) of different combined determinations were compared using z test. All statistical tests were two-sided, and $p<0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS 13.0 and MedCalc 9.5 software.

Table 1. Correlation between The Clinicopathologic Features and Serum Levels of VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA in NPC Patients [M(QL-QU)]

Characteristics	n	VCA/IgA (1:)	*p* value	EA/IgA (1:)	*p* value	Rta/IgG (rA)	*p* value	EBNA1/IgA (rA)	*p* value
Gender									
Male	156	160 (80-320)	0.72	40 (20-80)	0.64	1.40 (0.62-2.07)	0.83	0.76 (0.41-1.28)	0.07
Female	55	160 (80-320)		40 (20-80)		1.36 (0.65-2.07)		0.64 (0.37-0.96)	
Age (y)									
≤39	47	80 (40-160)	0.05	20 (10-40)	0.07	0.85 (0.60-1.58)	0.07	0.68 (0.32-1.15)	0.44
40-49	67	160 (40-320)		40 (10-80)		1.38 (0.64-2.15)		0.81 (0.46-1.25)	
50-59	67	160 (80-320)		40 (20-80)		1.56 (0.68-2.11)		0.68 (0.51-1.22)	
≥60	30	160 (80-320)		40 (20-80)		1.68 (0.67-2.15)		0.54 (0.35-1.06)	
T classification									
T1	14	80 (40-160)	0.23	15 (10-40)	0.26	1.10 (0.59-1.93)	0.4	0.29 (0.19-0.69)	0.01*
T2	59	160 (40-320)		40 (10-80)		1.15 (0.54-2.07)		0.81 (0.62-1.38)	
T3	53	160 (80-320)		40 (20-80)		1.61 (0.64-2.07)		0.68 (0.37-1.19)	
T4	85	160 (80-320)		40 (20-80)		1.36 (0.79-2.09)		0.67 (0.42-1.08)	
N classification									
N0	29	80 (40-320)	0.01*	40 (10-40)	0.01*	1.37 (0.61-2.08)	0.53	0.37 (0.25-0.94)	0.02*
N1	70	80 (80-320)		40 (20-80)		1.28 (0.68-1.94)		0.67 (0.39-1.26)	
N2	80	160 (80-320)		40 (20-80)		1.55 (0.71-2.14)		0.76 (0.53-1.15)	
N3	32	320 (160-640)		80 (40-160)		1.10 (0.52-2.09)		0.83 (0.52-1.37)	
Metastasis									
No	199	160 (80-320)	0.07	40 (20-80)	0.1	1.39 (0.63-2.07)	0.36	0.69 (0.38-1.20)	0.36
Yes	12	240 (160-480)		40 (40-120)		1.18 (0.52-1.81)		0.87 (0.47-1.27)	
Clinical stage									
I+II	29	80 (40-160)	0.02*	20 (10-40)	0.01*	1.35 (0.59-1.89)	0.27	0.46 (0.23-0.84)	0.01*
III+IV	182	160 (80-320)		40 (20-80)		1.40 (0.65-2.09)		0.74 (0.44-1.23)	

*Statistically significant.

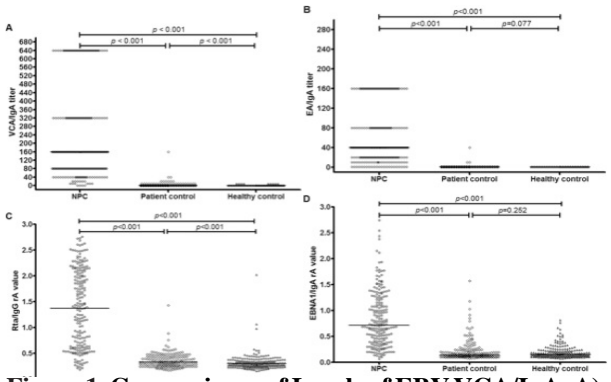

Figure 1. Comparisons of Levels of EBV VCA/IgA; A), EA/IgA; B), Rta/IgG; C) and EBNA1/IgA; D) in Sera in Patients with NPC (n=211), Patient Controls (n=203) and Healthy Controls (n=210). Horizontal bars indicate medians. Statistical analysis of overall comparison was done by *Kruskal-Wallis H* rank test, and that of multiple comparison was done by *Mann-Whitney U* rank test

Results

Comparison of EBV antibody levels in the clinicopathologic features of NPC

We analyzed the possible correlation between the serum levels of these four antibodies and various clinicopathological features of the NPC patients enrolled in this study. No statistical association with gender, age and distant metastasis could be found in all four antibodies (Table 1). The VCA/IgA and EA/IgA antibody titers were significantly lower in patients with early N classification and clinical stage compared with the later stage patients, but not statistically associated with T classification (Table 1). And the EBNA1/IgA antibody levels were significantly lower in patients with early T classification, N classification and clinical stage compared with the later stage patients (Table 1).

Table 2. Diagnostic Values of The Four EBV Antibodies in Detection for NPC

Antibody	Sensitivity (%)	Specificity (%)	Youden index	Area under ROC curve (95%CI)
VCA/IgA	98.1 (207/211)	82.8 (168/203)	0.81	0.98 (0.96-0.99)
EA/IgA	89.1 (188/211)	98.5 (200/203)	0.88	0.94 (0.92-0.96)
Rta/IgG	90.5 (191/211)	85.2 (173/203)	0.76	0.92 (0.89-0.95)
EBNA1/IgA	87.2 (184/211)	84.2 (171/203)	0.71	0.92 (0.89-0.95)

Comparison of levels of EBV antibodies in NPCs, patient controls and healthy controls

Levels of EBV antibodies in NPC group, patient control group and healthy control group were showed in Figure 1. For each antibody, the *Kruskal-Wallis H* rank test and *Mann-Whitney U* rank test showed that the mean levels among NPCs were all significantly higher than those in each subgroup of controls ($p<0.001$).

Diagnostic value of each EBV antibody independently

According to ROC curve, the cutoff values of Rta/IgG, EBNA1/IgA, VCA/IgA and EA/IgA were defined as 0.49, 0.26, 1:10 and 1:10, respectively. Comparison of diagnostic values for each EBV antibody (Table 2) showed that the highest sensitivity in VCA/IgA (98.1%) and the highest specificity in EA/IgA (98.5%). And the diagnostic accuracy of the ratio for VCA/IgA, defined by AUC was the highest (Table 2 and Figure 2).

Diagnostic accuracy of combinations of EBV antibodies

Table 3 showed that the diagnostic accuracy, defined

Table 3. Diagnostic Acuracy of Different Combined Determinations of EBV Antibodies Accessed with ROC Curve Based on Logistic Regression

Combination	Cutoff value of probability prediction	Sensitivity (95%CI)%	Specificity (95%CI)%	Youden index	Area under ROC curve (95%CI)%
Rta/IgG+EBNA1/IgA	0.32	93.4 (89.1-96.3)	90.6 (85.8-94.3)	0.84	0.97 (0.95-0.98)
VCA/IgA+EA/IgA	0.41	92.4 (88.0-95.6)	98.5 (95.7-99.7)	0.91	0.98 (0.96-0.99)
VCA/IgA+ EBNA1/IgA	0.47	94.3 (90.3-97.0)	98.0 (95.0-99.4)	0.92	0.98 (0.97-0.99)
VCA/IgA+Rta/IgG	0.36	94.8 (90.9-97.4)	98.0 (95.0-99.4)	0.93	0.99 (0.98-1.00)
VCA/IgA+EA/IgA+EBNA1/IgA	0.31	97.2 (93.9-98.9)	95.6 (91.7-97.9)	0.93	0.98 (0.97-0.99)
VCA/IgA+EA/IgA+Rta/IgG	0.65	92.9 (88.5-96.0)	99.5 (97.3-99.9)	0.92	0.99 (0.98-1.00)
VCA/IgA+Rta/IgG+EBNA1/IgA	0.52	94.8 (90.9-97.4)	98.5 (95.7-99.7)	0.93	0.99 (0.98-1.00)
VCA/IgA+EA/IgA+Rta/IgG+EBNA1/IgA	0.34	96.7 (93.3-98.6)	97.0 (93.7-98.9)	0.94	0.99 (0.98-1.00)

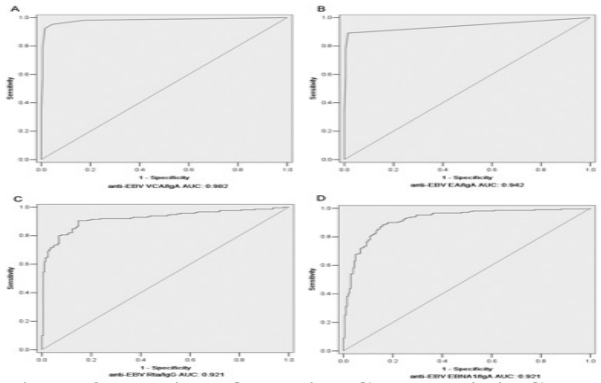

Figure 2. Receiver Operating Characteristic Curves (ROCs) for VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA

by AUC, showed significantly different between Rta/IgG+EBNA1/IgA combination with other combinations, respectively (p<0.05); likewise, there were significant differences between VCA/IgA+EA/IgA combination with VCA/IgA+Rta/IgG and VCA/IgA+EA/IgA+Rta/IgG combinations for AUC (p<0.05). And no significant differences were found among other combinations for AUC (p>0.05).

Discussion

In the endemic area of southern China, the incidence rate of NPC rapidly increases after age 30. Ninety three percent NPC patients are over 30 years at diagnosed. The peak age of NPC at diagnosis was 35 to 55 years old. The study subjects from Wuzhou City of Guangxi Province in southern China holds, perhaps, the highest recorded NPC incidence (Tang et al., 2010; 2012; Huang et al., 2012). NPC prefers males over females. The male to female ration is about 2/1 to 3/1 (Jia et al., 2006). In this study, the male to female ratio of NPC was 2.84 and the patients with NPC at the age from 30 to 60 accounted for 83.4% (176/211), which accorded with characteristic of NPC epidemiology above. The results of this study showed that EBV antibodies of VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA were not significantly associated with the gender and age of NPC.

The lowest antibody levels of EBNA1/IgA for the patients with NPC at T_1 classification, N_0 classification and clinical stage I have been shown in present study. These results suggested that EBNA1/IgA antibody expression was relatively low in serum of NPC at early stage or with minor clinical symptoms. We speculate that since EBNA1 gene is mainly expressed by most of NPC cells in which EBV persists in the latent stage, the low expression of EBNA1 antigen may be explained by the small size of primary lesion. On the other hand, there were the rising trends of VCA/IgA and EA/IgA titers with the degree of N classification. The infiltrating lymphocytes in NPC may also contribute significantly to the elevation of antibodies to the lytic antigens of EBV (Niedobitek, 2000). Belonging to be the EBV lytic antigens, VCA and EA may result expressing highly from the degree of neck lymph node metastasis. However, Rta/IgG antibody has shown no significantly associated with the TNM classification and clinical stage. One possible reason for this is that despite being the lytic antigen, Rta proteins are produced by the immediate early gene BRLF1 which expression might be relatively stable.

At the aspect of diagnostic value of EBV antibody independently, the sensitivity of VCA/IgA and the specificity of EA/IgA were the highest; these results were similar to our team previous studies (Li et al., 2007; Zhan et al., 2009) and were superior to the performances of the same seromarkers by IFA reported by (Yu et al., 2011; Liu et al., 2012). It suggests that on the current technological condition, VCA/IgA and EA/IgA detected by immunoenzymatic assay still play important roles in NPC screening and diagnosis.

Recently, combined determination of multiple markers for NPC serodiagnosis has become a popular tendency (Leung et al., 2004; Fachiroh et al., 2006; Hu et al., 2007). A previous study conducted by our group had been shown that combined detection of Rta/IgG, EA/IgA, VCA/IgA, and EBNA1/IgA serve as an optimal combination for NPC serodiagnosis (Cai et al., 2010). In this study, we evaluated the diagnostic value by ROCs and AUC in an extended study cohort; expect to provide a more suitable model for NPC serodiagnosis. Our results showed that AUC of VCA/IgA+EA/IgA were relatively lower in two-marker combinations evaluated by ROCs based on logistic regression. The collinearity of two variables due to strong correlation between VCA/IgA and EA/IgA (r=0.980, p<0.001) may partly weaken the fitting power of logistic regression. However, the diagnostic accuracy of VCA/IgA+Rta/IgG combination with the highest AUC and relatively higher Youden index was significantly higher than that of VCA/IgA+EA/IgA. Four NPC cases showed VCA/IgA negative. Among them, 2 patients were showed EBNA1/IgA positive, but all these 4 patients

were Rta/IgG positive. On the other words, none of NPC showed negative for VCA/IgA and Rta/IgG at the same time. It indicated that there was the complementarity with VCA/IgA and Rta/IgG for NPC serodiagnosis. Thus, the accuracy of VCA/IgA+Rta/IgG combination was the highest among that of two-marker combinations.

In three-marker combinations, both of the sensitivity and specificity of VCA/IgA+EA/IgA+ EBNA1/IgA and VCA/IgA+EA/IgA+Rta/IgG were not improved at the same time due to collinearity between VCA/IgA and EA/IgA so as to weaken the fitting power of logistic regression as well. The accuracies of these two three-marker combinations were similar or far worse than that of VCA/IgA+Rta/IgG. The specificity and Youden index of VCA/IgA+Rta/IgG+EBNA1/IgA were just slightly higher than those of VCA/IgA+Rta/IgG, and no significant difference was found in these two combinations. The four-marker combination did not appear more superiority than others; on the contrary, the economical burden of patients would obviously increase.

In conclusion, the expression of EBV antibodies to VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA are similar in different gender and age of NPC. No significant differences of the serum level of Rta/IgG are found among TNM classification and clinical stages of NPC. The EBNA1/IgA antibody is expressed relatively lowly at early NPC stage. The titers of VCA/IgA and EA/IgA are associated with the degree of neck lymph node metastasis, and may play a role in evaluating the stages of NPC. The expressions of EBV lytic antigens are reflected more widely through the combined detection of Rta/IgG against immediate early antigens and VCA/IgA against later antigens with excellent complementarity. In the aspect of cost-benefit, the combination of VCA/IgA+Rta/IgG may be more suitable model for NPC serodiagnosis.

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