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Expression of Epstein-Barr virus antibodies EA-IgG, Rta-IgG, and VCA-IgA in nasopharyngeal carcinoma and their use in a combined diagnostic assay

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ABSTRACT. Epstein-Barr virus (EBV) infection is closely associated with nasopharyngeal carcinoma, which can be monitored by the levels of Rta protein antibody IgG (Rta-IgG), early antigen antibody (EA-IgG), and viral capsid antibody (VCA-IgA). In the present study, we investigated the serum levels of Rta-IgG, EA-IgG, and VCA-IgA in nasopharyngeal cancer patients, and the diagnostic value of a combined assay that includes these antibodies in addition to the EBV-DNA. A total of 56 nasopharyngeal cancer patients were recruited as the study population, along with 48 benign rhinitis patients and 42 healthy individuals. Serum EA-IgG, Rta-IgG, and VCA-IgA levels were measured by enzyme-linked immunosorbent assay,

and EBV-DNA was quantified with PCR. The diagnostic value of these indices was further evaluated by ROC curve analysis. The expression levels of EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA were elevated in the nasopharyngeal cancer patients, who had higher levels of these antibodies than those in the rhinitis patients, followed by the healthy individuals. These indices were also increased with advanced TNM stage. The overall diagnostic efficacy was ranked as follows: VCA-IgA, Rta-IgA, EA-IgA, and EBV-DNA. The combined diagnosis using these four indices increased the sensitivity to 98.21% and the negative predictive value to 98.61%, without any significant compromise on the test specificity. In conclusion, EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA expression levels were elevated in nasopharyngeal patients. The combined diagnostic value of these serum indices has important implications in nasopharyngeal carcinoma.

Key words: Nasopharyngeal carcinoma; EBV DNA; Combined assay; VCA-IgA

INTRODUCTION

Nasopharyngeal carcinoma, which often originates in the top and lateral wall of nasopharyngeal cavity, is one of the leading malignant tumors in China, and is known to be closely associated with Epstein-Barr virus (EBV) infection (Chen et al., 2015; Peng et al., 2015). Most nasopharyngeal carcinoma patients have EBV DNA and anti-EBV antibodies detectable in their serum (Lian et al., 2015; Liang et al., 2015). The infectious cycle of EBV can be divided into the latent stage and the lysis stage (Cai et al., 2014; Chen et al., 2014). As the disease progresses, EBV inside the body becomes activated into the replicative stage, which is when the major viral proteins including the *BRLF1* gene coding Rta protein, early antigen (EA), and viral capsid antigen (VCA) are expressed (Luo et al., 2013; Ji et al., 2014). Therefore, immunoglobulins (Ig) against these proteins, including EA-IgG, VCA-IgA, and Rta-IgG can work as molecular biomarkers for predicting the prognosis of nasopharyngeal cancer (Luo et al., 2013; Chen et al., 2014).

Currently, there are many studies focusing on the expression of single antibodies during different stages of EBV infection, and their implications in serum screening and diagnosis of nasopharyngeal carcinoma. However, both sensitivity and specificity of single antibodies have been unsatisfactory thus far. Due to the subtle onset of nasopharyngeal cancer, and minor symptoms at early stages, nasopharyngeal cancer has a high rate of misdiagnosis. Therefore, we hypothesize that the combined use of serum EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA may better reflect the *in vivo* status of EBV, and thus be more predictive of the malignancy of nasopharyngeal cancer. In the current study, we investigated the levels of serum EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA in cancer patients, and discussed the implication of the combined assay in nasopharyngeal carcinoma diagnosis to provide insights for future clinical diagnosis and prognostic predictions.

MATERIAL AND METHODS

Clinical information

A total of 56 nasopharyngeal carcinoma patients, from Affiliated Shanghai Sixth

People's Hospital of Shanghai Jiaotong University, were recruited between September 2011 and October 2014. Another two cohorts of 48 rhinitis patients and 42 healthy individuals were recruited as control groups. All patients were confirmed to have nasopharyngeal cancer based on pathological examinations. Malignant tumor (TNM) stage was classified according to UICC standards (sixth edition, 2002) as low differentiated squamous carcinoma, of which 11 cases were stage I, 20 stage II, 17 stage III, and 8 stage IV. All patients had no other inflammatory diseases or used immune modulator drugs before surgery. Of the 56 cancer patients, there were 27 males and 29 females, with ages ranging from 21 to 68 years old (average age = 43.6 years). In the rhinitis group, there were 26 males and 22 females, with ages ranging from 22 to 67 years old (average age = 45.4 years). In the healthy control group, there were 20 males and 22 females, with ages ranging from 23 to 67 years old (average age = 46.7 years). Healthy control individuals were all recruited from those who underwent routine physical examinations in our hospital and had no history of rhinitis. All three groups had no significant differences in age or gender distribution ($P < 0.05$), and were thus comparable in these regards. This study was approved by the ethical committee of our hospital and written informed consent was obtained from all participants.

Serum assay

Peripheral blood samples were collected from all individuals, which were first centrifuged (14,000 *g*, 15 min) to separate serum and plasma, and was further anti-coagulation treated with EDTA. Serum Rta-IgG, EA-IgG, and VCA-IgA levels were quantified by enzyme-linked immunosorbent assay (ELISA) using commercially available test kits (Zhongshan Bio, China) according to the manufacturer instructions. Plasma EBV-DNA content was detected after DNA extraction with a commercial kit (Zhongshan Bio, China) followed by the PCR method using EBV-specific primers (Jikang, China).

Both specificity and sensitivity of the four parameters (serum Rta-IgG, EA-IgG, VCA-IgA, and plasma EBV-DNA) in nasopharyngeal carcinoma were compared. In brief, three of the four indices were first tested in combination. A positive result was scored based on any one or more positive index value using pre-specified criteria (EA-IgG > 0.05, VCA-IgA > 1.1, Rta-IgG > 30 U/mL, and EBV-DNA > 1 x 10^3 copies per mL). Overall, negative results were scored when all parameters showed negative results. We then performed a combined assay using all four indices. The critical values of these four parameters were then evaluated by ROC curve analysis.

Statistical analysis

The SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA) was used for analyzing all collected data, of which the comparison of ratios was done by chi-square tests with correction coefficients if necessary. All data were first tested for normality using the Kolmogorov-Smirnov test. Those that fit in the normal distribution curve are reported as means ± standard deviation. Analysis of variance (ANOVA) followed by the LSD test were used to compare means across groups. ROC analysis was used to evaluate the diagnostic efficacy of Rta-IgG, EA-IgG, VCA-IgA, and EBV-DNA. Statistical significance was defined as $P < 0.05$.

RESULTS

Expression levels of Rta-IgG, EA-IgG, VCA-IgA, and EBV-DNA

Nasopharyngeal carcinoma patients had higher Rta-IgG, EA-IgG, VCA-IgA, and EBV-DNA levels compared to those of rhinitis patients and healthy controls, and rhinitis patients also had elevated levels of all viral indices compared to those of healthy controls ($P < 0.05$ in all cases; Table 1).

Table 1. Expression levels of EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA.

Group	N	Rta-IgG (U/mL)	EA-IgG (against standard)	VCA-IgA (against standard)	EBV-DNA (copy per mL)
Control	42	1.51 ± 0.32	0.28 ± 0.08	0.62 ± 0.21	<500
Rhinitis	48	5.69 ± 1.35*	0.41 ± 0.14*	0.90 ± 0.24*	<500
Nasopharyngeal	56	149.37 ± 26.12*$^{\Delta}$	1.23 ± 0.34*$^{\Delta}$	2.54 ± 0.45*$^{\Delta}$	30865.46 ± 1728.21*$^{\Delta}$
F value	-	48.536	15.637	42.132	121.364
P value	-	0.00	0.00	0.00	0.00

*$P < 0.05$ compared to the control group; $^{\Delta}P < 0.05$ compared to the rhinitis group.

Expression levels across different TNM staged cancer patients

We further compared the viral indices across different TNM stages, and found that nasopharyngeal patients at advanced stages had higher expression levels of EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA than those in patients at earlier stages ($P < 0.05$; Table 2).

Table 2. EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA levels at different TNM classification of malignant tumor TNM stages.

TNM stage	N	Rta-IgG (U/mL)	EA-IgG (against standard)	VCA-IgA (against standard)	EBV-DNA (copy per mL)
Stage I	11	5.42 ± 2.15	0.39 ± 0.11	0.86 ± 0.11	<500
Stage II	20	45.11 ± 3.07*	0.81 ± 0.16*	1.24 ± 0.13*	<500
Stage III	17	96.16 ± 4.13*$^{\Delta}$	1.05 ± 0.13*$^{\Delta}$	2.46 ± 0.12*$^{\Delta}$	6068.36 ± 254.24*$^{\Delta}$
Stage IV	8	154.05 ± 12.36*$^{\Delta\#}$	1.31 ± 0.21*$^{\Delta\#}$	2.59 ± 0.24*$^{\Delta\#}$	38046.55 ± 2132.14*$^{\Delta\#}$
F value	-	51.207	16.585	41.155	118.588
P value	-	0.00	0.00	0.00	0.00

*$P < 0.05$ compared to stage I patients; $^{\Delta}P < 0.05$ compared to stage II patients, $^{\#}P < 0.05$ compared to stage III

ROC analysis

Using healthy controls and nasopharyngeal cancer patients, we performed a ROC analysis where the areas under the curves were 0.873, 0.914, 0.928, and 0.866 for EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA, respectively. This indicates that each of these factors had satisfactory diagnostic efficacy for nasopharyngeal carcinoma. The overall rank of efficacy was (from high to low) as follows: VCA-IgA, Rta-IgG, EBV-DNA, and EA-IgA (Figure 1 and Table 3).

ROC curves were drawn using true positive rates (sensitivity) as ordinate and false positive rates (1 - specificity) as abscissa. The more the ROC curves close to the top left corner, the higher the accuracy of the test. The area values under the curves (AUC) suggested the diagnostic performance of the test, the bigger the better. AUC were between 0.5 and 1.0. In the case of AUC > 0.5, the value closer to 1 indicated a better diagnosis performance. It was lower accuracy when of AUC was from 0.5 to 0.7. It was normal accuracy when AUC was from 0.7 to 0.9. It was high accuracy when AUC was above 0.9.

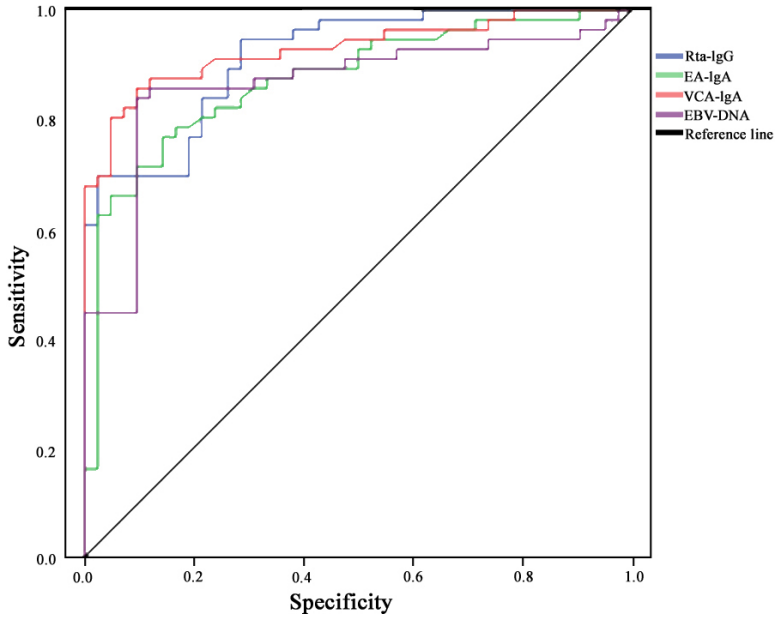

Figure 1. ROC curve analysis for EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA.

Table 3. ROC area under the curve.

Test parameter	Area	SD[a]	P value[b]	95% Confidence interval	
				Lower limit	Upper limit
EA-IgG	0.873	0.036	0.000	0.802	0.943
Rta-IgG	0.914	0.027	0.000	0.862	0.967
VCA-IgA	0.928	0.026	0.000	0.877	0.979
EBV-DNA	0.866	0.039	0.000	0.789	0.942

[a]Under non-parametric hypothesis; [b]null hypothesis: area = 0.5.

Diagnostic efficacy of test parameters

Based on the expression levels in both healthy controls and nasopharyngeal carcinoma patients, we specified the criteria for a positive result of a single index as follows: EA-IgG > 0.05, VCA-IgA > 1.1, Rta-IgG > 30 U/mL, and EBV-DNA > 1 x 10^3 copies per mL. The sensitivity and specificity of EA-IgG, Rta-IgG, EBV-DNA and VCA-IgA in nasopharyngeal cancer were 73.21 and 98.89%, 76.79 and 92.22%, 78.57 and 90.00%, and 66.07 and 96.67%, respectively. VCA-IgA had the highest test sensitivity compared to those of Rta-IgG or EA-IgA. When using only one parameter in the diagnosis of nasopharyngeal cancer, EA-IgG and EBV-DNA had the highest specificity (Table 4).

Combined assay and efficacy

We then compared the efficacy of the combined assay using three or four parameters together. Using the combined assay of all four indices, the sensitivity and negative predictive value were increased to 98.21 and 98.61%, respectively, without significant decrease of specificity (Table 5).

Table 4. Diagnostic efficacy of EBV viral parameters in terms of EA-IgG, Rta-IgG, VCA-IgA, EBV-DNA.

Index	Positive value	Sensitivity (%)	Specificity (%)	False negative (%)	False positive (%)
Rta-IgG	>30 U/mL	76.79 (43/56)	92.22 (83/90)	23.21 (13/56)	7.78 (7/90)
EA-IgG	>0.5	73.21 (41/56)	98.89 (89/90)	26.79 (15/56)	1.11 (1/90)
VCA-IgA	>1.1	78.57 (44/56)	90.00 (81/90)	21.43 (12/56)	10.00 (9/90)
EBV-DNA	>1 x 10^3 copies/mL	66.07 (37/56)	96.67 (87/90)	33.93 (19/56)	3.33 (3/90)
χ^2	-	2.6461	8.4706	2.6461	8.4706
P	-	>0.05	<0.05	>0.05	<0.05

Table 5. Combined assay of EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA.

Index	Sensitivity (%)	Specificity (%)	Positive predictive (%)	Negative predictive (%)
Rta-IgG + EA-IgG + VCA-IgA	94.64 (53/56)	82.22 (74/90)	76.81	96.10
Rta-IgG + EA-IgG + EBV-DNA	87.50 (49/56)	86.67 (78/90)	80.33	91.76
Rta-IgG + VCA-IgA + EBV-DNA	92.86 (52/56)	80.00 (72/90)	74.29	94.74
EA-IgG + VCA-IgA + EBV-DNA	89.29 (50/56)	85.56 (77/90)	79.37	92.77
Rta-IgG + EA-IgG + VCA-IgA + EBV-DNA	98.21 (55/56)	78.8 9(71/90)	74.32	98.61

DISCUSSION

EBV infection is the most important factor in nasopharyngeal carcinoma development, as EBV-coding proteins can interact with genes in epithelial cells to initiate a series of oncogenic molecular events that lead to nasopharyngeal cancer. After EBV infection, the body can express various related antibodies including EA-IgA, EA-IgG, and VCA-IgA, all of which can be used in the diagnosis of nasopharyngeal cancer (Chen et al., 2013). Due to its subtle onset and unique occurrence site, nasopharyngeal cancer is usually difficult to identify during routine examination. Serum indices can reflect the progression of cancer at its very early stages, and thus play an important role in screening and early diagnosis, which can improve survival rates (Ai et al., 2013; Cai et al., 2013; Sun et al., 2014). Moreover, the gold standard for diagnosing nasopharyngeal cancer is tumor biopsy followed by pathological examination. However, this method may not be feasible by some patients. Serum indices including SA, VCA-IgA, EA-IgA, Rta-IgG, and EA-IgG have thus been used for early-diagnosis and population screening of nasopharyngeal cancer (Crowley et al., 2012; Xing et al., 2013). In the present study, we further describe a combined assay that includes EA-IgG, Rta-IgG, VCA-IgA, and EBV-DNA in the diagnosis of nasopharyngeal cancer.

The lytic stage of EBV can be subdivided into immediate early, early, viral DNA replicative, and late stages. Rta protein, which is coded by the immediate early gene *BRLF1*, plays a critical role in the transition of EBV from latent stage to replicative stage. Rta protein expressed in the viral particle can be recognized by cytotoxic T lymphocytes, and can induce the production of anti-Rta antibodies such as IgG and IgA, which may thus have diagnostic value. However, previous studies have suggested that Rta-IgG-based tests of have higher sensitivity in diagnosing nasopharyngeal cancer (Baizig et al., 2012; Liu et al., 2012). EBV mainly infects epithelial cells of the oral or pharyngeal cavity and B lymphocytes, where the virus exists in a circular DNA form in the cytoplasm. Therefore, the presence of the EBV genome can be detected in most infected patients. As EBV only replicates in B lymphocytes, serum and plasma copy numbers of viral DNA can be used to monitor the progression of nasopharyngeal cancer (Cai et al., 2010; Koidl et al., 2011). During the progression of nasopharyngeal carcinoma, the EBV genome begins to transcribe related genes coding EBV antigens. In clinical practice, it is thus useful to quantify serum levels of antibodies

including EA-IgG, VCA-IgA, and EA-IgA in order to gain insight into the replicative status of EBV inside the body, and thus monitor the progression of nasopharyngeal cancer (Qin et al., 2011). Here, we simultaneously tested the diagnostic value of various viral indices for their diagnostic values in nasopharyngeal cancer. Our results showed elevated Rta-IgG, EA-IgG, VCA-IgA, and EBV-DNA levels in nasopharyngeal cancer patients compared to those in rhinitis patients, who further had elevated levels of these viral indices compared to those in healthy controls, suggesting the importance of EBV-antibodies and EBV-DNA in cancer diagnosis. At advanced TNM stages, these indices were also increased, suggesting consistency between these serum indices and TNM staging. This provides the first evidence that serum EBV antibody levels have predictive value in nasopharyngeal carcinoma TNM staging.

The combined assay described herein has unique advantage over a single index as it improved the accuracy of diagnosis. The ROC curves showed that VCA-IgA had the best diagnostic efficacy among all parameters examined. In clinical practice, this may be a gold standard to adopt in lieu of pathological examination. However, a single positive result in serum may not lead to a confirmed diagnosis. In evaluating the efficacy of single parameters, we found that VCA-IgA had the highest sensitivity, whereas EA-IgG and EBV-DNA had higher specificity. These results suggest the production of EA at the time of EBV replication. All parameters examined herein had relatively higher false negative rates, as being negative of antibodies against VCA, EA, or Rta cannot rule our cancer. Therefore, clinical diagnosis needs to be made based on the conjunction of serum laboratory and clinical symptoms. We also analyzed the efficacy of the combined assay using all four parameters. We found that there was elevated sensitivity and negative predictive value in the combined assay without depression of specificity, suggesting the complementary role of single indices in a combined scenario. This combined approach may help to improve the early diagnosis of nasopharyngeal cancer and improve the value of serum markers. In practice, considering the current limitations of a single index, multiple assays, and biopsies, it is still necessary develop methods to decrease false-negative and false-positive rates.

In summary, Rta-IgG, EA-IgG, VCA-IgA, and EBV-DNA levels were elevated in nasopharyngeal carcinoma patients. The combined assay to evaluate serum EA-IgA, Rta-IgG, VCA-IgA, and plasma EBV-DNA levels had clinical implications in diagnosing nasopharyngeal cancer, and may be useful for population screening and monitoring the progression of cancer.

Conflicts of interest

The authors declare no conflict of interest.

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