Original Article
Expression of EBV antibody EA-IgA, Rta-IgG and VCA-IgA and SA in serum and the implication of combined assay in nasopharyngeal carcinoma diagnosis

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Abstract: Epstein-Barr virus (EBV) is an important non-invasive index for nasopharyngeal carcinoma. Serum sialic acid (SA) level was known to be related with tumor progression. Rta protein antibody IgG (Rta-IgG), early antigen antibody (EA-IgA) and viral capsid antibody (VCA-IgA) levels in serum can also be used to effectively monitor the progression of cancer. This study investigated serum level of SA, Rta-IgG, EA-IgA and VCA-IgA in nasopharyngeal cancer patients and the diagnostic value of combined assay. A total of 64 nasopharyngeal cancer patients were recruited, in parallel with 60 benign rhinitis and 60 healthy individuals. Serum SA, EA-IgA, Rta-IgG and VCA-IgA levels were measured by enzyme-linked immunosorbent assay (ELISA). The diagnostic value of these indexes was further evaluated by ROC curve analysis. Logistic regression model was used to analyze the diagnostic implication of combined assay. The expression levels of SA, EA-IgA, Rta-IgG, and VCA-IgA were highest in nasopharyngeal cancer patients. Those indexes were also increased with advanced TNM stage of cancer. The overall diagnostic efficacy was ranked as: VCA-IgA, Rta-IgA, EA-IgA and SA. The combined diagnosis increased the sensitivity to 98.44% and the negative predictive value to 99.03%, without compromising specificity. SA, EA-IgA, Rta-IgG and VCA-IgA expression levels were elevated in nasopharyngeal patients. The combined diagnosis of those serum indexes may improve the diagnostic efficacy of nasopharyngeal carcinoma.

Keywords: Nasopharyngeal carcinoma, sialic acid, combined assay

Introduction

Nasopharyngeal carcinoma is the leading malignant tumor of ear-nose-throat department [1, 2]. Due to its insidious onset and complicated mechanism, patients are mostly already at the late or terminal stage. The golden standard of diagnosis currently still use pathological examination following biopsy sampling, which, however, may not be cooperated by potential patients due to the surgical procedures. Epstein-Barr virus (EBV) has been confirmed to be closely related with occurrence and progression of nasopharyngeal cancer [2, 3]. Most cancer patients had plasma expression of EBV-DNA and anti-EBV antibodies [2, 4], making EBV-related antigen and antibody as important non-invasive index for nasopharyngeal cancer. Current studies, however, mostly focus on the implication of single antibody

expression for serology examination and screening [3, 5]. Such single index may not obtain satisfactory diagnostic sensitivity and specificity. Major proteins expressed by EBV include BRLF1 gene-coding Rta protein, early antigen (EA) and viral capsid antigen (VCA) [6, 7]. EA-IgA and VCA-IgA thus can work as important indexes predicting the prognosis of nasopharyngeal cancer [5-8]. Re-occurrence and lymph node metastasis are major reasons causing the failure the primary treatment of cancer. Serum sialic acid (SA) level has been known to be related with tumor progression and metastasis. Previous findings suggested elevated SA levels, which were positively correlated with VCA-IgA titer, making SA as one index predicting metastatic condition [9, 10]. Due to its insidious onset and high rate of misdiagnosis, the early and accurate diagnosis and nasopharyngeal carcinoma is of great importance. This

Table 1. Expression level of EA-IgA, Rta-IgG, VCA-IgA and SA

Group	N	Rta-IgG (U/ml)	EA-IgA (against standard)	VCA-IgA (against standard)	SA (mg/L)
Control	60	1.58±0.22	0.53±0.09	0.65±0.13	568.23±41.57
Rhinitis	60	15.21±3.24*	0.81±0.14*	0.89±0.22*	599.34±43.28
Nasopharyngeal	64	132.34±25.26*,Δ	1.23±0.34*,Δ	2.55±0.46*,Δ	658.41±62.45*,Δ
F value	-	93.657	98.617	133.937	15.663
P value	-	0.000	0.000	0.000	0.000

Note: *, P<0.05 compared to control group; Δ, P<0.05 compared to rhinitis group.

Table 2. EA-IgA, Rta-IgG, VCA-IgA and SA levels at different TNM stage

TNM stage	N	Rta-IgG (U/ml)	EA-IgA (against standard)	VCA-IgA (against standard)	SA (mg/L)
Stage I	13	14.21±6.05	0.88±0.11	0.90±0.17*	615.31±45.26
Stage II	22	60.91±17.61*	0.96±0.17*	1.37±0.18*	647.28±42.56
Stage III	19	97.54±14.58*,Δ	1.12±0.33*,Δ	2.24±0.52*,Δ	674.22±53.18*,Δ
Stage IV	10	146.51±21.72*,Δ,#	1.36±0.25*,Δ,#	2.58±0.36*,Δ,#	708.11±63.23*,Δ,#
F value	-	31.566	6.390	15.306	3.757
P value	-	0.000	0.001	0.000	0.015

Note: *, P<0.05 compared to stage I patients; Δ, P<0.05 compared to stage II patients, #, P<0.05 compared to stage III patients.

study thus detected serum levels of Rta-IgG, VCA-IgA, EA-IgA and SA, all of which can reflect the status of EBV and malignancy of nasopharyngeal cancer, in an attempt to investigate the significance of combined assay in diagnosis.

Materials and methods

Clinical information

A total of 64 nasopharyngeal carcinoma patients between February 2012 and December 2014 were recruited in this study from our hospital. Another two cohorts of 60 benign rhinitis patients and 60 healthy individuals were recruited as control groups. All nasopharyngeal cancer patients received confirmed diagnosis by pathological examinations. TNM stage was confirmed by UICC standards (Sixth edition, 2002) as low differentiated squamous cell carcinoma, with 13 cases of stage I, 22 cases of stage II, 19 stage III patients, and 10 stage IV patients. All patients did not use any immune modulating drugs, no other inflammatory diseases or any radio-/chemo-therapy before the surgery. This study has been approved by the ethical committee of Second Affiliated Hospital and has obtained written consents from all participants. Within all 64 cancer patients, there were 31 males and 33 females, with aging between 21~70 years old

(average age = 45.6 years). In the rhinitis group, there were 29 males and 31 females, aging between 22~69 years old (average age = 47.4 years). In the healthy control group, there were 32 males and 28 females, aging between 21~71 years old (average age = 44.7 years). Healthy controlled individuals were all recruited from those who underwent routine body checks in our hospital and had no history of rhinitis. All three groups had no significant difference regarding age or sex distribution (P<0.05) and were thus comparable.

Serum assay

All individuals were collected for peripheral blood samples, which were firstly centrifuged (14 000 g, 15 min) to separate serum and plasma. Serum Rta-IgG, EA-IgA, VCA-IgA and SA levels were quantified by enzyme-linked immunosorbent assay (ELISA) using test kits (Jiancheng Bio, China) following the manual instruction.

The critical values of Rta-IgG, EA-IgA, VCA-IgA and SA levels were determined by ROC curve analysis. Positive criteria were: EA-IgA>1.1, VCA-IgA>1.1, Rta-IgG>30 U/mL, SA>650 mg/L. Both specificity and sensitivity of these four parameters in nasopharyngeal carcinoma were compared. In brief, three out of all four indexes were firstly tested in combination. The positive
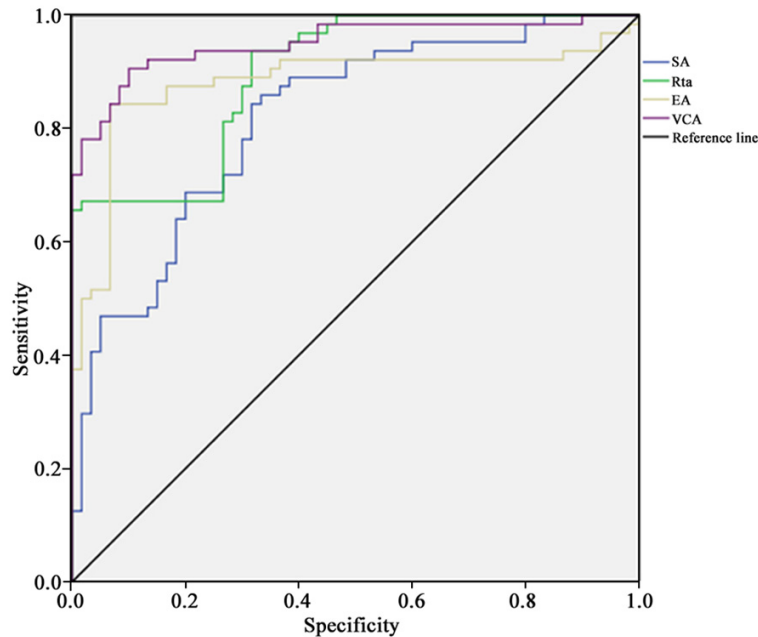
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Figure 1. ROC analysis of EA-IgA, Rta-IgG, VCA-IgA and EBV-DNA.

Table 3. Area under curve of ROC

Test parameter	Area	SD[a]	P value[b]	95% Confidence interval	
				Lower limit	Upper limit
Rta-IgG	0.897	0.026	0.000	0.846	0.949
VCA-IgA	0.951	0.019	0.000	0.912	0.989
EA-IgA	0.882	0.035	0.000	0.813	0.950
SA	0.818	0.038	0.000	0.744	0.891

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

statistical analysis was defined when $P<0.05$.

Results

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

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

Expression level across different TNM stage cancer patients

We further compared those indexes across different TNM stages and found nasopharyngeal patients at advanced stages had further elevated expression levels in EA-IgA, Rta-IgG, VCA-IgA and SA ($P<0.05$, **Table 2**).

ROC analysis

Using both healthy controls and nasopharyngeal cancer patients as objects, we performed ROC analysis and showed the under-curves-areas were 0.882, 0.897, 0.951 and 0.818 for EA-IgA, Rta-IgG, VCA-IgA and SA, respectively. All those factors had satisfactory diagnostic efficacy of nasopharyngeal carcinoma. The overall rank of efficacy was (from higher to lower): VCA-IgA, Rta-IgG, EA-IgA and EA-IgA (**Figure 1**; **Table 3**).

Diagnostic efficacy of test parameters

With reference to expression levels in both healthy people and nasopharyngeal carcinoma patients, we specified the criteria of positive results of single index (EA-IgA>1.1, VCA-IgA>1.1, Rta-IgG>30 U/mL, SA>650 mg/L). Using Youden index (J = Sensitivity + Specificity-1), the sensitivity and specificity of Rta-IgG, EA-IgA, VCA-IgA and SA in nasopharyngeal cancer were 75.00% and 71.88%, 79.69%

result was made based on any one or more positive indexes using pre-specified criteria. Overall negative result only occurred when all those parameters showed negative results. We then performed a combined assay using all four indexes.

Statistical analysis

SPSS 20.0 software package was employed for analyzing all collected data, of which the comparison of ratios was done by chi-square test with correction coefficient if necessary. Those fitted normal distribution were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by LSD test were used to compare means across groups. ROC curve analysis was used to evaluate the diagnostic effect of Rta-IgG, EA-IgA, EBV-DNA and SA by Logistic regression model and z-test. A

Table 4. Diagnostic efficacy of parameters

Index	Positive criteria	Sensitivity/%	Specificity/%	False negative /%	False positive/%
SA	≥650 mg/L	68.75 (44/64)	86.63 (103/120)	31.25 (20/64)	14.17 (17/120)
Rta-IgG	>30 U/ml	75.00 (48/64)	90.83 (109/120)	25.00 (16/64)	9.17 (11/120)
EA-IgA	>1.1	71.88 (46/64)	96.67 (116/20)	28.13 (18/64)	3.33 (4/120)
VCA-IgA	>1.1	79.69 (51/64)	95.00 (114/120)	20.31 (13/64)	5.00 (6/120)
χ^2	-	2.163	21.110	2.163	21.110
P	-	>0.05	<0.05	>0.05	<0.05

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

| Index	Sensitivity/%	Specificity/%	Positive predictive/%	Negative predictive/%
SA + Rta-IgG + EA-IgA	89.06 (57/64)	88.33 (106/120)	80.28 (57/71)	93.81 (106/113)
SA + Rta-IgG + VCA-IgA	93.75 (60/64)	85.83 (103/120)	77.92 (60/77)	96.26 (103/107)
SA + EA-IgA + VCA-IgA	90.63 (58/64)	89.17 (107/120)	81.69 (58/71)	94.69 (107/113)
Rta-IgG + EA-IgA + VCA-IgA	95.31 (61/64)	92.50 (111/120)	87.14 (61/70)	97.37 (111/114)
SA + Rta-IgG + EA-IgA + VCA-IgA	98.44 (63/64)	85.00 (102/120)	76.83 (63/82)	99.03 (102/103)
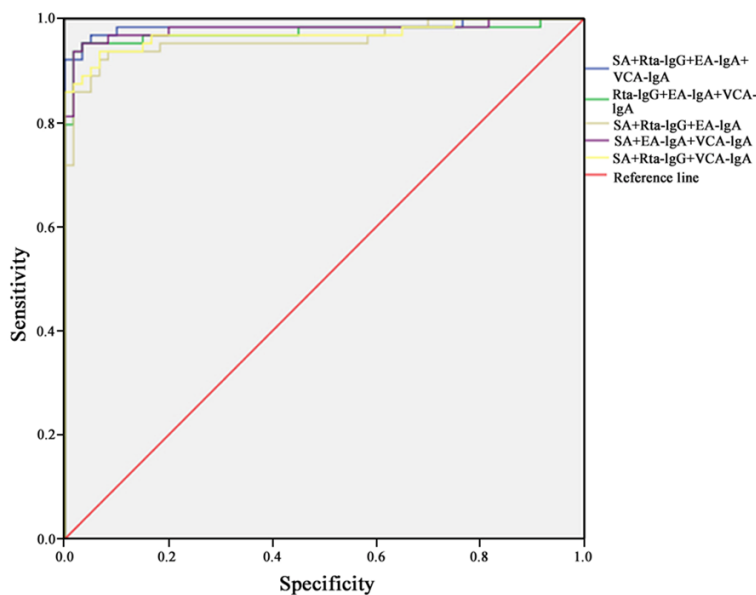
Figure 2. ROC curve of combined assay.

and 68.75%, 90.83% and 96.67%, 95.00% and 86.63%, respectively. VCA-IgA had the highest test sensitivity compared to Rta-IgG, SA or EA-IgA. VCA-IgA had the highest sensitivity, while EA-IgA had the highest specificity. Significant difference existed in both specificity and false positive rate across these indexes (P<0.05, **Table 4**).

Combined assay and efficacy

We then compared the efficacy of combined assay using four parameters altogether. Such combined assay increased the sensitivity and negative predictive value to 98.44% and 99.03%, respectively, without significant decrease of specificity (**Table 5**). The area under ROC curve was 0.989 (95% CI: 0.980~0.998, **Figure 2**).

Discussion

Major risk factors causing nasopharyngeal carcinoma include genetic, EBV infection and diet habit, with little correlation with patients' sex or age [11]. EBV genome can encode a series of viral proteins, which are interacting with epithelial genes to express various biological molecules during the progression of nasopharyngeal cancer. Clinical diagnosis can be made based on the serum level of EBV cellular immunity related antibodies such as EA-IgA and VCA-IgA [12, 13]. The early screening and diagnosis of nasopharyngeal cancer is of critical importance for improving treatment efficacy [14, 15]. However, due to the insidious onset and lack of significant features at early stage, the sensitivity and specificity of serological examination is not satisfactory. The golden standard currently used for nasopharyngeal cancer prognosis is pathological examina-

tion following biopsy at the lesion site. The complicated and painful procedures of biopsy, however, impede the promotion of this approach. Currently, serological indexes including VCA-IgA and EA-IgA have been used for screening and primary diagnosis [16, 17]. EBV-related antigens and antibodies are the most important non-invasive index for nasopharyngeal. This study thus investigated the diagnostic value of SA, Rta-IgG, EA-IgA and VCA-IgA in a combined scenario.

EBV infection plays a critical role in the progression of nasopharyngeal carcinoma, as body can produce lots of EBV-related antibodies, which can be detected in serum at the early stage. Clinical assay of EA-IgA, EA-IgA and VCA-IgA have been used to reflect the condition of nasopharyngeal cancer [16, 18]. The lytic stage of EBV includes immediate early, early, viral DNA replicative and late stages. The immediate early gene BRLF1 encodes Rta protein, which plays an important role in the early viral replication. The presenting of Rta protein antigen site and recognition by CTL cells lead to the production of anti-Rta antibodies, mainly in the form of IgA and IgA. Some studies suggested a better test sensitivity of Rta-IgG in diagnosing nasopharyngeal cancer compared to IgA [17, 18]. A time sequence of EBV proteins is: Rta protein, EA and VCA. During the malignancy development, EBV genome begins to transcribe related EBV antigens, which can be detected by serum antibody levels including Rta-IgA, EA-IgA and VCA-IgA, all of which can reflect the *in vivo* viral replication and cancer progression [19, 20]. SA is widely distributed across body tissues and participates in cell surface physiological functions. It is recognized as one of tumor markers in a complex form with glycolipids or glycoproteins. The dynamic alternation of SA expression level is known to be related with development of malignant tumors [10]. Results of this study showed significantly elevated serum SA levels in nasopharyngeal cancer patients, when compared to rhinitis patients or healthy individuals. The sensitivity and specificity of SA in cancer diagnosis are 68.75% and 86.63%, respectively, suggesting the application value of SA serology in nasopharyngeal diagnosis.

This study investigated the diagnostic value of combined assay including SA, Rta-IgA, EA-IgA and VCA-IgA. Results showed elevated serum levels of those parameters in nasopharyngeal

patients compared to those in rhinitis patients, which also had higher levels compared to control people. These results suggested the correlation between serum antibody levels of EBV and the disease progression, in addition to the crucial role of EBV-related antibodies in early monitor of nasopharyngeal cancer. We also showed the elevated expression of SA, Rta-IgA, EA-IgA and VCA-IgA with advanced clinical stages. This may not be consistent with previous studies showing no significant correlation between EA-IgA and VCA-IgA with clinical stages [1, 3].

Using the single index as the diagnostic criteria, VCA-IgA had the highest sensitivity while EA-IgA had the best specificity, suggesting the production of EA at the very early stage of EBV replication. ROC curve analysis showed satisfactory diagnostic power of all these four indexes, with the sequence (from high to low): VCA-IgA, Rta-IgA, EA-IgA, and SA. Positive results of single index cannot make confirmative diagnosis, so does the combined negative results of Rta-IgA, EA-IgA and VCA-IgA for ruling out cancer. Due to the possible interference for the final conclusion from single index, the combined assay could improve sensitivity and accuracy of tumor diagnosis. In this study, the diagnostic value of SA, Rta-IgA, EA-IgA and VCA-IgA are simultaneously tested, resulting in elevated sensitivity and negative predictive values, without significant decrease of specificity. These results supported the complementary role of combined assay in early diagnosis of nasopharyngeal cancer. In clinical practice, it is thus beneficial to apply the combined assay of multiple serological indexes and pathological examinations, if necessary, to avoid misdiagnosis or false positive.

In summary, SA, Rta-IgG, EA-IgA and VCA-IgA levels were elevated in nasopharyngeal carcinoma patients. The combined assay of serum SA, Rta-IgG, EA-IgA and VCA-IgA can improve the sensitivity and accuracy for diagnosing nasopharyngeal cancer, and can be used for population screening and monitoring the cancer progression.

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Disclosure of conflict of interest

None.

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