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ORIGINAL ARTICLE

Antibodies Against Epstein-Barr Virus Glycoprotein gp42 for the Diagnosis of Nasopharyngeal Carcinoma

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SUMMARY

Background: **Assessment of immunoglobulin A (IgA) antibody responses to Epstein-Barr virus (EBV) antigen is important for the early diagnosis of nasopharyngeal carcinoma (NPC). EBV glycoprotein gp42 has been shown to play an essential role in membrane fusion with B cells. The aim of the present study was to assess whether the antibodies to EBV glycoprotein gp42 in serum could be a novel marker for diagnosis of NPC.**
Methods: **EBV glycoprotein gp42 expressed in the recombinant baculovirus system was used in an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to gp42 in serum. The blood samples were obtained from 406 participants (n = 208 patients with NPC and 198 healthy controls). Receiver operating characteristics (ROC) was used to calculate diagnostic accuracy.**
Results: **The ROC curves showed that IgA-gp42 ELISA had a sensitivity of 76.4%, specificity of 78.3% and an area under the curve (AUC) of 0.856 (95% CI, 0.82 - 0.891) to diagnose NPC. Furthermore, gp42 maintained diagnostic capacity in NPC patients who were IgA-viral capsid antigen (VCA) negative (87.5%, 64.1% and 0.844 [95% CI, 0.776 - 0.912]). Combining gp42 and VCA improved the diagnostic capacity compared with the individual tests (89.9%, 94.4% and 0.973 [95% CI, 0.959 - 0.987]).**
Conclusions: **The EBV glycoprotein complex gp42 acts as a novel biomarker for diagnosis of NPC and improves identification of patients with VCA-negative NPC.**
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KEY WORDS

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INTRODUCTION

In southern China, where nasopharyngeal carcinoma is endemic, EBV serology has been used for population screening [1]. In comparison with healthy EBV carriers, NPC patients typically show strong immunoglobulin G (IgG) and especially IgA reactivities to lytic antigens. This response can be used for detecting NPC and might be used as an indicator for treatment outcome [2].

In 1978, Zeng et al. carried out screening for NPC using a combination of IgA against VCA and IgA antibodies against EBV early antigen (IgA-EA) by immunofluorescence assay (IFA) in the NPC-endemic area [3]. This method was subsequently established as the standard tool for NPC screening [4]. Irrespective of the high sensitivity and specificity of IgA-VCA in the diagnosis of NPC, there were still 4 - 24% of patients [4-8] with undetectable IgA-VCA, which led to a missed diagnosis. Therefore, reliable diagnostic biomarkers to complement IgA-VCA are required to improve the early diagnostic rate.

The entry of EBV into B lymphocytes involves at least five viral glycoproteins, the EBV gH, gL, gB, gp350, and gp42 proteins, with gp42 providing a key recognition step in the activation of membrane fusion [9]. EBV gp42 is present on the surface of infected cells and in the virion envelope [10], which has been shown to bind the cell-surface major histocompatibility complex (MHC) class II molecules [11,12]. Furthermore, EBV glycoproteins play an important role in humoral immune responses, and sera against gp42 can neutralize the virus [13]. Therefore, we proposed that gp42 has the potential to serve as a diagnostic biomarker for NPC. In the present study, we developed a novel ELISA using a baculovirus-expressed gp42 (aa 33-223) protein to test the feasibility of antibodies against gp42 in the detection of patients with NPC and compare the sensitivity and specificity of these antibodies separately or in combination with IgA-VCA or circulating EBV DNA in the detection of patients with NPC.

MATERIALS AND METHODS

Study population

A total of 208 serum samples were collected from histologically confirmed NPC patients before treatment; all of the patients were diagnosed at the Sun Yat-sen University Cancer Center (SYSUCC), histologically diagnosed with WHO type II or III NPC [14], and had definite clinical stage NPC according to the seventh American Joint Committee on Cancer TNM staging manual [15]. The characteristics of the patients are presented in the Supplementary Table 1. We randomly selected 198 healthy controls, including 138 males and 60 females ranging in age from 30 to 60 years (mean age = 44.1 years), from the hospital staff who participated in the medical examination projects. This study was approved by the Clinical Research Ethics Committee of the Sun Yat-sen University Cancer Center, and all of the participants provided written informed consent.

Cell culture

High Five™ insect cells (gift from Yun Wang, Wuhan Institute of Virology, Chinese Academy of Sciences, Hubei, China, wangyun@wh.iov.cn) were used for protein expression and grown in shaker flasks in Express Five serum-free medium (Invitrogen, Grand Island, NY, USA). Sf9 insect cells used for baculovirus production were grown in 150-cm^2T flasks in Sf-900 II serum-free medium (Invitrogen, Grand Island, NY, USA). All of the media contained penicillin-streptomycin and amphotericin B.

EBV gp42

P2089 EBV plasmid (gift from Wolfgang Hammerschmidt, German Research Center for Environmental Health, Munich, Germany, hammerschmidt@helmholtz-muenchen.de) was used as the template in a PCR refer to EBV gp42 . The construction of soluble EBV gp42 has been previously described [11]. In the expression constructs, gp42 (aa 33-223) was fused to the baculovirus gp64 signal sequence, and a flag epitope tag was inserted into the carboxyl termini of each protein. The related primers and gp64 are listed in Supplementary Table 2. pFastBacHTB and DH10Bac™ *E. coli* were kindly provided by Yun Wang.

Protein expression and purification

One liter of High Five™ insect cells at a density of 2 x 10^6 cells/mL in shaker flasks was infected with the P3 gp42 baculovirus at 27°C while stirring at 100 rpm/min. Cell supernatants of gp42 were harvested at 96 hours post infection according to the optimal experiments. The supernatants were concentrated and sterile filtered. The expressed six-His-tagged protein was purified from the supernatants by Ni chelate-Sepharose (Qiagen-Sample & Assay Technologies, Hilden, Germany) chromatography according to the manufacturer's protocol. After washing the combined agarose with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the purified recombinant proteins were eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 300 mM imidazole, pH 7.5). The eluted proteins were exchanged into PBS by ultrafiltration. The purified proteins were concentrated 10-fold, dialyzed, and then stored at -80°C until required.

SDS/PAGE and western blot analysis of the EBV gp42

Ten microliters of the eluted protein was lysed in 10 µL 2x sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) loading buffer (0.2% Bromophenol Blue, 20% (v/v) glycerol, 4% (w/v) SDS, 100 mmol/L Tris/HCl, pH 6.8, 200 mmol/L dithiothreitol) by boiling at 98°C for 10 minutes. The samples were subjected to 10.5% acrylamide resolving gels and stained with Coomassie Blue for at least 30 minutes and

then destained in methanol-acetic acid. After the SDS/PAGE, the proteins were transferred onto PVDF membranes at 200 mA for 3 hours. The membranes were blocked for 45 minutes at room temperature with 5% non-fat milk in PBS-T buffer (containing 0.1% Tween 20) and then incubated at 1:1000 dilutions of the primary antibody (anti-flag tag mouse polyclonal antibody, flag-M2, Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight and followed by secondary antibody (anti-mouse IgG secondary antibody) coupled to horseradish peroxidase (HRP) for at least 45 minutes.

ELISA detection of antibodies to recombinant gp42

The purified recombinant gp42 was dissolved in 10 mM PBS at pH 7.4. The 96-well microtiter plates were coated with 450 ng of gp42 protein per well at 37°C for 2 hours. After incubation, the unoccupied sites of the plates were blocked with 3% BSA in PBS. The plates were washed five times with PBS containing 0.1% Tween-20 (PBS-T). After washing, serum samples at a dilution of 1:100 in PBST containing 3% BSA were added and incubated for 1 hour at 37°C. After five washes, horseradish-peroxidase-labelled goat anti-human IgA antibodies (Boster Corporation, Wuhan, China) diluted 1:6000 for gp42 in PBS-T were added. The plates were again incubated at 37°C for 30 minutes and washed before adding a tetramethylbenzidine reagent (Sigma-Aldrich, St Louis, MO, USA) for 10 minutes at 37°C. The reaction was stopped with 2 M H_2SO_4, and the optical density (OD) at 450 nm was determined using an ELISA reader.

IgA-VCA immunofluorescence assay (IFA) and plasma EBV assay

The EBV-specific IgA-VCA antibody was assessed using a previously described immunofluorescent method [4,16], a titer ≥ 1/40 was considered to be positive. As described in previous studies [7,17], the patients' plasma EBV DNA concentrations were measured by qPCR; a detection of 100 copies/mL was chosen as the cutoff level.

Statistical analysis

The results were analyzed using the statistics software SPSS for Windows (version 16.0), MedCalc (version 13.0.2.0), and GraphPad Prism (version 5.0). The participants' characteristics and NPC risk factors were compared using chi-squared tests. The Mann-Whitney U test was used to determine the statistical significance of the differences between patients with NPC and healthy controls. The ROC curves were constructed to assess the sensitivity, specificity, and areas under the curves (AUCs) using 95% CI. The cutoff value for the biomarker was defined as the value with the highest sensitivity and specificity selected from the respective ROCs. Chi-squared tests were used to compare the mean OD of IgA-gp42 among patients at different cancer stages. McNemar's paired-sample test or chi-squared test was applied to determine whether the sensitivity, specificity, positive predictive values, and negative predictive values of gp42, VCA, EBV DNA and their combinations were significantly different, and AUC comparisons were assessed using Z tests. The binary logistic regression models of all possible combinations of biomarkers were used to select the optimal combination with diagnosis of NPC. A value of $p < 0.05$ was considered to indicate statistical significance, and all of the statistical tests were two-sided.

RESULTS

Production and purification of soluble EBV gp42 protein

After purification with Ni chelate-Sepharose chromatography, the proteins were quantified and their purity was evaluated. The SDS/PAGE gels revealed a major band of molecular masses of approximately 36 kDa (Supplementary Figure 1). Immunoblotting with an anti-flag monoclonal antibody revealed specific bands of the same size, which suggests that the soluble gp42 proteins are the major components of the purified proteins (Supplementary Figure 1). Thus, the purified proteins were considered to be a decent antigen and could be used in a subsequent ELISA assay.

Elevated levels of antibodies against gp42 in NPC patients

To determine whether gp42 could be applied in the diagnosis of NPC, we developed an ELISA assay to detect anti-gp42 antibodies in the serum of patients with NPC and in serum of healthy controls. A comparison of the basic information (Supplementary Table 1) between patients with NPC and the healthy controls showed that differences in age ($p = 0.973$), gender ($p = 0.388$), and smoking history ($p = 0.622$) were not statistically significant, but the difference in family history was statistically significant ($p < 0.001$).

To evaluate the distribution of blood IgA antibodies against EBV gp42 in patients with NPC and in the healthy populations, we analyzed the level of antibodies to glycoprotein with the OD values (median ± IQR [IQR, interquartile range]). As shown in Figure 1A, the antibody titers against gp42 were elevated in a majority of patients with NPC compared with the controls. The median OD value of gp42 for the patients with NPC was 0.828 ± 0.409, which was higher than that of the healthy controls (0.514 ± 0.222). The Mann-Whitney U test showed that the difference was statistically significant ($p < 0.001$).

To determine whether the serological reactivities correlated with the clinical characteristics of patients with NPC, the distribution of IgA-gp42 levels according to the individual cancer stage is shown in Figure 1B. We found that the median OD value of IgA-gp42 for patients with stage IV NPC was 0.86 ± 0.36, which was higher than that of the early stage (I + II) (0.77 ± 0.48) and stage III (0.82 ± 0.39), but it was not statistically

Table 1. Associations of EBV IgA-gp42 and clinicopathological parameters of patients with NPC.

Parameters	OD Value			No.	OD ≥ 0.66		OD < 0.66		p [a]
	Maximum	Minimum	Median		No.	(%)	No.	(%)	
Age, years									
< 46	2.531	0.476	0.822	111	87	78.4	24	21.6	0.482
≥ 46	3.022	0.429	0.844	97	72	74.2	25	25.8	
Gender									
Male	3.022	0.429	0.828	153	117	76.5	36	23.5	0.981
Female	1.584	0.435	0.828	55	42	76.4	13	23.6	
Smoking history									
Yes	3.022	0.429	0.807	98	74	75.5	24	24.5	0.765
No	2.1	0.435	0.847	110	85	77.3	25	22.7	
Tumor stage									
I + II	1.934	0.447	0.77	44	31	70.5	13	29.5	0.307
III	3.015	0.429	0.818	110	83	75.5	27	24.5	
IV	3.022	0.494	0.859	54	45	83.3	9	16.7	
VCA									
Positive	3.022	0.429	0.829	176	138	78.4	38	21.6	0.117
Negative	2.152	0.435	0.806	32	21	65.6	11	34.4	
EBV DNA									
Positive	3.022	0.435	0.846	149	117	78.5	32	21.5	0.261
Negative	2.19	0.429	0.805	59	42	71.2	17	28.8	

OD - optical density at 450 nm, VCA - immunofluorescence assay for IgA antibodies against viral capsid antigen, EBV DNA - qPCR test for plasma circulating EBV DNA.
The OD value of 0.66 was the cutoff value of the IgA-gp42 ELISA test, a titer ≥ 1/40 was considered a positive result in the IgA-VCA IFA test, a detection of 100 copies/mL was chosen as the cutoff level for the EBV DNA test. [a] - Chi-squared test.

significant (p = 0.307). Additionally, we did not observe correlations between the level of antibodies and other patient clinical characteristics, such as age, gender, smoking history, and IgA-VCA or EBV DNA status (Table 1).

Diagnostic values of IgA-gp42 for NPC

To determine whether IgA-gp42 could be a potential marker for the diagnosis of NPC, the diagnostic value of the gp42 antibody was then evaluated using a ROC (the receiver operating characteristic) analysis based on the OD values. The results are presented in Figure 2A and Table 2. Using a cutoff OD value of 0.66 for the gp42 test, the IgA-gp42 ELISA had a sensitivity of 76.4%, specificity of 78.3%, positive predictive value (PPV) of 78.7%, negative predictive value (NPV) of 76%, and AUC (area under the curve) of 0.856 (95% CI, 0.82 - 0.891). Compared to IgA-gp42, the IgA-VCA IFA had a higher sensitivity of 84.6% (p= 0.037), higher specificity of 93.4% (p < 0.001), and AUC of 0.933 (95% CI, 0.906 - 0.959) (p < 0.001). However, circulating EBV DNA had the lowest sensitivity at 71.6%, AUC of 0.849 (95% CI, 0.810 - 0.889) and highest specificity of 94.9% (p < 0.001).

Validation of logistic regression model combining IgA-gp42 and IgA-VCA

Because IgA-gp42 alone did not show an advantage in the detection of NPC in comparison to IgA-VCA, we attempted to determine whether the combination of different EBV markers could improve the diagnostic efficiency. We used a binary logistic regression to investigate whether combining this novel marker with the previous traditional marker could improve the diagnostic capacity (Figure 2B, Table 2). The combination of IgA-gp42 and IgA-VCA achieved similar sensitivity (89.9%), specificity (94.4%), PPV (94.4%) and NPV (89.9%), and an AUC of 0.973 (95% CI, 0.959 - 0.987) with the combination of IgA-VCA and EBV DNA (87.5%, 97.4%, 97.3%, 88.1%, and 0.967 [95% CI, 0.949 - 0.985]), which was superior to the combination of IgA-gp42 and EBV DNA (77.9%, 92.4%, 91.5%, 80%, and 0.931 [95% CI, 0.908 - 0.954]). We selected the logistic regression model that combines IgA-VCA

Table 2. AUC, sensitivity, specificity, PPV and NPV of IgA-gp42, IgA-VCA, EBV DNA and their combinations for detection of NPC.

Test	AUC (95%)	p [a]	Sensitivity		p [b]	Specificity		p [b]	PPV		p [c]	NPV		p [c]
			%	95% CI		%	95% CI		%	95% CI		%	95% CI	
gp42	0.856 (0.820 - 0.891)		76.4	70.6 - 82.2		78.3	72.6 - 84.0		78.7	73.1 - 84.3		76.0	70.1 - 81.9	
VCA	0.933 (0.906 - 0.959)		84.6	79.7 - 89.5		93.4	89.9 - 96.9		93.1	89.5 - 96.7		85.3	80.6 - 90.0	
EBV DNA	0.849 (0.810 - 0.889)		71.6	65.5 - 77.7		94.9	91.8 - 98.0		93.7	89.9 - 97.5		76.1	70.8 - 81.4	
gp42 vs. VCA		< 0.001			0.037			< 0.001			< 0.001			0.016
gp42 vs. EBVDNA		NS			NS			< 0.001			< 0.001			NS
VCA vs. EBVDNA		< 0.001			0.001			NS			NS			0.013
gp42 + VCA	0.973 (0.959 - 0.987)		89.9	85.8 - 94.0		94.4	91.2 - 97.6		94.4	91.2 - 97.6		89.9	85.8 - 94.0	
gp42 + EBV DNA	0.931 (0.908 - 0.954)		77.9	72.3 - 83.5		92.4	88.7 - 96.1		91.5	87.4 - 95.6		80.0	74.8 - 85.2	
VCA + EBV DNA	0.967 (0.949 - 0.985)		87.5	83.0 - 92.0		97.4	95.2 - 99.6		97.3	95.0 - 99.6		88.1	83.8 - 92.4	
gp42 vs. gp42 + VCA		< 0.001			< 0.001			< 0.001			< 0.001			< 0.001
VCA vs. gp42 + VCA		< 0.001			0.007			NS			NS			NS
EBV DNA vs. gp42 + VCA		< 0.001			< 0.001			NS			NS			< 0.001
gp42 + VCA vs. gp42 + EBV DNA		< 0.001			< 0.001			NS			NS			0.004
gp42 + VCA vs. VCA + EBV DNA		NS			NS			NS			NS			NS

PPV - positive predictive value, NPV - negative predictive value, NS - not significant, gp42 - enzyme-linked immunosorbent assay detection of IgA antibodies against EBV glycoprotein complex gp42, VCA - immunofluorescence assay for IgA antibodies against viral capsid antigen, EBV DNA - qPCR test for plasma circulating EBV DNA, [a] - Z test, [b] - McNemar's paired-sample test, [c] - Chi-squared test.

Table 3. AUC, sensitivity, specificity, PPV and NPV of IgA-gp42, EBV DNA in the diagnosis of IgA-VCA-negative patients with NPC.

Test	AUC (95%)	p [a]	Sensitivity		p [b]	Specificity		p [b]	PPV		p [c]	NPV		p [c]
			%	95% CI		%	95% CI		%	95% CI		%	95% CI	
gp42	0.844 (0.776 - 0.912)		87.5	76.0 - 99.0		64.1	57.4 - 70.8		28.3	19.4 - 37.2		96.9	93.9 - 99.9	
EBV DNA	0.750 (0.637 - 0.863)		53.1	35.8 - 70.4		94.9	91.8 - 98.0		63.0	44.8 - 81.2		92.6	89.0 - 96.2	
gp42 vs. EBV DNA		0.05			0.003			< 0.001			0.001			NS

PPV - positive predictive value, NPV - negative predictive value, NS - not significant, gp42 - enzyme-linked immunosorbent assay detection of IgA antibodies against EBV glycoprotein complex gp42, VCA - immunofluorescence assay for IgA antibodies against viral capsid antigen, EBV DNA - qPCR test for plasma circulating EBV DNA, [a] - Z test, [b] - McNemar's paired-sample test, [c] - Chi-squared test.

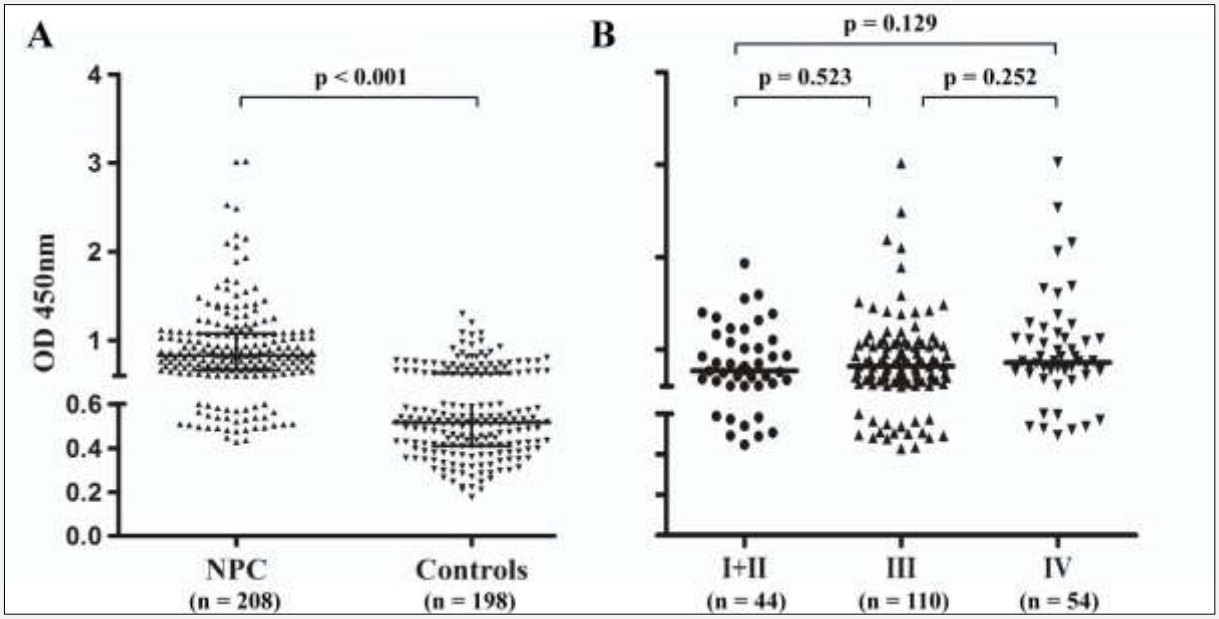

Figure 1. Characteristics and diagnostic values of IgA-gp42.

A. Scatter plots of the distribution of IgA-gp42 ELISA for NPC cases (n = 208) and healthy controls (n = 198). The vertical axis for markers tested by ELISA shows the optical density (OD) at wave length 450 nm. The medium black horizontal lines are medians. The upper black horizontal line indicates the 75th percentile of the data set, and the lower black horizontal line indicates the 25th percentile. B. The ELISA results for pretreatment serum of patients with NPC who have stage I (n = 10), II (n = 34), III (n = 110), and IV (n = 54) disease are shown. The black horizontal lines are medians. The distribution of OD values of IgA-gp42 was not significantly different between stages.

with the IgA-gp42 ELISA as the new optimal combination for NPC detection. The following formula was established:

$$\text{Log}\left(\frac{p}{1-p}\right) = -6.268 + 0.057 \times \text{VCA} + 6.168 \times \text{gp42}$$

where VCA represents the reciprocal transformation of the titer of IgA to VCA and gp42 represents the OD at a wave length of 450 nm of IgA to gp42.

In this model, the ROC analysis (Figure 2B) showed that testing of both VCA and gp42 increased the diagnostic capacity for NPC (AUC, 0.973; 95% CI, 0.959 - 0.987) compared to them individually. A total of

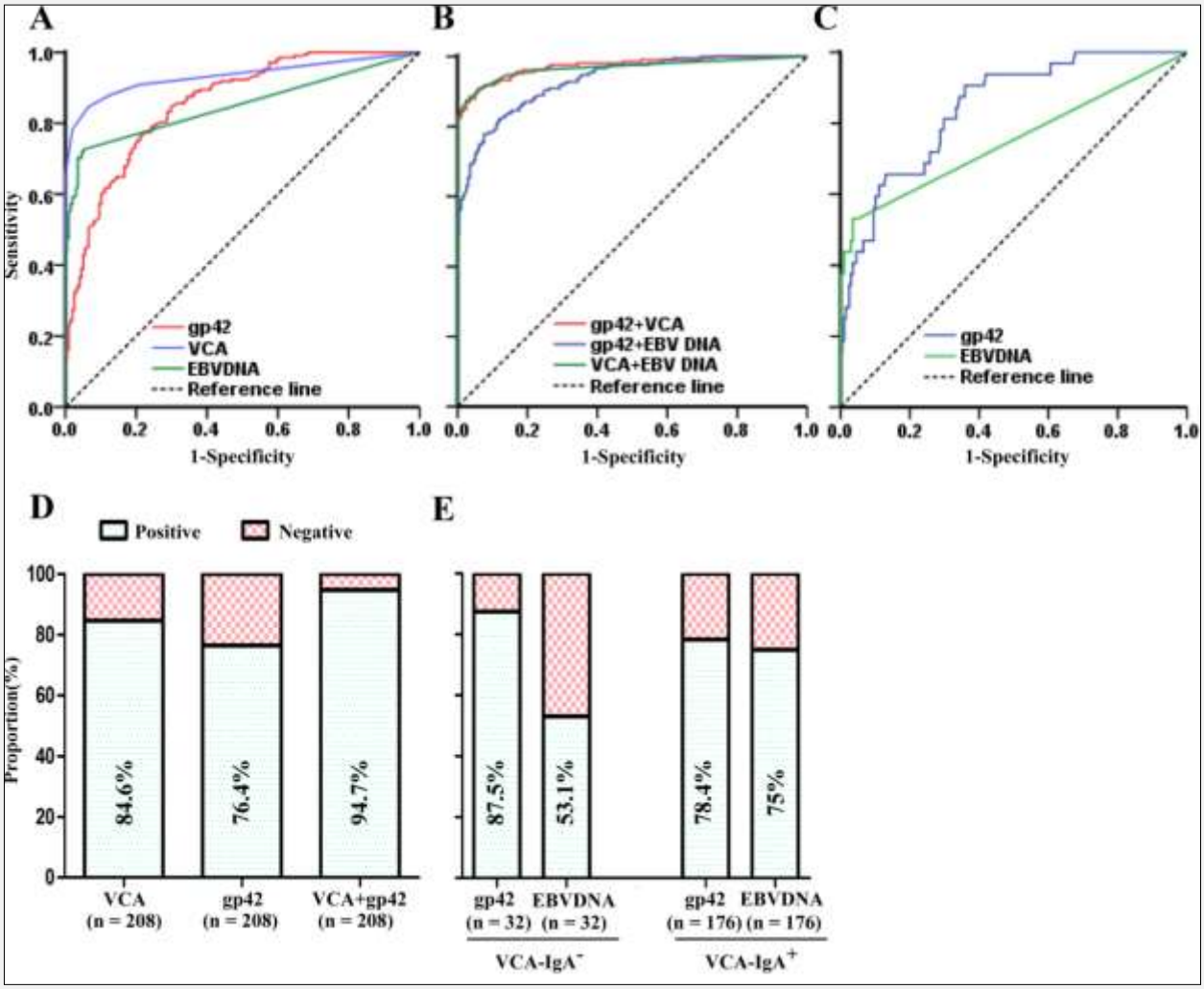

Figure 2. Diagnostic outcomes for gp42, VCA, EBV DNA and their combinations in the diagnosis of NPC.

A. ROC curve for gp42, VCA or EBV DNA for all patients with NPC vs. the control. B. ROC curve for combination of gp42 and VCA, gp42 and EBV DNA, or VCA and EBV DNA for all patients with NPC versus the control. C. ROC analysis of antibody responses to gp42 or plasma EBV DNA concentrations in VCA-negative patients with NPC showing an AUC of IgA-gp42 of 0.844 (95% CI, 0.776 - 0.912) and EBV DNA of 0.750 (95% CI, 0.637 - 0.863). D. The rates of positive results for VCA, gp42 or both in all of the patients with NPC. E. The rates of positive results for gp42 and EBV DNA in the VCA-negative or VCA-positive patients.

197 (94.7%) of the 208 patients with NPC had positive results when VCA and gp42 were tested together (Figure 2D). Furthermore, 28 (87.5%) of 32 VCA-negative patients with NPC had positive gp42 results (Figure 2E). The rate was similar (138 [78.4%] of 176) to that observed in the VCA-positive patients (Figure 2E). The ROC curves for gp42 indicated a diagnosis of NPC in patients with negative VCA (Figure 2C), which could achieve a sensitivity of 87.5% and AUC of 0.844 (95% CI, 0.776-0.912). In the case of EBV DNA, the most specific assay with the highest positive predictive value would be the combination of IgA-VCA with EBV DNA, but only 17 (53.1%) of the 32 VCA-negative patients with NPC had positive EBV DNA results (Figure 2E). The rate was lower than that (132 [75%] of 176) of the VCA-positive patients (Figure 2E). EBV DNA could achieve a sensitivity of 53.1% and AUC of 0.750 (95% CI, 0.637 - 0.863); these values are inferior to that of gp42 (Table 3).

DISCUSSION

The presence of high titers of antibody to gp42 in the plasma of EBV seropositive donors raises the possibility for a new approach to minimally invasive detection and monitoring of NPC [13]. Because NPC has a close association with EBV, measuring antibodies to gp42 in serum should be a feasible method to detect these tumors. In the present study, the sensitivity and specificity of IgA-gp42 for NPC diagnosis both exceeded 75%, comparable to IgA-VCA and EBV DNA. Moreover, as a primary screening test, high sensitivity should have the priority to not miss potential patients, with researchers showing that combination tests can marginally improve diagnostic efficacy compared with single marker tests [4]. The logistic regression analysis showed that IgA-VCA combined with IgA-gp42 ELISA was a good model for discriminating between patients with NPC and the controls, and it had a sensitivity of 89.9%, specificity of 94.4%, PPV of 94.4%, NPV of 89.9%, and AUC of 0.973 (95% CI, 0.959 - 0.987). EBV IgA-VCA derived from the IFA has been considered one of the most conventional and frequently used biomarkers for primary screening of NPC in endemic areas. However, the EBV serological spectrum differs among individuals, and 4% - 24% of patients with NPC remain negative for VCA [4-8]. Consequently, a combination of different markers, especially novel markers that maintain high diagnostic capacity among VCA-negative patients, is extremely important. A combination of circulating EBV DNA and IgA-VCA, which was studied by Leung et al. [18] showed that EBV DNA identifies almost all false-negative IgA-VCA cases and gives a 99% diagnostic sensitivity when combined with IgA-VCA. Similarly, Dardari et al. [19] reported that 89% of the IgA-VCA- and 92% of the IgA-early antigen (EA)-negative sera from young NPC patients are positive for IgG-EBV transactivator protein (ZEBRA). More recently, detection of the antibodies to VCA-p40+18 [20] using an ELISA assay illustrated that positive IgA/VCA-p40+18 reactivity using ELISA for 63.6% of the nasopharyngeal carcinoma samples was missed by peptide-based IgA/EBV-ELISA (peptides derived from immunodominant epitopes of EBNA1 and VCA-p18). In our report, 15.4% of all patients with NPC were IgA-VCA negative; however, gp42 maintained diagnostic capacity (28 of the 32 VCA-negative patients with NPC had positive gp42 results), and 197 (94.7%) of the 208 patients with NPC had positive results when VCA and gp42 were tested together. Therefore, the detection of IgA-gp42 complemented the IgA-VCA test for the diagnosis of NPC.

In the past decade, many discoveries of non-protein serum markers have been documented; e.g. Plasma EBV DNA has also been reported to perform well [18,21]. However, practical considerations support our choice of the combination of VCA with gp42 instead of EBV DNA. First, the sensitivity and specificity of EBV DNA are not duplicative in different laboratories by different investigators, which was explained by the PCR-based techniques in EBV DNA assay that vary in sensitivities when different segments of viral genes or different viral genes are assayed [22]. Secondly, EBV DNA owned a low diagnostic value in the detection of stage I NPC and local relapse [5]. Moreover, in our report, the positive rate in VCA-negative patients was lower than that of gp42.

Our observation supports the suggestion of others that no single serological marker is capable of screening all NPC patients; therefore, we have designed a new NPC-screening strategy, as follows. Participants were examined by indirect nasopharyngoscopy, and blood samples were drawn for testing of seromarkers. Subjects were identified as high-risk and were invited for a fiberoptic endoscopic examination of the nasopharynx if they met either or both of the following sets of criteria: IgA-VCA greater than or equal to 1:40, positive results when testing of both VCA and gp42, based on the optimal logistic regression model; or positive gp42 results among VCA-negative serum samples. Individuals with an abnormal appearance by the fiberoptic endoscopic examination were biopsied.

Interestingly, the application of other EBV glycoproteins in NPC screening has been previously reported. Several studies have detected IgA-gp350/220 in patients with NPC who had higher titers than did the healthy controls [23,24]. Although its function remains unknown to date, gp78 has confirmed its value for the diagnosis of NPC, and the sensitivity and specificity of IgA-gp78 and IgG-gp78 have both exceeded 70% [25]. The glycoprotein gp125, is believed to be a dominant immunogen of the VCA complex [26,27], and several ELISA kits based on VCA-gp125 have been commercialized for testing NPC [4].

Our study had several limitations. The sample size was small, and our data are for a single cohort of patients, to confirm the efficacy of IgA-gp42. These results need to be confirmed in another cohort, also a multi-center study is required.

CONCLUSION

This study demonstrated that serum IgA-gp42 could potentially be used to diagnosis NPC and will help to resolve the deficiencies of VCA in the testing VCA-negative patients.

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Declaration of Interest:

No conflicts.

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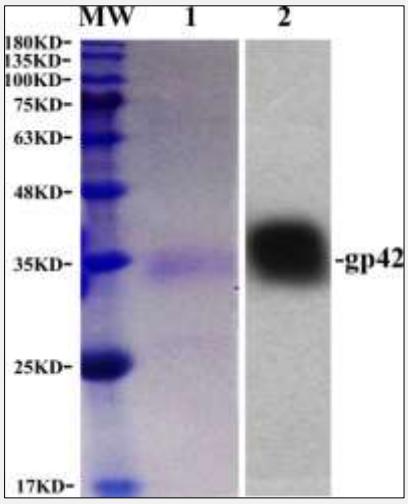
Supplementary Table 1. Clinical characteristics of NPC patients and healthy controls.

Characteristic	NPC (208)		Controls (198)		P [a]
	No.	%	No.	%	
Age, years					
< 46	111	53.4	106	53.5	0.973
≥ 46	97	46.6	92	46.5	
Gender					
Male	153	74	138	70	0.388
Female	55	26	60	30	
Family history of NPC					
Yes	27	13	6	3	< 0.001
No	181	87	192	97	
Smoking history					
Yes	98	47	89	45	0.662
No	110	53	109	55	
IgA-VCA					
Positive	176	85	13	7	< 0.001
Negative	32	15	176	93	
Tumor stage					
I	10	4.8			
II	34	16.3			
III	110	52.9			
IV	54	26			

IgA-VCA, immunofluorescence assay for IgA antibodies against, viral capsid antigen, A titer ≥ 1/40 was taken as positive result in IgA-VCA IFA test. [a] - Chi-square test.



Supplementary Figure 1. Production and purification of soluble EBV gp42 proteins. SDS-PAGE showing gel filtration-purified samples of soluble gp42 (line 1). Western blotting of the recombinant protein identified by anti-Flag antibody (line 2). The values on the left are molecular sizes in kilodaltons.