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Improved Accuracy for Diagnosis of Nasopharyngeal Carcinoma by the Combination of Recombinant EBV Proteins ZEBRA/IgA and LMP2A/IgG ELISA

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a common cancer in Malaysia and elevated serum antibodies to Epstein-Barr virus (EBV) proteins are useful diagnostic markers of the NPC. Coding sequences of EBV proteins LMP2A, EA-D and ZEBRA were cloned from RNA of B95.8 cell line, an EBV-transformed marmoset cell line, into yeast *Saccharomyces cerevisiae* expression vectors. In this study, ELISA was used to immobilize the recombinant EBV proteins for the detection of serum antibodies in NPC patients. The sensitivities and specificities of serum IgG and IgA against recombinant EBV proteins LMP2A, EA-D and ZEBRA in 124 histopathologically diagnosed NPC and 124 age, gender and ethnic-matched healthy individuals were determined. ZEBRA/IgA was found to be the most sensitive single test, which correctly predicted 90.3% of the NPC cases, followed by LMP2A/IgG (77.4%) and EA-D/IgG (73.4%). For specificity, ZEBRA/IgA, EA-D/IgG and EA-D/IgA were each able to exclude 96.0% of the non-NPC cases. The combination of ZEBRA/IgA and LMP2A/IgG ELISA achieved a sensitivity of 95.2% and a specificity of 99.2%. Among the 124 NPC patients recruited in this study, 100 (80.6%) had elevated VCA/IgA determined by the reference method of indirect immunofluorescence assay (IFA). Thus, a higher sensitivity (95.2%) was achieved by the combination of ZEBRA/IgA and LMP2A/IgG. In addition, the combined ELISA could distinguish the 24 NPC sera which had VCA/IgA titers not detectable by IFA.

Keywords: Nasopharyngeal carcinoma, Epstein-Barr virus, ZEBRA, LMP2A, enzyme-linked immunosorbent assay

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a disease with remarkable geographical and ethnic distribution (Busson *et al*., 2004). It is common in the Chinese populations, particularly in South China and Southeast Asian countries, including Malaysia. NPC is difficult to diagnose in the early stages, and much research has been carried out to identify diagnostic markers of the NPC.

Epstein-Barr virus (EBV) is believed to be an important etiologic agent of the NPC. Elevated serum antibody levels to EBV antigens detected in the NPC patients are useful in the serodiagnosis of this malignancy. The detection of serum antibodies to the EBV viral capsid antigen (VCA) and an early antigen (EA) by indirect immunofluorescence assay (IFA) were among the earliest tests developed (Henle and Henle, 1976; Ho *et al*., 1976).

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IFA remains the gold standard for the EBV serodiagnosis of NPC (Leung *et al*., 2004; Raab-Traub, 2000). However, IFA is rather time-consuming and unsuitable for a large-scale testing or automated handling. Enzyme-linked immunosorbent assay (ELISA) provides an alternative with the potential for automation and mass screening.

The EBV antigens which have been reported as the markers of the NPC, detectable by ELISA, include thymidine kinase (Connolly *et al*., 2001), DNase (Stolzenberg *et al*., 1996), ribonucleotide reductase (Fones-Tan *et al*., 1994), ZEBRA (Chan *et al*., 2003; Dardari *et al*., 2001), and EA (Dardari *et al*., 2001). However, a single EBV antigen may not be sufficient to identify all the individual NPC patients in view of the observed diversity of antibody reactivity among the NPC patients. The combined testing of two EBV antigens yielded more sensitive results in the diagnosis of NPC (Chan *et al*., 2003; Dardari *et al*., 2001).

Three diagnostically relevant recombinant EBV proteins had been produced and evaluated by the researchers in the serodiagnosis of NPC. They are LMP2A from the EBV latent cycle; EA-D from the lytic cycle; and ZEBRA, a transactivator protein which switches on the lytic cycle. LMP2A (expressed from a highly spliced mRNA, containing exons located at both ends of the linear EBV genome), is an integral membrane protein which mimics the B-cell receptor to enable EBV to escape from the host immunity (Kieff and Rickinson, 2001; Caldwell *et al*., 1998). EA-D (encoded by full-length BMRF1) corresponds to a dominant immunogen of the diffuse EA complex (Henle *et al*., 1971). The EBV BZLF1 is an immediate-early gene product, while the ZEBRA is a transactivator protein which is capable of disrupting EBV latency when it is expressed in latently infected cells (Rooney *et al*., 1989).

MATERIALS AND METHODS

Clinical and Biological Samples

Peripheral blood samples were collected from 124 patients with histologically confirmed NPC from the otorhinolaryngology clinic at the University Malaya Medical Centre (UMMC). Serum isolation was performed for each blood sample immediately after the receipt in the laboratory, heat-inactivated at 56˚C for 30 minutes, and stored at -20˚C until use. Sera from age, gender and ethnic-matched healthy individuals served as controls. The collection of clinical samples was carried out with prior ethical clearance from the UMMC Ethics Committee (Ethics Committee Reference No.: 471.2) and an informed consent was also obtained.

B95.8 (ATCC CRL-1612) and P3HR1 (ATCC HTB-62) cell lines were used in this study. These cells were maintained in a RPMI-1640 medium containing 10% of fetal calf serum (v/v), 100 IU/ml penicillin and 100 µg/ml of streptomycin at 37˚C in a humidified 5% CO_2 atmosphere (NuAire water jacketed CO_2 incubator, USA). All reagents used were purchased from Flowlab, Australia.

Cloning and Expression of Recombinant EBV Proteins

Total RNA from B95.8 cells were extracted using the RNeasy Protect Mini Kit (Qiagen, Germany), following the protocol suggested by the manufacturer. The coding sequences of EBV-ZEBRA, EA-D and LMP2A were amplified by the reverse-transcriptase polymerase chain reaction (RT-PCR) from the B95.8 RNA, using gene-specific primers (Table 1) and Superscript III One-Step RT-PCR system with Platinum® *Taq* DNA polymerase (Invitrogen, USA), also following the manufacturer's recommendation. The primers used were designed in the laboratory, based on the known sequences of the EBV genes (Baer *et al*., 1984).

The amplified EBV transcripts were cloned into yeast expression plasmid vectors pYES2.1/V5-His-TOPO® (Invitrogen, USA) and transformed into yeast *Saccharomyces cerevisiae* (INV*Sc*1 strain, Invitrogen, USA). The EBV-ZEBRA, EA-D and LMP2A recombinant proteins were expressed and purified from the yeast *Saccharomyces cerevisiae* transformed by the recombinant pYES2.1/V5-His-TOPO®

TABLE 1
Gene-specific primers used in RT-PCR

Transcript	Primer designation	Genome co-ordinates[a]	Oligonucleotides sequence (5' to 3')	Fragment Size (bp)
ZEBRA	Forward	103155-103136	ATGATGGACCCA AACTCGAC	738
	Reverse	102212-102231	AGAAATTTAAGA GATCCTCG	
EA-D	Forward	79899-79918	ATGGAAACCACT CAGACTCT	1215
	Reverse	81113-81094	TTAAATGAGGGG GTTAAAGG	
LMP2A	Forward	166561-166580	ATGGGGTCCCTA GAAATGGT	1494
	Reverse	1679-1660	TATACAGTGTTGC GATATGG	

[a] Genome co-ordinates are given with reference to the B95.8 genomic sequence (Baer *et al*., 1984).

plasmids, containing the respective EBV genes under the control of the galactose-inducible *GAL1* promoter.

Enzyme-linked Immunosorbent Assay (ELISA)

The serum IgG and IgA against EBV-ZEBRA, EA-D and LMP2A recombinant proteins were determined by ELISA. Individual wells of 96-well microtiter plates (MaxiSorp Nunc, Denmark) were coated with 1µg/ml of purified recombinant EBV proteins. Non-specific binding was blocked with phosphate-buffered saline (PBS) containing 5% milk diluent (KPL Inc., USA) for two hours at room temperature. Plates were incubated with diluted sera (200× dilutions for IgG assays; 100× dilutions for IgA assays) for one hour at the room temperature, followed by five washes with PBS/Tween-20 (0.05%). Bound serum antibodies were incubated with alkaline phosphatase (AP)-conjugated goat anti-human IgG or IgA (5000× dilutions) for two hours at the room temperature, followed by five washes with PBS/Tween-20 (0.05%) and detected by BluePhos® microwell phosphatase substrate reagent (KPL Inc., USA). Negative control wells, comprising of blocking buffer, were included in every test. The cut-off values were calculated as the mean optical density (OD) at 630 nm of the negative samples, plus two standard deviations (SD) for each test. The sensitivity of the test was defined as the percentage of NPC individual detected positive, and specificity was defined as the percentage of the negative scored among healthy individuals.

Indirect Immunofluorescence Assay (IFA)

All sera were titrated for IgA antibodies to VCA by the in-house IFA assay. The expression of the VCA in P3HR1 cell line was induced with 20 ng/ml 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, Sigma) and 3 mM sodium butyrate (NBA, Merck). These induced P3HR1 cells were fixed on the Teflon-coated multi-wells slides in cold acetone-methanol (1:1) and air dried. The fixed cells in each well were then incubated with a serially diluted serum samples and later, incubated with the fluorescein-conjugated goat anti-human IgA which allowed indirect detection of VCA-specific antibody, using a UV microscope. A sample was considered IgA-VCA positive when the titre was ≥ 1:10.

Statistical Analysis

Data analyses were performed using the statistical software SPSS version 11.5 (SPSS Inc., USA). In addition, student's *t*-test was also used to compare the mean OD_{630} values of the anti-EBV recombinant proteins between the NPC patients and the healthy controls. A p value of < 0.05 was considered as statistically significant.

RESULTS

In this study, three EBV antigens from the different phases of the virus infection cycle (i.e. LMP2A in the latent phase, ZEBRA in the immediate early phase of active replication, and EA-D in the lytic phase) were cloned from B95.8 RNA (*Fig. 1*) and transformed into yeast *Saccharomyces cerevisiae* strain INV*Sc*1 for expression. The optimum yields of the recombinant EBV proteins in *S. cerevisiae* strain INV*Sc*1 were obtained 24-hour post induction with 2% galactose. Purified recombinant EBV proteins were coated onto 96-well microtiter plates, at 1µg/ml for the detection of specific serum IgG and IgA.

One hundred and twenty four NPC sera and 124 age, gender and ethnic-matched healthy individuals were also tested. Significant differences (p value ranged from < 0.0001 and 0.005 were shown between the NPC patients and the healthy controls for all the three recombinant EBV proteins in this study (Table 2). All the tests were found to be significant at 0.0001 level, except for the ZEBRA/IgG with p value being 0.005.

ZEBRA/IgA was the most sensitive single test, which correctly predicted 90.3% of the NPC cases, followed by LMP2A/IgG (77.4%) and EA-D/IgG (73.4%). For specificity, the ZEBRA/IgA, EA-D/IgG and EA-D/IgA were able to exclude 96.0% of the non-NPC cases (Table 3).

Higher sensitivities were achieved using the combination of ZEBRA/IgA and EA-D/IgG (93.6%), as well as the combination of ZEBRA/IgA and LMP2A/IgG (95.2%). The combination of EA-D/IgG and LMP2A/IgG showed a sensitivity of 86.3%, which was higher than the sensitivities shown by either of the individual tests involved: EA-D/IgG (73.4%) and LMP2A/IgG (77.4%). The specificity was generally higher by combining two ELISA: 96.8% for EA-D/IgG and LMP2A/IgG; 99.2% for ZEBRA/IgA and EA-D/IgG, as well as for ZEBRA/IgA and LMP2A/IgG, as compared to the best specificity achieved by individual ELISA test, which was 96.0%.

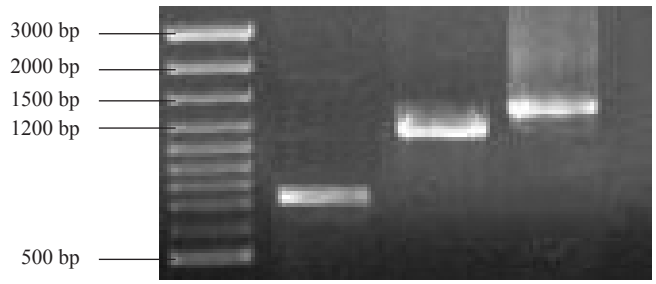

Fig. 1: Three EBV genes amplified by RT-PCR (1% agarose stained with ethidium bromide) that were used to clone into yeast pYES2.1/V5-His-TOPO® vectors

Lane 1: Gene-Ruler™ 100bp DNA Ladder Plus
2: ZEBRA transcript (738 bp)
3: EA-D transcript (1215 bp)
4: LMP2A transcript (1494 bp)

TABLE 2
Comparison of the serum IgG and IgA to the EBV recombinant proteins, between the NPC patients and healthy controls

Reactivity of Recombinant EBV protein	Mean value of OD_{630} ± standard deviation		Student's *t*-test (*p* value)	Significance Level
	NPC Patients	Healthy Controls		
ZEBRA/IgG	1.232±0.587	0.407±0.246	0.00239	< 0.005*
ZEBRA/IgA	0.586±0.376	0.147±0.071	0.00000	< 0.0001*
EA-D/IgG	1.398±0.607	0.392±0.250	0.00000	< 0.0001*
EA-D/IgA	0.524±0.407	0.126±0.080	0.00000	< 0.0001*
LMP2A/IgG	1.186±0.492	0.364±0.202	0.00000	< 0.0001*
LMP2A/IgA	0.465±0.338	0.133±0.086	0.00008	< 0.0001*

* Statistically significant.

TABLE 3
Sensitivities and specificities of the recombinant EBV proteins

Reactivity of Recombinant EBV protein	Sensitivity	Specificity
Single test		
ZEBRA/IgG	66.9	94.4
ZEBRA/IgA	90.3	96.0
EA-D/IgG	73.4	96.0
EA-D/IgA	72.6	96.0
LMP2A/IgG	77.4	95.2
LMP2A/IgA	66.9	93.6
Combination of two tests		
ZEBRA/IgA and EA-D/IgG	93.6	99.2
ZEBRA/IgA and LMP2A/IgG	95.2	99.2
EA-D/IgG and LMP2A/IgG	86.3	96.8

All the sera tested by ELISA were also tested for VCA/IgA by the in-house IFA. Among the 124 NPC sera used in this study, 100 (80.6%) had elevated VCA/IgA determined by the in-house IFA (titers ≥ 1:10), but 24 (19.4%) had undetectable levels of VCA/IgA by IFA (titers < 1:10). All healthy controls (n =124) were found to be negative for VCA/IgA by IFA (< 1:10).

Thus, a higher sensitivity (95.2%) was achieved using the combination of ELISA of ZEBRA/IgA and LMP2A/IgG; furthermore, the combination of ELISA ZEBRA/IgA and LMP2A/IgG could distinguish the 24 NPC sera which were not detected to have elevated IgA/VCA by the IFA.

DISCUSSION

In this study, the potential use of three recombinant proteins of EBV antigens, expressed in the different phases of virus infection (i.e. LMP2A in the latent phase, ZEBRA in the immediate early phase of active replication, and EA-D in the lytic phase) to detect the NPC cases, were determined. It was found that the serum IgA to ZEBRA is the best marker in the serodiagnosis of the NPC, with the sensitivity of 90.3% and specificity of 96.0%. Other studies reported by Yoshizaki *et al*. (2000) had also shown that the antibodies to ZEBRA could significantly be increased ($p < 0.05$) in the newly diagnosed NPC patients. Thus, ZEBRA should be included in the panel of the EBV proteins for the serodiagnosis of the NPC.

A much better performance in the serodiagnosis of NPC could be achieved using the combination of two ELISA tests, as shown in Table 3. The best performance was achieved by the combination of ZEBRA/IgA and LMP2A/IgG ELISA, with a sensitivity of 95.2% and a specificity of 99.2%, respectively. The combined ELISA was found to achieve better sensitivity and specificity. The studies from the other groups such as Dardari *et al*. (2001) and Chan *et al*. (2003) also reported that no single test of the EBV antibody was sufficient to detect every patient with the NPC.

The performance of anti-LMP2A was average in the single ELISA test, but it may help to detect the NPC individuals which are not recognized by either anti-ZEBRA or EA-D recombinant EBV proteins. A recent work by Chen *et al*. (2005) found three sera negative for VCA/IgA were positive for LMP2A/IgA in NPC patients, suggesting that the LMP2A specific antibodies might have the potential significance for the diagnosis of the NPC. The above findings demonstrate that the antibody re-activities among the NPC patients are diverse and that the use of combinations of EBV proteins will lead to a wider coverage of immunodominant epitopes recognised by different subset of antibodies.

The detection of serum antibodies to EBV VCA in the sera of NPC patients is traditionally done by IFA. Although the IFA of IgA against VCA has been the mainstay of the EBV serodiagnosis of NPC, there remain 15% to 20% of the NPC patients who have undetectable levels of IgA antibody to VCA (Sam *et al*., 1989). In the present study, 24 of the 124 NPC patients (19.4%) did not exhibit elevated levels of serum IgA to VCA determined by IFA. The combination of ZEBRA/IgA and LMP2A/IgG ELISA was found to be able to distinguish the 24 NPC patients.

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