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Epstein Barr Virus (EBV) Antibodies in the Diagnosis of NPC – Comparison Between IFA and Two Commercial ELISA Kits

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ABSTRACT

Background: Antibodies to Epstein Barr Virus (EBV) antigens have been used for the diagnosis of nasopharyngeal carcinoma (NPC). While immunofluorescence assays (IFA) of IgA anti-viral capsid and early antigens have been the mainstay of this diagnosis, enzyme immunoassays (ELISA) of various EBV antigens are now available. However in almost all of these assays, the sensitivities and specificities have been calculated using blood donors and normal hospital staff as controls, who may not be the most appropriate controls. We wanted to evaluate the usefulness of IFA and ELISA of various EBV antigens in a clinical setting to distinguish between patients with NPC and those suspected of NPC but being biopsy negative.

Methods: Between January 1987 and June 1988, 322 consecutive patients suspected of NPC and who had a post-nasal biopsy were studied. Blood was taken for EBV tests before diagnosis. Tests included IFA and ELISA IgA anti-VCA and anti-EA and ELISA IgA and IgG anti-ribonucleotide reductase, a cloned EA antigen.

Results: IFA IgA anti-VCA together with IFA IgA anti-EA both at a cut-off of 1:10 gave the best discrimination between patients with NPC and those suspected of NPC but were biopsy negative.

Conclusion: The ELISA IgG anti-ribonucleotide reductase test is convenient to perform and looks very promising. An ELISA using a cocktail of cloned EA peptides may be even better.

Keywords: NPC, EBV, IgA VCA, IgA Ea, IgG anti-ribonucleotide reductase

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INTRODUCTION

The aetiology of nasopharyngeal carcinoma (NPC) is multifactorial and implicated agents include genetic (eg. HLA, T cell receptors etc), viral (EBV) and other environmental (preserved salted food, lack of fresh fruit and vegetables etc) factors[1]. The association between NPC and EBV include elevated antibody titres to EBV antigens particularly IgA to viral capsid (VCA) and early (EA) antigens, decreased T cytotoxic cells to EBV and EBV genome within the tumour, regardless of the histological type. Pre-invasive lesions are already infected by the virus; within the tumour, it is mainly episomal and clonal in nature[2].

In Singapore, EBV infection occurs early in life. Seroprevalence studies in the early 1970s and 1990s showed that EBV infection has not changed in the last 20 years despite changes in other herpesviral infections over this period[3]. Once infected, the virus remains latent in B lymphocytes and possibly also in epithelial cells. Possible viral products expressed during latency are 6 nuclear proteins, termed EBNA 1-6 and 3 membrane proteins LMP-1, 2A and 2B. In NPC tumours, only EBNA 1 and the 3 LMPs are expressed[4]. The switch from latent to replicative infection is triggered by the BamH1 Z EBV replication activator (ZEBRA) protein. This results in extensive transcription of the viral genome and expression of the early (EA, non-structural) and late (VCA, MA, structural) antigens. The early antigen is made up of a group of enzymes and other proteins. Even though ZEBRA protein can be found in NPC cells, there is no early and late antigens suggesting that replication is aborted and no virus is being made. However, there must be high viral replication in NPC patients because of the high antibody response and it is not certain as to the site of this replication.

EBV can be divided into 2 different types, A and B and there are differences in their genomes. Chinese NPC is associated with EBV type A, the prototype being the B95-8.

EBV serology for the diagnosis of NPC

The high antibody titres to EBV antigens in NPC patients serve as a marker for the diagnosis of NPC. There are many tests available detecting both IgG and IgA responses to VCA, EA, membrane (MA), EBNA 1, ZEBRA, DNase and ribonucleotide reductase[5], the last two being part of the complex EA. They have varying sensitivities and specificities. In almost all of these assays, the sensitivity and specificity are calculated using normal blood donors, medical and hospital staff as controls. The usefulness of these assays is in the clinical setting where an Ear Nose & Throat (ENT) surgeon has to distinguish between a patient with NPC from those with the same symptoms and signs but are NPC negative.

Even though the WHO Collaborating Centre in Immunology has been performing EBV serology for research and service since 1972, with in-house prepared EBV-B95-8 antigens using the immunofluorescence assay (IFA), the usefulness of

these assays in an ENT clinic setting has not been documented. Recently, EBV serology by ELISA has become available commercially. In this paper, we evaluated the usefulness of these assays in a clinical outpatient setting for the diagnosis of NPC.

MATERIAL AND METHODS

Between January 1987 and June 1988, consecutive patients suspected of NPC attending ENT clinics at two major hospitals in Singapore and who had a post-nasal biopsy were included in this study. Blood was taken at the same time for EBV serology and HLA typing and EBV IFAs performed before diagnosis or therapy. IgA antibodies to EBV-VCA and EBV-EA were tested using the in-house IFA and also at a later date by a first generation commercial ELISA (EBVIRAL ELISA, Singapore Biotech Pte Ltd). Some of these sera were also tested using a second generation ELISA (IgG anti-Ribonucleotide reductase, Innovative Biotech, Singapore). In the first generation ELISA, EBV VCA and EA extracted from EBV cell lines were used to coat ELISA plates. In the second generation ELISA, a recombinant protein, ribonucleotide reductase (RiRe, part of EA) was used to coat the plates. Subsequently, the patients were grouped according to the result of the biopsy into 64 NPC, 19 other head and neck malignancies, 75 epitaxis, 25 otitis media, 18 upper respiratory tract infection (URTI), 45 lymph node (LN) enlargement, 9 vasomoto rhinitis and 67 miscellaneous patients. The miscellaneous group consisted mainly of cranial nerve palsies, pulmonary TB etc. All NPC patients and controls were tested by IFA and first generation ELISA. An additional 100 blood donors were tested by IFA. In the second generation ELISA, some of the controls (those not exhausted by previous assays) and a different set of 100 NPC sera were tested. The NPC sera used in the second generation ELISA came from ENT clinics and a radiotherapy department in a private hospital, representing a mixed variety of NPC (newly diagnosed, post-therapy and those with recurrences).

RESULTS

Table I shows the anti-VCA results by IFA and ELISA of the 64 NPC patients, various groups of NPC negative patient and normal controls. With IFA, 11% of normal blood donors had a titre of $\geq$ 10 and 4%, $\geq$ 40. In marked contrast, 90.7% of NPC patients had titres of $\geq$ 10 and 81.3%, $\geq$ 40 (Table I). However in the NPC negative patients, some also had high titres, especially patients with other head and neck cancers, otitis media and vasomotor rhinitis. When combined, 26.8% of NPC negative patients had titres of $\geq$10 and 11.3%, $\geq$40. With the first generation ELISA anti-VCA, NPC patients also showed a higher frequency of positivity compared to NPC negative controls (Table I). However compared to IFA, positive results were more common with ELISA in the controls. IgA anti-EA was a much more specific test (Table II). With IFA 51/64 (79.7%) of NPC patients had titres $\geq$ 10 compared to 11/258 (4.3%) combined NPC negative patient controls. None of the 100 blood donors tested positive. This higher degree of specificity was also reflected in the first generation ELISA, but again was lower than that by IFA. The second generation ELISA tested for IgG anti-RiRe. This assay was evaluated on a different set of 100 NPC patients and was positive in 81 compared to 17/72 (23.6%) combined NPC negative patients.

Table III compares the sensitivities and specificities of the 5 tests. The comparison group was the total NPC negative patient controls, that is ENT patients suspected of NPC but were biopsy negative. In IFAs the most discriminating cut-off titre appeared to be IgA anti-VCA titre at 1:10 and IgA anti-EA titre of also 1:10. NPC patients had significantly ($p < 0.0001$) higher frequencies of positivities in all 5 assays, which also showed relatively good sensitivities and specificities. The IFA IgA anti-VCA had the highest sensitivity and the IFA IgA anti-EA, the highest specificity. Positivity in the IFA IgA anti-EA was associated with the highest relative risk (88.1) and this assay together with the ELISA IgG anti-RiRe had the highest positive predictive values. Of the ELISAs, the IgG anti-RiRe also had the best sensitivity. All assays except the IgG anti-RiRe had negative predictive values of over 90%.

DISCUSSION

The present study compared the value of various EBV serology tests in the diagnosis of NPC in a clinical setting. Even though ELISAs are simpler to handle, less subjective and can do a larger number of tests,

Table I – EBV IgA anti-VCA by IFA and first generation ELISA in NPC and Controls

		IFA (Titre)						ELISA
Groups	**No**	**≥640**	**160**	**40**	**10**	**5**	**-ve**	**+ve**
NPC	64	19 (29.7)*	16 (25.0)	17 (26.6)	6 (9.4)	1 (1.6)	5 (7.8)	50 (78.1)
H&N Ca	19	0	3 (15.8)	4 (21.4)	1 (5.3)	1 (5.3)	19 (52.6)	9 (47.4)
Epitaxis	75	0	2 (2.7)	4 (5.3)	14 (18.7)	6 (8.0)	49 (65.3)	28 (37.3)
Otitis media	25	0	2 (8.0)	1 (4.0)	6 (24.0)	1 (4.0)	15 (60.0)	11 (44.0)
URTI	18	0	0	1 (5.6)	2 (11.1)	0	15 (83.3)	7 (38.9)
LN	45	0	1 (2.2)	4 (8.9)	4 (8.9)	2 (4.4)	34 (75.6)	14 (31.1)
VMR	9	0	1 (11.1)	0	0	1 (11.1)	7 (77.8)	2 (22.2)
Misc	67	0	1 (1.5)	5 (7.5)	13 (19.4)	0	48 (71.6)	19 (28.4)
NPC-ve Total	258	0	10 (3.9)	19 (7.4)	40 (15.5)	11 (4.3)	178 (69.0)	90 (34.9)
Normals	100	0	0	4 (4.0)	7 (7.0)	3 (3.0)	86 (86.0)	-

* Number positive (%)

Table II – EBV IgA anti-EA by IFA and first generation ELISA in NPC and Controls

Groups	No	IFA (Titre) ≥640	160	40	10	5	-ve	ELISA +ve
NPC	64	10 (15.6)*	10 (15.6)	19 (29.7)	12 (18.8)	0	13 (20.3)	44 (68.8)
H&N Ca	19	0	0	0	4 (21.1)	1 (5.3)	14 (78.9)	7 (36.8)
Epitaxis	75	0	0	0	1 (1.3)	2 (2.7)	72 (96.0)	10 (22.2)
Otitis media	25	0	0	1 (4.0)	1 (4.0)	0	23 (92..0)	7 (37.5)
URTI	18	0	0	0	0	0	18 (100)	3 (37.5)
LN	45	0	0	0	1 (2.2)	1 (2.2)	43 (95.6)	9 (20.0)
VMR	9	0	0	1 (11.1)	0	0	8 (88.9)	1 (11.1)
Misc	67	0	0	0	2 (3.0)	0	65 (97.0)	15 (22.4)
NPC-ve Total	258	0	0	2 (0.8)	9 (3.5)	4 (1.6)	243 (94.2)	52 (20.2)
Normals	100	0	0	0	0	0	100 (100)	-

* Number positive (%)

Table III – Comparison of different EBV assays in the diagnosis of NPC

Tests	Sensitivity	Specificity	p	RR	95% CL	Predictive Value Positive	Negative
IF anti-VCA IgA >1:10	90.6%	73.3%	<0.0001	26.5	10.9-64.1	45.7%	96.9%
IF anti-EA IgA >1:10	79.7%	95.7%	<0.0001	88.1	37.4-207.7	82.3%	95.0%
ELISA IgA anti-VCA	78.1%	65.1%	<0.0001	6.7	3.5-12.7	35.7%	92.3%
ELISA IgA anti-EA	68.8%	79.8%	<0.0001	8.7	4.7-16.0	45.8%	91.2%
ELISA IgG anti-RiRe	81.0%	76.4%	<0.0001	13.7	6.6-28.9	82.7%	74.3%

RR=Relative Risk; 95% CL=95% confidence limit

the IFAs appeared to be better in discriminating between NPC positive patients and those patients suspected of NPC but proven to be NPC negative on biopsy. Another advantage is that the IFAs are reported in titres and the level of the antibody can be monitered. The most discriminatory IFA titre for the diagnosis of NPC were IgA anti-VCA 1:10 and IgA anti-EA 1:10. At these IFA titre cut-offs, the IgA anti-VCA was the most sensitive and the IgA anti-EA, the most specific. At the WHO collaborating centre for Immunology, these 2 tests are used as aids for routine diagnosis of NPC.

There were 11 NPC negative patients who had an IFA IgA anti-EA titre of 10 or more, 9 with a titre of 10 and 2 with a titre of 40 (Table II). Of the 9 patients with a titre of 10, four had cancer of the parotid or tongue, some of these cancers may be associated with EBV, 3 were lost to follow-up and 2 remained cancer-free. Of the two patients with titres of 40, one developed NPC and cancer of the colon diagnosed 1 year later and the other died soon after the study; but the cause of death was not recorded. These data strongly suggest that NPC negative patients with an IFA IgA anti-EA titre of 10 or more should be closely followed-up.

Regardless as to whether EBV causes NPC, antibodies to EBV antigens are obviously good markers for the diagnosis of NPC. However the IFA is tedious, quality control of the slides absolutely important and results are very subjective. ELISA is preferable. The first generation ELISA looks promising and the second generation ELISA appears better. However, their sensitivities and specificities need to be improved. Since RiRe is part of the EA complex, a cocktail together with other cloned EAs may be an even better test. Hopefully this will increase the sensitivity of the assay. A higher cut-off value will improve specificity. IgAs are secreted and testing of saliva for these antibodies should be more convenient, especially if screening and follow-up tests are required.

CONCLUSION

Antibodies to EBV VCA and EA are useful tests for the diagnosis of NPC. Currently IFA IgA anti-VCA together with IFA IgA anti-EA both at a cut-off of 1:10 gave the best discrimination between patients with NPC and those suspected of NPC but were biopsy negative. The ELISA IgG anti-ribonucleotide reductase test is convenient to perform and looks promising. An ELISA using a cocktail of cloned EA peptides may be even better, but the sensitivities and specificities of the current commercial assays need to be improved.

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