ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (1999) 18: 221-226

Specific IgA Antibody to Epstein-Barr Viral Capsid Antigen: A Better Marker for Screening Nasopharyngeal Carcinoma than EBV-DNA Detection by Polymerase Chain Reaction

Wannee Kantakamalakul[1], Cheerasook Chongkolwatana[2], Preechaya Naksawat[1], Sorranart Muangsomboon[3], Sanya Sukpanichnant[3], Samut Chongvisal[2], Choakchai Metheetrairat[2], Uraiwan Kositanont[1] and Pilaipan Puthavathana[1]

SUMMARY **Nasopharyngeal carcinoma (NPC) is strongly associated with Epstein-Barr virus (EBV) infection. To assess whether EBV DNA detection by polymerase chain reaction (PCR) or presence of specific serum antibody to viral capsid antigen (VCA) was a better marker for screening NPC, nasopharyngeal tissues and blood samples from 58 NPC patients and 24 non-NPC patients (23 with laryngotracheal stenosis and 1 with chronic tonsillitis) were tested for the presence of EBV DNA and serum specific VCA antibodies, respectively. EBV DNA was detected in 56 (96.5%) of NPC patients and 15 (62.5%) of non-NPC controls, with predominantly EBV type A in both groups. On the other hand, specific VCA IgA antibody was detected in the majority of NPC patients: 52 (89.7%) while only 4 (16.7%) were detected in non-NPC controls. Therefore, specific VCA IgA antibody may serve as a better marker for screening NPC than EBV DNA detected by PCR.**

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Southern China and South-East Asia including Thailand.[1-4] Epstein-Barr virus (EBV) has been found to be closely associated with this cancer. Pathmanathan *et al.*[5] showed new evidence confirming that EBV is the primary etiological agent in the development of NPC by demonstrating dysplastic lesions and carcinoma *in situ* in premalignant clones of EBV-infected cells. There are two different types of EBV: types A and B (or types 1 and 2). Although both types have been found in nasopharyngeal tissues, type A is more common.[6,7]

The presence of serum antibodies to EBV antigens have been used for the diagnosis of NPC.[8-11] IgG and IgA anti-viral capsid antigen (VCA) antibody titers in NPC patients were found to be higher than those of healthy cases.[11-13] In addition, IgA anti-VCA was useful for the early detection of NPC in a field survey in China.[14] However, 15-20% of NPC patients were not identified using this antibody.[15,16] Other specific antibodies to EBV antigens have also been shown to be useful for the early detection of NPC.[17-19] So far, no single serological marker has been identified to be applicable as a screening assay for all NPC patients. Meanwhile, detection of EBV DNA in tissue by polymerase chain reaction (PCR) has recently been used as a supplement to the serologic screening of NPC[20] and as a screening method for NPC in a high risk group.[21,22] However, EBV DNA detected by PCR technique was also found in oropharyngeal cells, nasopharyngeal tissues and exfoliated cells from the postnasal space of a high number of healthy individuals.[23-25] The purpose of this study was to evaluate whether specific anti-VCA antibody or EBV DNA detection by PCR was a better marker for screening patients for NPC.

From the Departments of [1]Microbiology, [2]Otolaryngology, and [3]Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Correspondence: Wannee Kantakamalakul

MATERIALS AND METHODS

Subjects and clinical specimens

Fifty-eight histologically confirmed NPC patients and 24 non-NPC controls who attended the Department of Otolaryngology, Siriraj Hospital, Bangkok, Thailand were enrolled in this study. Out of 24 non-NPC controls, 23 patients had laryngotracheal stenosis and one patient chronic tonsillitis. Nasopharyngeal tissue biopsies of approximately 30 mg and blood samples were collected from patients and controls prior to any clinical treatment following the protocol and after obtaining written informed consents. Tissue samples and sera were kept at -70°C and -20°C, respectively, until tested.

Preparation of clinical samples

The frozen tissue biopsies were thawed and DNA was extracted by the modified procedure described by Feinmesser *et al.*[26] Briefly, 0.5-1 ml of lysis buffer C (1 x PCR buffer [20 mM Tris pH 8.3, 50 mM KCl, 2 mM $MgCl_2$], 0.45% Nonidet-P40, 0.45 % Tween 20, and 100 µg of proteinase K [Sigma-Aldrich, Singapore] per milliliter) were used to digest the biopsies for 4 hours at 56°C and subsequently the samples were boiled at 95°C for 10 minutes. Two percent of the total volume of the cell lysates were directly used for DNA amplification in the nested PCR experiment.

The B95-8 (ATCC CRL 1612) and Jiyoye (ATCC CCL87) cell lines were used as the positive DNA amplification controls for EBV types A and B, respectively. These cell lines were similarly treated as tissue biopsies.

Nested PCR for detection of EBV DNA

Three sets of primers were synthesized based on the published oligonucleotide sequence primers of EBNA 2 for EBV typing.[27] The first set contained the common EBNA 2 primers (5'-AGGGATGC-CTGGACACAAGA-3' and 5'-TGG-TGCTGCTGGTGGTGGCAAT-3') which yielded 596 base pairs (bp) amplified product for both types of EBV. The second set contained the EBV type A specific primers (5'-TCTTGATAGGGATCCGCTAGGATA-3' and 5'-ACCGTGGTTCTGGAC-TATCTGGATC-3') which yielded 497 bp amplified product; and the third set contained EBV type B specific primers (5'-CATGGTAG-CCTTAGGACATA-3' and 5'-AG-ACTTAGTTGATGCCCTAG-3') which yielded 150 bp amplified product.

Target DNA was first amplified in 50 µl of a reaction mixture containing 10-20 µl of the sample; 25 pmole of each primer from the first set; 400 µM of each deoxynucleotide triphosphate (dNTP); 1 x PCR buffer and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA) for 30 cycles (denaturation for 1.5 minutes at 94°C, primer annealing for 1 minute at 60°C, and extension for 2 minutes at 72°C) in a DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA). One microliter of the PCR product was then used for the nested reaction for EBV typing under the same condition as mentioned above except using the second and the third sets of primers. After the nested reaction, 20 µl of the DNA amplified product was analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide solution and visualized under UV light.[27] Amplification of β-globulin gene using primers described elsewhere[28] was used to confirm the presence of cellular DNA in the treated tissue.

Immunofluorescence test

The B95-8 cell line was used as a source of VCA. Details of B95-8 culture, slide preparation and staining procedure were described elsewhere.[16] The test sera were serially two fold diluted in PBS starting at 1:10. FITC-conjugated goat anti-human IgG or IgA heavy chain specific antibodies (Hyland Diagnostics, IL, USA) were used as the secondary antibody. The titer was defined as the reciprocal of the highest dilution that gave a positive reaction. A titer of ≥ 10 was considered to be positive.

Statistical analysis

Data were analyzed with statistical software (SPSS, Chicago). Two independent groups were compared by the Mann-Whitney U test. The level of critical significance was assigned at $p < 0.05$. Sample proportions were compared by χ^2 test. When the minimum estimated expected value was < 5, Fisher's exact test was used. The validity of a screening test was evaluated in terms of sensitivity and specificity using clinical diagnosis with histologically confirmed NPC as a gold standard.

RESULTS

The tissue biopsies from the NPC patients were categorized into distinct histological types according to the WHO classification. Three (5.2%), 39 (67.2%) and 16 (27.6%) were classified as WHO type 1 (squamous cell carcinoma), WHO type 2 (non-keratinizing carcinoma) and WHO type 3 (undifferentiated carcinoma), respectively.

Table 1 Different PCR specific EBV in biopsy tissues from NPC patients and controls

Histological types	No. biopsy tissues (%)	No. biopsy tissues with PCR specific EBV			
		Type A (%)	Type B (%)	Types A+B (%)	Not found (%)
NPC					
WHO type 1	3 (5.2)	2	1	0	0
WHO type 2	39 (67.2)	33	3	1	2
WHO type 3	16 (27.6)	13	2	1	0
Total	58	48 (82.7)	6 (10.3)	2 (3.5)	2 (3.5)
Controls	24	14 (58.3)	1 (4.2)	0	9 (37.5)

Table 2 EBV specific VCA IgG antibody in NPC patients and controls

Group	EBV type specific	Total	No. of cases with reciprocal titer of												Reciprocal GM titer
			<10	10	20	40	80	160	320	640	1,280	2,560	5,120	10,240	
NPC	**A**	48	0	0	0	0	0	3	7	16	14	4	3	1	871.0
	B	6	0	1	0	0	0	2	0	3	0	0	0	0	199.5
	A+B	2	0	0	0	0	0	0	0	0	1	0	1	0	2,570.4
	Not found	2	0	0	0	0	0	0	0	1	0	1	0	0	1,288.2
	Total	58	0	1	0	0	0	5	7	20	15	5	4	1	955.0
Controls	**A**	14	0	0	0	0	0	2	6	3	0	3	0	0	524.8
	B	1	0	0	0	0	0	0	0	1	0	0	0	0	640.0
	A+B	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
	Not found	9	0	0	0	0	0	2	4	2	1	0	0	0	371.5
	Total	24	0	0	0	0	0	4	10	6	1	3	0	0	457.1

Table 3 EBV specific VCA IgA antibody in NPC patients and controls

Group	EBV type specific	Total	No. of cases with reciprocal titer of												Reciprocal GM titer
			<10	10	20	40	80	160	320	640	1,280	2,560	5,120	10,240	
NPC	**A**	48	5	9	6	7	13	4	4	0	0	0	0	0	36.3
	B	6	0	0	1	2	2	1	0	0	0	0	0	0	56.2
	A+B	2	0	0	0	1	0	1	0	0	0	0	0	0	79.4
	Not found	2	1	0	0	1	0	0	0	0	0	0	0	0	14.1
	Total	58	6	9	7	11	15	6	4	0	0	0	0	0	38.0
Controls	**A**	14	12	1	0	0	1	0	0	0	0	0	0	0	6.5
	B	1	1	0	0	0	0	0	0	0	0	0	0	0	5.0
	A+B	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
	Not found	9	7	1	1	0	0	0	0	0	0	0	0	0	6.3
	Total	24	20	2	1	0	1	0	0	0	0	0	0	0	6.3

EBV DNA was detected in 56 (96.5%) of 58 NPC patients: 48 (82.7%) with type A, 6 (10.3%) with type B, and 2 (3.5%) with both types (Table 1). There was an association between EBV DNA detection and NPC (χ^2 test at $p < 0.001$). However, EBV DNA was also found in 15 (62.5%) of 24 non-NPC controls in which 14 (58.3%) were type A. The sensitivity and specificity of EBV DNA detection by PCR for NPC patients were 96.6% and 37.5%, respectively.

All NPC patients and controls showed specific VCA IgG antibody in their sera (Table 2). Specific VCA IgG titers of NPC patient sera were significantly higher than those of control sera (Mann-Whitney U test at $p = 0.006$). The geometric mean (GM) titers were somewhat higher in the NPC patients. However, 45 (77.6%) of 58 NPC patient sera and 10 (41.7%) of 24 control sera had IgG titers of 1:640 or above. There was an association between VCA IgG titers of ≥ 1:640 and NPC (χ^2 test at $p = 0.004$). The sensitivity and specificity of anti-VCA IgG testing with titers of ≥ 1:640 for NPC patients were 77.6% and 58.3%, respectively. Specific VCA IgA antibody was detected in the sera of 52 (89.7%) of 58 NPC patients but only 4 (16.7%) of 24 control sera (Table 3). The specific IgA titers of NPC patient sera were significantly higher than those of control sera (Mann-Whitney U test at $p < 0.001$). The GM titers were somewhat higher in the NPC patients. There was a high association between VCA IgA detection and NPC (χ^2 test at $p < 0.001$). The sensitivity and specificity of VCA IgA detection for NPC patients were 89.7% and 83.3%, respectively.

DISCUSSION

NPC is strongly related to EBV infection.[5] All histological types of NPC were present in this study; however, only 5.2% were WHO type 1 since this type was not common in Thailand.[7,29] Laboratory diagnosis of EBV infection for screening NPC has been based on serological assays[13,30-32] and recently on EBV DNA detection by PCR.[20-22] In this study, EBV DNA was detected in 96.5% (56/58) of NPC patients and 62.5% (15/24) of controls, both with predominantly type A. The percentage of EBV DNA detection in NPC patients in this study was similar to that of our previous report[7] in which EBV DNA was detected in 94.1% (32/34) of cases. However, EBV DNA was previously found in only 20% (1/5) of the non-NPC controls. The higher number of EBV DNA detection in the non-NPC controls in the present study (62.5% *vs* 20%) may be due to the larger number of controls and a more sensitive PCR technique used. This high number of positive EBV DNA in the controls was similar to the previous findings of other groups, i.e., 70%-80% of the nasopharyngeal tissues from healthy individuals were shown to have EBV DNA by PCR technique.[23,24] In addition, Diaz-Matoma, *et al.*[25] reported that 56% of healthy individuals had detectable levels of EBV DNA in oropharyngeal cells. These findings, including ours, support the concept that although EBV has been implicated in the pathogenesis of NPC, other factors such as environmental and genetic factors must be considered to be involved in the etiology of NPC. EBV infection is ubiquitous in all human population. The virus replicates in epithelial cells and establishes a latent infection in lymphoid cells. Oropharyngeal epithelial cells are the source of the virus found in the throat.[33,34] Nasopharyngeal tissues in non-NPC individuals could possibly be infected with EBV that is periodically excreted in the oropharynx. The specificity of the EBV DNA detection in this present study was only 37.5%. Therefore, we conclude in this study that EBV DNA detection by PCR might not be a suitable method for screening for NPC.

On the other hand, we demonstrated that the titers of both serum IgG and IgA antibodies against EBV VCA in the NPC patients were significantly higher than those of the non-NPC controls. These results were similar to those of our previous study.[16] Although serum IgG anti-VCA was found in non-NPC controls with various titers, the GM titers were somewhat lower in the controls. The reciprocal GM titer in this study was similar to that of our previous report (457.1 and 371.5, respectively). In this study, we found that an IgG anti-VCA titer of ≥ 1:640 was associated with NPC. However, the specific VCA IgG antibody titer of ≥ 1:640 may not be used as a marker for screening NPC because of the low specificity (58.3%). Interestingly, the findings that serum IgA anti-VCA antibody found in 89.7% (52/58) of NPC patients but only in 16.7% (4/24) of the controls were similar to 84.6% (77/91) in the NPC patient sera and 15.5% (22/142) in the control sera reported in our previous study.[13] This specific IgA anti-VCA antibody was highly associated with NPC. This result is consistent with earlier studies showing a relationship between the specific IgA antibody and NPC.[35,36]

In conclusion, EBV DNA detection by PCR may not be a use-

ful tool for screening NPC because of its low specificity. On the other hand, the high specificity and sensitivity of specific IgA anti-VCA antibody appears to confirm the use of this antibody as a marker for screening or diagnosis of NPC.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund, Grant #PDF66/2540. The authors would like to thank Dr. Rutt Chuachoowong for statistical assistance and Mrs. Suthatip Sri-nguirai for specimen processing.

REFERENCES

1. Ho J. An epidemiologic and clinical study of nasopharyngeal carcinoma. Int J Radiat Oncol Biol Phys 1978; 4: 182-98.
2. Hirayama T. Descriptive and analytical epidemiology of nasopharyngeal cancer. IARC Sci Publ 1978; 20: 167-89.
3. Garmjana-Goonchorn S. Chantarakul N. Nasopharyngeal cancer at Siriraj Hospital, Dhonburi, Thailand. UICC Monograph Series 1965; 1: 33-7.
4. Titapant V, Vatanasapt V, Tangvoraponkchai V, *et al.* Cancer incidence in Khon Kaen province 1985-1987. Thai Cancer J 1989; 15: 55-63.
5. Pathmanathan R, Prasad U, Sadler R, Flym K, Raab-Traub N. Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N Engl J Med 1995; 333: 693-8.
6. Shu CH, Chang YS, Liang CL, Liu ST, Lin CZ, Chang P. Distribution of type A and type B in normal individuals and patients with head and neck carcinomas in Taiwan. J Virol Methods 1992; 38: 123-30.
7. Kositanont U, Kondo K, Chongkolwatana C, *et al.* Detection of Epstein-Barr virus DNA and HHV-6 DNA in tissue biopsies from patients with nasopharyngeal carcinoma by polymerase chain reaction. Southeast Asian J Trop Med Public Health 1993; 24: 455-60.
8. Chan SH, Soo MY, Gan YY, *et al.* Epstein-Barr virus (EBV) antibodies in the diagnosis of NPC comparison between IFA and two commercial ELISA kits. Singapore Med J 1998; 39: 263-5.
9. Zong YS, Sham JS, Ng NH, *et al.* Immunoglobulin A against viral capsid antigen of Epstein-Barr virus and indirect mirror examination of the nasopharyngeal carcinoma. Cancer 1992; 69: 3-7.
10. Mendoza J, Rojas A, de la Rosa M, Amador JM, Exposito J, Esquivias J. Evaluation of IgG and IgA type antibodies to capsid and early antigens of the Epstein-Barr virus in the diagnosis of nasopharyngeal carcinoma. Med Clin (Barc) 1991; 97: 609-11.
11. Ho HC, Ng MH, Kuan HC, Chau JC. Epstein-Barr-virus-specific IgA and IgG serum antibodies in nasopharyngeal carcinoma. Br J Cancer 1976; 34: 655-60.
12. Lynn TC, Tu SM, Kaeamura A. Long term follow-up of IgG and IgA antibodies against viral capsid antigens of Epstein-Barr virus in Nasopharyngeal Carcinoma. J Laryngol Otol 1985; 99: 567-72.
13. Puthavathana P, Kositanont U, Chongkolwatana C, *et al.* Prevalence of IgA specific antibodies to Epstein-Barr virus capsid and early antigens in nasopharyngeal carcinoma. Asian Pac J Allergy Immumol 1993; 11: 39-43.
14. Zeng Y, Zhang LG, Li HY, *et al.* Serological mass survey for early detection of nasopharyngeal carcinoma in Wuzhou city, China. Int J Cancer 1982; 29: 139-41.
15. Sam CK, Prasad U, Pathmanathan R. Serological markers in the diagnosis of histopathological types of nasopharyngeal carcinoma. Eur J Surg Oncol 1989; 11: 17-20.
16. Puthavathana P, Sutthent R, Vitavasiri A, Wasi C, Chantarakul N, Thongcharoen P. Epstein-Barr virus serological markers for nasopharyngeal carcinoma in Thailand. Southeast Asian J Trop Med Public Health 1991; 22: 326-31.
17. Chen JY, Chen CJ, Liu MY, *et al.* Antibody to Epstein-Barr virus-specific DNase as a marker for field survey of patients with nasopharyngeal carcinoma in Taiwan. J Med Virol 1989; 27: 269-73.
18. Liu MY, Chou WH, Nutter L, Hsu MM, Chen JY, Yang CS. Antibody against Epstein Barr virus DNA polymerase activity in sera of patients with nasopharyngeal carcinoma. J Med Virol 1989; 28: 101-5.
19. Gorgievski-Hrisoho M, Hinderer W, Nebel-Schickel H, *et al.* Serodiagnosis of infectious mononucleosis by using recombinant Epstein-Barr virus antigens and enzyme-linked immunosorbent assay technology. J Clin Microbiol 1990; 28: 2305-11.
20. Sheen TS, Ko JY, Chang YL, *et al.* Nasopharyngeal swab and PCR for the screening of nasopharyngeal carcinoma in the endemic area: a good supplement to the serologic screening. Head Neck 1998; 20: 732-8.
21. Luxamechanporn T, Neimhom S, Kunachak S, Kulapaditharom B. Polymerase chain reaction study of Epstein-Barr virus in high risk group of nasopharyngeal carcinoma: A preliminary report. J Med Assoc Thai 1999; 82: 593-7.
22. Chang YS, Tyan YS, Lui ST, Tsai MS, Pao CC. Detection of Epstein-Barr virus DNA sequences in nasopharyngeal carcinoma cells by enzymatic DNA amplification. J Clin Microbiol 1990: 28: 2398-402.
23. Lees JF, Goodeve AC, Arrand JE, Ghosh AK, Jones PH, Arrand JR. Detection of EBV DNA in post-nasal space biopsy tissue from asymptomatic EBV-seropositive individuals. J Med Virol 1992; 37: 30-8.
24. Hording U, Albeck H, Katholm M, Kristensen HS. Epstein-Barr virus in exfoliated cells from the postnasal space. Viral detection by polymerase chain reaction is not a useful means of screening for nasopharyngeal carcinoma in high-risk populations. APMIS 1994; 102: 367-70.
25. Diaz-Mitoma F, Ruiz A, Flowerdew G, *et al.* High levels of Epstein-Barr virus in the oropharynx: a predictor of disease progression in human immunodeficiency virus infection. J Med Virol 1990; 31: 69-75.
26. Feinmesser R, Miyazaki I, Cheung R, Freeman J, Nover AM, Dosch HM. Diagnosis of nasopharyngeal carcinoma by DNA amplification of tissue obtained by fine-needle aspiration. N Engl J Med 1992; 326: 17-21.
27. Telenti A. PCR detection and typing of Epstein-Barr Virus. In: Persing DH, ed. Diagnostic Molecular Microbiology. Berne, Switzerland 1994; pp. 344-9.
28. Saiki RK, Scharf S, Faloona F, *et al.* Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985; 230: 1350-4.
29. Niemhom S, Maeda S, Raksakait K, Petchclai B. Epstein-Barr virus DNA in nasopharyngeal carcinoma in Thai patients at Ramathibodi Hospital, Bangkok. Southeast Asian J Trop Med Public Health 1995; 26: 325-8.

30. Zeng Y. Seroepidemiological studies on nasopharyngeal carcinoma in China. Adv Cancer Res 1985; 44: 121-38.
31. Hadar T, Rahima M, Kahan E, *et al.* Significance of specific Epstein-Barr virus IgA and elevated IgG antibodies to viral capsid antigens in nasopharyngeal carcinoma patients. J Med Vior 1986; 20: 329-39.
32. Wara WM, Wara DW, Phillips TL, Ammann AJ. Elevated IgA in carcinoma of the nasopharynx. Cancer 1975; 35: 1313-5.
33. Sixbey JW, Nedrud JG, Rabb-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. N Engl J Med 1984; 310: 1225-30.
34. Lemon SM, Hutt LM, Shaw JE, Li JL, Paano JS. Replication of Epstein-Barr virus DNA in epithelial cells *in vivo*. IARC Sci Publ 1978; 24: 739-44.
35. Wara WM, Wara DW, Phillips TL, Ammann AJ. Elevated IgA in carcinoma of the nasopharyngeal carcinoma cells. Nature 1973; 244: 245-7.
36. Henle W, Henle G. Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int J Cancer 1976; 17: 1-7.