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Serum Responses to the Combination of Epstein-Barr Virus Antigens from both Latent and Acute Phases in Nasopharyngeal Carcinoma: Complementary Test of EBNA-1 with EA-D[1]

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Abstract

Elevated serum IgA to antigens of EBV is associated with nasopharyngeal carcinoma (NPC). We have tested 620 NPC sera by ELISA for the presence of antibodies to EBV-encoded DNA binding protein, EBV-specific DNA polymerase, early antigen-diffused (EA-D), EBV nuclear antigen 1 (EBNA-1), EBV-specific thymidine kinase, and *Bam*HI Z fragment EBV replication antigen. Sensitivity of these proteins was in the range of 51.5–79.5% for IgA and 69.4–82.8% for IgG. The complementary use of EBNA-1 with EA-D, however, could increase the sensitivity significantly to 98.1%. Western blot analysis further showed that the combination of EBNA-1 and EA-D is most useful for the detection of NPC.

This is the first report of using double biomarkers including EBV gene products from both latent and active infections. The results of this study suggest that EBV in NPC may not be latent alone and that the method may be valuable for the early detection, early treatment, and better survival rate of patients with NPC. Because the application of recombinant EBV protein in ELISA is cost-effective and feasible for mass screening, the method may be of worth for further clinical investigation.

Introduction

NPC[3] is a common tumor in Southeast Asia, including Southern China (1), Taiwan (2), Hong Kong (3), Singapore (4), and Malaysia (5), where a large population of Chinese are gathered. The annual incidence rate varies from $3/10^5$ to $60/10^5$. Radiotherapy is a curative treatment for early stage NPC. For a patient who is treated with radiotherapy at an early stage (I or II) of the disease, the 5-year survival rate can be more than 70%, whereas at a later stage (III or IV), the survival rate declines significantly to 41 and 17%, respectively (6). Unfortunately, most NPC patients present with advanced disease. Therefore, a method for detecting NPC at an early stage is important to initiate early treatment and to increase the survival rate.

EBV DNA (7–9) and EBV gene products (10–12) have been frequently detected in biopsies from patients with NPC. Serum IgA (5, 9) and IgG (5) to EBV antigens in those patients were also high. The application of viral antigens, such as alkaline DNase (13), DBP (14), EA-D (15), EBNA-1 (16), EDP (17), ribonucleotide reductase (18), membrane antigens (19), TK (20), VCA (21), and ZEBRA (22), in indirect immunofluorescence and enzyme neutralization assays for the early detection and follow-up of NPC patients has been reported. However, both of these methods are expensive and difficult to apply for mass screening. The sensitivity and specificity of these individual proteins are also only in the range of 60–83% (13–22). A new method with higher sensitivity and specificity for accurate detection of NPC is urgently required.

Because of the simple handling and the automatic readout (17, 23), ELISA using recombinant EBV antigen may be the best choice for mass screening. Several systems have been used to express various EBV proteins. Nevertheless, the application capacity of each prospective antigen for mass screening is limited (12–22). The reason could be due in part to the difficulties of producing consistent EBV antigens from EBV-infected lymphoblastoid cells for the conventional test (23) and to the confounding fact that the virus in cancer cells may not be latent alone.

To examine the hypothesis that EBV in NPC cells may be a mixture of latent and active infection, we have cloned six EBV-encoded proteins that were expressed at different phases of virus infection into an *Escherichia coli* recombinant system. We further mixed the purified EBV antigens in the ELISA as well as the Western blot to find the best combination of EBV antigens for early detection of NPC. We describe here the combined use of EBV antigens ENBA-1 and EA-D in Western blot and ELISA to evaluate the serum IgA in patients with NPC and to investigate the primary evidence that EBV infection in NPC may be a mixture of latent and active infection.

Materials and Methods

Human Sera. From June 1980 to September 1995, sera from 620 patients with newly diagnosed NPC were collected, recorded, divided into small aliquots, and properly stored in a

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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; DBP, DNA binding protein; EA-D, early antigen-diffused; EBNA-1, EBV nuclear antigen 1; EDP, EBV-specific DNA polymerase; TK, thymidine kinase; VCA, virus capsid antigen; ZEBRA, Z fragment EBV replication antigen; CMV, cytomegalovirus; HSV-1, herpes simplex virus-1.

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Table 1 Primer sequences for PCR to EBV antigens

Primer sequences for EA-D
5'-ATGGAAACCACTCAGACTCT-3'
5'-GCTGCTTGCACGAGAACATG-3'
Primer sequences for EBNA-1
5'-GATACATATGTCCGGTGGCCGCGGTCGAGGTGGTTCCGGTGGTCGCGGTCG-3'
5'-CTAGGATCCTTACTCCTGCCCTTCCTCACCCTCATCTCCATCACC-3'
Primer sequences for EDP
5'-ATGCAGATCTCTAAGGAGGAATAACCATATGTCTGGCGGTCTGTTCTATAACCC-3'
5'-CTACTCTAGAGGGCACCCTCACTTTGGGTCTTAGAATGGTGGCCGGGCTGT-3'
Primer sequences for EBV-specific DBP
5'-ATGTACGGATCCTACTACCTGCAGTTCTGCCAGGG-3'
5'-CTACGGATCCGGGTCTCTAGACCTCGAGTCC-3'
Primer sequences for ZEBRA
5'-ATGCAGATCTCTAAGGAGGAATAACATATGATGGACCCGAACTCTACTTCATGAAG-3'
5'-CTACTCTAGAGGATCCAATCACGGGAGTTAGAAATTTAAGAG-3'

−70°C freezer. All patients had pathologically confirmed NPC. Stage of disease progression was classified according to the Unio International Contre Cancer system.

Sera from 340 healthy donors with equivalent distributions of age and sex were collected as normal controls. The sensitivity and specificity of the assay were calculated from the result of NPC sera and that of normal controls. Sera from 122 patients with high titers IgG to CMV or to HSV-1 were used to test cross-reactivity. Titers of antiserum to CMV or HSV-1 were determined by an ELISA kit sensitized with the respective antigen (Merck, Darmstadt, Germany).

Plasmid Construction and EBV Antigen Purification. Construction of the plasmid expressing recombinant EBV antigen in *E. coli* was described previously (17, 20). Briefly, a cDNA (from P3HR1 cells) or an EBV genomic library (from B95-8) was subjected to PCR by a unique pair of primers (primer sequences are listed in Table 1). The amplified DNA fragment was cloned into *E. coli* expression vector pET3C (Novagen, Madison, WI) or into expression vector pET3CP. Recombinant protein was confirmed by molecular weight determination and partial amino acid sequencing (17) or by the measurement of increased enzyme activity (20). The molecular weight of the recombinant EBV proteins as determined by gel electrophoresis is 110,000 for EDP, 76,000 for DBP, 73,000 for TK, 46,000 for EA-D, 37,000 for EBNA-1, and 27,000 for ZEBRA (as shown in Fig. 1).

Recombinant EBV antigen was purified stepwise from the *E. coli* cell lysate by ion exchange, gel filtration, and SDS-PAGE. The purity of the recombinant protein was determined by gel electrophoresis and gel scanning densitometer GS300 (Hoeffer Scientific Instruments, San Francisco, CA).

ELISA. The ELISA plate sensitization and ELISA protocol have been described previously (20). Briefly, a 96-well microtiter plate was coated with 50 ng of the purified antigen in TBS [20 mM Tris (pH 7.5) and 150 mM NaCl]. A set of plates was sensitized with two or three different EBV antigens. Before proceeds for ELISA, sera were diluted 1:200 into ELISA buffer (1% BSA, 0.1% Tween 20, and Tris-buffered saline), and the reaction was incubated at 37°C for 60 min. Plates were washed with distilled water 10 times before the addition of peroxidase-conjugated goat anti-human antisera (1:12,000 dilution). The reaction was incubated at 37°C for 30 min before washing. Positive reaction was identified by developing with 3,3',5,5'-tetramethylbenzidine in 50 mM sodium acetate (pH 6.5) at room temperature for 15 min and by reading at $A_{450\ nm}$. The individual serum sample was done in triplicate. Some serum samples were aliquoted and stored for the further repeated measurement to test the stability of the ELISA. Data were recorded, tabulated, and compared to those of the Western blot. A single-blind procedure was followed to carry out both ELISA and Western blot protocols.

Western Blot Analysis. Six purified recombinant proteins, EA-D, EBNA-1, EDP, DBP, TK, and ZEBRA, were mixed and subjected to SDS-PAGE. Proteins on the gel were electrotransferred to a nitrocellulose membrane. The corresponding lane containing all six proteins on the membrane was sectioned into thin strips for immunostaining with sera from NPC patients. Positive reaction was identified by alkaline phosphatase-conjugated goat anti-human IgA (DAKO, Kyoto, Japan), and chromogen nitro blue tetrazolium with 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO). Positive reaction of each strip was recorded, tabulated, and compared to that of ELISA.

DNA Extraction and Signal Amplification. High molecular weight cellular DNA was extracted from Ficoll-Hypaque (Sigma) isolated WBCs. A total volume of 50 μl containing 0.5 unit of Taq DNA polymerase (Perkin Elmer, Norwalk, CT), reaction buffer, 20 pmol of primers, 100 ng of DNA, and appropriate amount of deoxynucleotide triphosphates was subjected to 40 cycles of PCR, and the amplified product was resolved in a 2% agarose-ethidium bromide gel. The specificity of the amplified product was confirmed by Southern hybridization using sequences internal to those bracketed by the primer sequences listed below (24). Primer sequences for the *Bam*HI L region were 5'-GGCTGGTGTCACCTGTGTTA-3' and 5'-CCTTAGGAGGAACAAGTCCC-3'. Primer sequences for the *Bam*HI W region were 5'-GCCAGAGGTAAGTGGACTTT-3' and 5'-TGGAGAGGTCAGGTTACTTA-3'.

Table 2 Age and sex distribution of patients with NPC, $n = 620$

Age (yrs)	Male	Female	Subtotal
0–20	4 (0.09%)	2 (1.15%)	6 (0.97%)
21–30	14 (3.14%)	28 (16.09%)	42 (6.77%)
31–40	69 (15.47%)	46 (26.43%)	115 (18.55%)
41–50	124 (27.8%)	57 (32.76%)	181 (29.19%)
51–60	124 (27.8%)	25 (14.37%)	149 (24.03%)
≥60	111 (24.89%)	16 (9.20%)	127 (20.48%)
Total	446	174	620

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Table 3 Seropositive rate of immunoglobulin subtypes of sera from NPC patients to EBV antigen in ELISA, n = 297

EBV antigen	IgA		IgG		IgM	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
DBP	76.8	89.5	82.8	91.4	37.4	95.2
EA-D	73.1	96.2	78.4	94.3	44.8	97.1
EDP	51.5	96.2	69.4	84.8	18.5	91.4
EBNA-1	79.5	93.3	78.8	96.2	11.8	96.2
TK	71.7	78.1	82.1	82.8	32.3	89.5
ZEBRA	75.4	80.9	79.1	86.6	36.0	92.4

Table 4 Immunoreactivity of serum IgA from patients with NPC to EBV antigens in ELISA, n = 32

Patient no.	EBV antigens					
	DBP	EDP	EA-D	EBNA-1	TK	ZEBRA
1	–[a]	–	–	–	+	++
2	+	+	+	++	+	–
3	+++	+	+++	+	++	++
4	–	–	+	+	–	–
5	+	–	++	+	+	+
6	+	+	+++	+	+	+
7	+	–	+	+++	–	++
8	–	+	–	+	–	–
9	–	–	+	+	+	–
10	–	–	–	+	–	–
11	+	–	++	+++	+	+++
12	++	–	++	+	+++	+
13	–	–	–	–	–	–
14	+	–	–	+	+	+
15	–	–	+++	+++	–	++
16	+	+	++	–	+++	+++
17	+	–	++	–	+	–
18	+++	–	++	+	+++	+
19	+	–	++	+++	+	+++
20	+++	+	+++	++	+++	++
21	–	–	–	+	–	–
22	–	–	+	–	–	–
23	–	–	–	+	+	–
24	+	–	–	++	+++	–
25	+++	–	–	+	+	++
26	–	–	+	+++	–	+
27	+++	++	+++	+	+++	+++
28	++	–	++	++	++	+
29	–	–	–	+	+	–
30	++	–	+	++	++	+++
31	+	+	++	–	+	–
32	++	+	+	+	+++	+
% positive	62.5	28.1	68.8	81.3	75	59.4

[a] Signal strength of immunoreactivity. –, negative; +, weak; ++, medium; +++, strong.

Results

Characteristics of Patients. The average age of male patients (n = 446) is 50.3 ± 12.5 years and that of female patients is 42.1 ± 13.2 years (n = 174). Respective distributions of age and gender are listed in Table 2. Of 620 NPC patients tested, 25 were at stage I (4%), 69 were at stage II (11%), 143 were at stage III (23%), and 383 were at stage IV (62%).

Immunoreactivity of NPC Sera to EBV Antigens Determined by ELISA. For the pilot study, 297 serum samples randomly selected from 620 NPC patients were used to test immunoreactivity to various recombinant EBV antigens. Results are shown in Tables 3 and 4. To EBV antigens, IgG is more sensitive than IgA, and IgA is more sensitive than IgM in NPC sera. A moderate to high titer of IgA antibody to DBP was present in 228 of 297 NPC serum samples as well as in 11 of 105 normal control sera (Table 3). The sensitivity and specificity of IgA/DBP for NPC were 76.8 and 89.5%, respectively. To DBP, IgG antibodies were present in 246 of 297 NPC sera and in only 9 of 105 normal control sera. The sensitivity and specificity of IgG/DBP for NPC were 82.8 and 91.4%. For IgM, sensitivity was 37.4% and specificity was 95.2%. The sensitivity and specificity for the other recombinant EBV proteins were calculated as were those of DBP (Table 3). However, for determining the presence of antibodies to EBV in NPC patients, some antigens are more sensitive than others. The sensitivity of each EBV protein varied.

On the other hand, when EBV antigens were complemented with each other, the detection rate increased significantly. For example (as shown in Table 4), when ENBA-1 was

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Table 5 Complementation of seropositive rate in immunoreactivity of NPC serum IgA to double EBV antigens in ELISA, n = 210[a]

EBV antigens	DBP	EDP	EA-D	EBNA-1	TK	ZEBRA
DBP	81.9	81.9	81.9	90.5	89.5	81.9
EDP	81.9	57.1	82.4	91.0	89.5	79.5
EA-D	81.9	82.4	78.6	97.6	89.5	91.0
EBNA-1	90.5	91.0	97.6	89.5	97.1	93.3
TK	89.5	89.5	89.5	97.1	89.5	89.5
ZEBRA	81.9	79.5	91.0	93.3	89.5	75.2

[a] The cut-off value was 0.200.

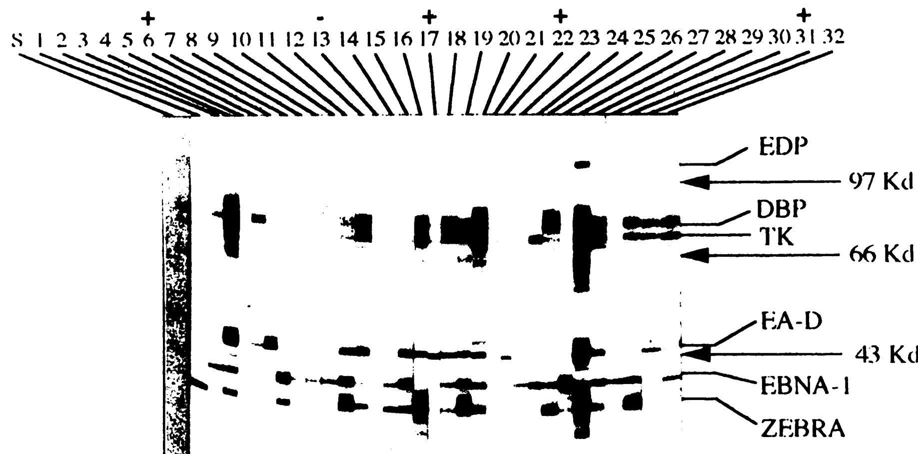

Fig. 1. Western blot analysis of recombinant EBV antigens to serum IgA from patients with NPC. Each nitrocellulose membrane strip contained six purified recombinant EBV proteins: DBP, 0.25 μg; EA-D, 0.25 μg; EBNA-1, 0.1 μg; EDP, 0.25 μg; TK, 0.25 μg; and ZEBRA, 0.25 μg. The position of the six EBV proteins in the gel is shown in *Lane S*, which was stained with 0.25% Coomasie Blue (Life Technologies, Inc., Bethesda, MD). The respective position of each EBV protein is labeled on the *right*. *Arrow*, position of the protein molecular weight standard. +, serum that is negative for EBNA-1 but positive for EA-D. −, serum that is negative for all six EBV antigens.

Western Blot Analysis of Recombinant EBV Antigens to Sera from Patients with NPC

used as the major test, six cases were negative. When the EBNA-1-negative case (Table 4, column 5) was aligned with positive immunoreactivity to EA-D (Table 4, column 4), the negative case became positive. In this case, the immunoreactivity of EBNA-1 was complemented with that of EA-D. The number of negative cases thus decreased to two (sensitivity increased from 81.3 to 93.8%). Sensitivity complementation, however, did not significantly affect the specificity. The result of sensitivity complementation of NPC serum IgA to the other EBV antigens is listed in Table 5.

Immunoreactivity of NPC Serum IgA to EBV Antigens Determined by Western Blot. To confirm the ELISA results, we applied six EBV antigens for Western blot analysis to determine the immunoreactivity of serum IgA from the same NPC patients. As shown in Table 4, sera from these NPC patients also revealed different patterns of immunoreactivity to the recombinant EBV antigens (Fig. 1). Serum IgA to EBNA-1 was present in 154 of 210 samples.

Interestingly, serum IgA to EBNA-1 was also complementary with the other antigens that were expressed during the active replication cycle of EBV. Among 210 cases tested, 31 cases (14.8%) expressed antibodies to all 6 antigens (as shown in *Lanes 3, 20,* and *27* in Fig. 1). The result of sensitivity complementation in the Western blot is shown in Table 6. Although the sensitivity of the respective antigen varies in either ELISA or Western blot (Tables 5 and 6), fortuitously, results from both ELISA and Western blot indicate that the best combination of antigen complementation is EBNA-1 and EA-D. Sensitivity was 98.1% and specificity was 93.3%. The combination of EBNA-1 and EA-D was thus chosen for the next-step test in ELISA, and the sensitivity as well as specificity was consistent (Table 7) in three groups of NPC sera. From repeated measurements, the reading of serum IgA from the same patient was also agreeable (Table 8).

The average $A_{450\ nm}$ value of the ELISA reading was 0.97 ± 0.37 for patients at stage I, 1.45 ± 0.62 for patients at

Table 6 Complementation of seropositive rate in immunoreactivity of NPC serum IgA to double EBV antigens in a Western blot, n = 210

EBV antigens	DBP	EDP	EA-D	EBNA-1	TK	ZEBRA
DBP	63.3	65.7	78.1	90.9	87.6	78.1
EDP	65.7	20.9	79.0	90.9	87.6	73.3
EA-D	78.1	79.0	76.7	98.1	91.4	91.9
EBNA-1	90.9	90.9	98.1	90.9	97.1	95.7
TK	87.6	87.6	91.4	97.1	87.6	90.0
ZEBRA	78.1	73.3	91.9	95.7	90.0	72.4

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Table 7 Seropositive rate in immunoreactivity for different groups of NPC serum samples to EBNA-1 and EA-D in ELISA

Immunoreactivity in groups B (summary of Table 4), C, and D was determined by ELISA. Group C was a next-step test in ELISA by using the total number of 620 NPC sera and the same control group as in group B. Group D was a repeat measurement of group C by using 581 of 620 NPC sera and expanded the number of normal control sera from 180 to 340. The routine cut-off value used in group B was 0.220. The cut-off value used in group C and group D was 0.200.

Group	Sensitivity	Specificity
A[a]	98.1% (n = 210)	93.3% (n = 105)
B	98.1% (n = 297)	93.9% (n = 180)
C	96.8% (n = 620)	93.9% (n = 180)
D	97.6% (n = 581)	97.3% (n = 340)

[a] Immunoreactivity in group A was determined by Western blot (summary of Table 5).

stage II, 1.53 ± 0.83 for patients at stage III, and 1.93 ± 0.78 for patients at stage VI. No statistical significance of sensitivity variation was observed when the data were stratified by means of disease stage, gender, or age (data not shown).

Among 105 normal control sera tested for Western blot, 7 were positive for all 6 EBV antigens. DNA isolated from Ficoll-Hypaque-enriched WBCs was analyzed by PCR for the presence of the EBV signal. A 239-bp (*Bam*HI L region) or a 240-bp (*Bam*HI W region) PCR product (24) was detected in 5 samples (data not shown). Because of the presence of EBV DNA in the blood sample, they were categorized as seropositive carriers. The other two that were seropositive by Western blot, but negative by PCR, were categorized as seropositive normal. Among 122 patients with high tier IgG to CMV or to HSV-1, 10 were positive for combined EBNA-1 and EA-D. Ficoll-Hypaque-enriched WBC DNA isolated from these patients was also analyzed by PCR. The presence of EBV was detected in these 10 samples (data not shown).

Discussion

Although NPC has been associated with latent infection of EBV, serological studies of NPC patients have provoked questions about the persistent high titer of antibodies to the early and late gene products of EBV (25–30). High titer of IgA to a specific antigen implicates the continuous stimulation of the host immune system with that specific antigen from certain mucosal origin (1, 26). The unusual immunological response of NPC patients to EBV could be the result of aberrant viral gene expression (10–12, 26–30) or of the reactivation of virus infection (5) or a mixture of both. Using a single EBV antigen that was expressed during a certain phase of viral infection to study the disease may have its intrinsic limitations.

Based on this concept, we cloned several EBV-encoded antigens that were expressed in different phases of virus infection, *e.g.*, EBNA-1 in the latent phase and DBP, EA-D, EDP, TK, and ZEBRA in the early phase of active replication, to examine a concurrent presentation of serum antibodies to these antigens. All six recombinant proteins are useful for diagnosing NPC. However, sensitivity and specificity varied among different proteins (as shown in Tables 3 and 4 as well as in Fig. 1). The difference may not be due to the different antigenicity of recombinant proteins, because they are all nuclear proteins, and, furthermore, by protein sequence analysis, they also contain hydrophilic stretches that could provide reasonable antigenicity for the immune response.

The difference may be due to the duration and the quantity of proteins presented to the patient's immune system during disease progression. Serum IgA specific to EBNA-1 and EA-D appears early in stage I of NPC, and the titer of IgA increases

Table 8 Immunoreactivity of repeated measurement of NPC serum IgA to EBNA-1 and EA-D by ELISA at different times, n = 24

Immunoreactivity of ELISA was determined by routine procedure. The number shown is the $A_{450\ nm}$.

Patient no.	Routine	QC11[a]	QC12	QC21	QC22	Reading
1	0.217	0.216	0.173	0.129	0.194	−
2	0.826	0.847	0.646	0.541	0.720	+
3	0.178	0.157	0.152	0.133	0.147	−
4	1.570	1.222	1.449	1.360	1.165	+
5	0.702	0.995	1.257	0.982	0.860	+
6	0.890	0.877	0.926	0.787	0.838	+
7	1.196	1.460	1.583	1.438	1.243	+
8	1.010	1.524	1.517	1.277	1.278	+
9	1.183	0.989	1.407	1.248	0.996	+
10	0.598	0.322	0.324	0.300	0.402	+
11	0.758	0.521	0.516	0.424	0.405	+
12	0.545	0.741	0.817	0.672	0.658	+
13	1.138	1.251	1.371	1.285	1.140	+
14	0.857	0.858	1.089	0.960	0.979	+
15	0.005	0.117	0.079	0.077	0.114	−
16	1.120	0.918	1.097	1.030	1.048	+
17	0.632	0.754	0.711	0.549	0.721	+
18	0.724	0.583	0.645	0.295	0.564	+
19	0.408	0.461	0.472	0.292	0.465	+
20	0.011	0.077	0.072	0.060	0.081	−
21	0.202	0.272	0.269	0.225	0.239	−
22	0.829	0.986	1.056	0.840	0.849	+
23	0.935	0.962	0.863	0.846	0.884	+
24	0.845	0.760	0.781	0.616	0.605	+

[a] Immunoreactivity of batch 1 ELISA plate was determined by quality controller 1 (QC11) or quality controller 2 (QC12), and that of batch 2 ELISA plate prepared 3 months apart was determined by quality controller 1 (QC21) and quality controller 2 (QC22).

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after progression of the disease (data not shown). However, no statistical significance of sensitivity variation was observed when the data were stratified by means of disease stage, gender, or age (data not shown). Combined recombinant EBNA-1 and EA-D could therefore provide a better option than a single antigen of EBV in detecting NPC at an early stage. The efficacy for mass screening is being evaluated in an ongoing study. Sensitivity complementation of EBNA-1 with that of the lytic cycle related EBV antigens could then reflect, at least in part, the difference of immunological reaction to the active processes of the virus in each patient (31). In NPC sera, the presence of antibodies to EBV gene products at various phases of infection could therefore suggest that viral gene expression in patients is a mixture of latent and activated infections. The result is consistent with the fact that EBNA-1 is essential for maintaining the episomal EBV genome (25, 30), whereas virus reactivation could be initiated by a yet-to-be determined mechanism (32).

A cross-reactivity study showed that protein sequence homology between EA-D of EBV and UL42 of HSV-1 (15) did not affect the antigenic specificity of NPC sera. Sequence homology among DNA polymerases of EBV, CMV, and HSV-1 (17) or that between TK of EBV and HSV-1 (33) did not affect immunological reactivity. Among 122 patients who had IgG titer to CMV or to HSV-1, 10 tested positive for EBNA-1 and EA-D. Antibody titer in these seropositive carriers was generally low when compared to that in patients with NPC. Differential ELISA, by using EBNA-1 and EA-D separately, showed the same result. PCR analysis of WBC fractions indicated that these patients may have dual viral infection.

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