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Evaluation of Antibodies against Different Epstein-Barr Virus Nuclear Antigen 1 Peptides in Diagnosis of Nasopharyngeal Carcinoma▽

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Epstein-Barr virus nuclear antigen 1 (EBNA1) is a protein expressed consistently in nasopharyngeal carcinoma (NPC). Although antibody levels against three different EBNA1 peptides were all high in NPC patients, the correlation between any two biomarkers was low. Therefore, the selection of distinct EBNA1 peptides could render different results in serological detection for individuals with NPC.

Epstein-Barr virus (EBV) infection is closely associated with several malignant diseases, including nasopharyngeal carcinoma (NPC). NPC is highly prevalent in southern China, and NPC patients usually have higher EBV antibody levels than healthy controls. Examination of the serum immunoglobulin A (IgA) antibodies against EBV capsid antigen, diffuse early antigens (EA-D), and EBV nuclear antigen 1 (EBNA1) are widely used for NPC diagnosis and prognosis (3).

EBNA1 plays a role in the maintenance of latent EBV infection and is expressed in all EBV-associated malignant tissues (7), resulting in the hypothesis that EBNA1 is critical for initiating and developing these tumors (6). Encoded by the BamHI K fragment of the EBV genome, EBNA1 contains a Gly-Ala repeat domain flanked by unique regions (1). The repeat region, C terminus, and N terminus are antigenic (2, 8). Thus, peptides with these motifs may be useful for EBNA1 serology.

To evaluate the commercial EBNA1 proteins for EBV serological examination, two EBNA1 peptides, a recombinant full-length peptide (rEBNA1) (Biodesign, Saco, ME) and a recombinant fusion fragment containing amino acids 1 to 90 and 408 to 498 (fEBNA1) (ProSpec Co., Rehovot, Israel), were chosen to compare antibody responses in NPC patients and healthy controls. Furthermore, to test if a synthesized EBNA1 peptide could substitute for the recombinant EBNA1 proteins in the serological examination, we analyzed the immunodominant epitopes of EBNA1 as described before (4). Briefly, the protein sequences were examined according to the reported EBV proteomes by using DNAStar software, and a sequence with a high possibility of hydrophilicity, surface orientation, and flexibility was chosen. Finally, we selected amino acids 61 to 78 in the BamHI K fragment to be chemically synthesized (sEBNA1, GSGPRHRDGVRRPQKRPS) by adding a biotinylated linker to the N terminus (Hanyu, Shenzhen, China).

Ninety-five patients with newly diagnosed and pathologically confirmed NPC were recruited from Sun Yat-sen University Cancer Center. The stage of disease progression was classified according to the 1996 Union International Cancer Control classification. The NPC case group, including 4 patients with stage I, 10 with stage II, 58 with stage III, and 23 with stage IV cancer, had 72 males and 23 females with an age range of 17 to 68 (mean ± standard deviation, 45.6 ± 10.9) years. Eighty-eight healthy volunteers were also recruited as healthy controls, including 78 males and 10 females with an age range of 25 to 71 (mean, 46.6 ± 13.1) years. Written informed consent was obtained from all participants.

Coupling of rEBNA1 and fEBNA1 to the carboxylated beads (Luminex Corp., Austin, TX) was performed according to our protocols as described previously (5). sEBNA1 was coupled to LumAvidin microspheres (Luminex Corp., Austin, TX) according to the manufacturer's instructions. Serum samples diluted to 1:21 in storage buffer (20 μl/well) were added to the 96-well filtration system (Millipore, Billerica, MA) and incubated with the conjugated beads for 30 min at room temperature in the dark. After three washes, 150 μl of R-phycoerythrin-conjugated goat anti-human IgA or IgG (1:200 in phosphate-buffered saline; SouthernBiotech, Birmingham, AL) was added to each reaction well and incubated for 30 min. The detection analysis was performed by using the Luminex multianalytic 100 system (Bio-Rad, Hercules, CA). All tests were carried out in duplicate.

As shown in Table 1, the IgA values against the three peptides were significantly higher for samples from the NPC patients than from the healthy controls ($P < 0.0001$). The areas under the concentration-time curve for IgA xMAP assays were all above 0.8, and the sensitivities and specificities ranged from 80 to 88% for NPC diagnosis according to the optimal cutoff values. The IgG levels against the fusion fragment or synthesized peptide were higher in samples from the NPC patients. However, the IgG levels against full-length EBNA1 were higher in samples from the healthy controls. These results might be due to the nonspecific response to the Gly-Ala repeat region presented in the full-length peptide.

The correlation between any two biomarkers was low, as the correlation coefficient (r) ranged from 0.314 to 0.456. Moreover, the levels of IgG-rEBNA1 had no correlation with the levels of IgG-sEBNA1 ($r = -0.066$, $P = 0.537$) or IgG-fEBNA1

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TABLE 1. Analysis of antibodies against different EBNA1 peptides in samples from NPC patients and healthy controls

Anti-EBNA1 peptide	No. of serum samples from:		FI[a] value (mean ± SEM) for:		Receiver operating characteristic analysis result for:			
	NPC patients	Healthy controls	NPC patients	Healthy controls	AUC[b] (95% CI)	Cutoff	% Sensitivity (95% CI)	% Specificity (95% CI)
IgA-rEBNA1	95	88	2,804.7 ± 296.4	441.8 ± 50.0	0.890 (0.825–0.955)	7,000	81.8 (72.2–89.2)	80.9 (71.6–88.2)
IgA-fEBNA1	95	88	9,806.2 ± 873.0	5,030.7 ± 892.3	0.914 (0.858–0.971)	400	88.6 (80.1–94.4)	80.9 (71.6–88.2)
IgA-sEBNA1	47	44	2,676.7 ± 394.6	802.1 ± 164.9	0.842 (0.758–0.926)	800	81.8 (67.3–91.8)	83.7 (74.2–90.8)
IgG-rEBNA1	95	88	17,700.0 ± 496.4	20,300.0 ± 543.0	0.302 (0.192–0.412)			
IgG-fEBNA1	95	88	7,070.5 ± 520.7	4,806.3 ± 498.8	0.688 (0.577–0.799)	4,000	63.6 (52.7–73.6)	68.1 (58.4–77.8)
IgG-sEBNA1	47	44	11,202.5 ± 502.5	4,528.4 ± 409.1	0.745 (0.641–0.849)	4,000	65.9 (50.1–79.5)	68.1 (52.9–80.9)

[a] FI, fluorescence intensity.
[b] AUC, area under the concentration-time curve.

($r = 0.072$, $P = 0.333$), indicating that the serum samples recognized the EBNA1 peptides variously. This may be due to various individual immune responses to EBNA1 after EBV infection. Alternatively, the peptides might have different conformations, consequently altering the immunogenic regions and resulting in distinct affinities with the same serum.

Therefore, the selection of distinct EBNA1 peptides could render different results in serological detection for individuals with NPC, and it might be more efficient for NPC screening and diagnosis in regions where the disease is endemic if any combination of these peptides is analyzed. Indeed, when IgA-rEBNA1 and -fEBNA1 were combined, only 7 of 95 NPC patients had IgA levels below both cutoff values, and the sensitivity of NPC diagnosis increased to 92.6% (95% confidence interval [CI], 85.4 to 97.0%), providing a better strategy for NPC screening and diagnosis. As for other combinations, the sensitivities of IgA-rEBNA1 and -sEBNA1, IgA-fEBNA1 and -sEBNA1, and IgG-fEBNA1 and -sEBNA1 reached 93.6% (95% CI, 82.5 to 98.7%), 97.9% (95% CI 88.7 to 99.9%), and 80.9% (66.7 to 90.9%), respectively, with better discriminatory values than the individual peptides. Since the EBV EBNA1 serology examination was performed with members of the Cantonese population, which has the highest NPC incidence, the diagnostic values of these methods await further confirmation in regions where NPC is not endemic.

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