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SHORT COMMUNICATION

SEROLOGICAL FOLLOW-UP STUDY ON THE ANTIBODY LEVELS TO EPSTEIN-BARR VIRUS-DETERMINED NUCLEAR ANTIGEN (EBNA) PATIENTS WITH NASOPHARYNGEAL CARCINOMA (NPC) AFTER RADIATION THERAPY

MISUZU SHIMAKAGE and NOBUKO IKEGAMI

Clinical Research Institute, Osaka National Hospital, Higashi-ku, Osaka 540, Japan

MASASHI CHATANI[1], KUNITOSHI YOSHINO[2] and TAKEO SATO[2]

[1] Department of Radiation Therapy, The Center for Adult Diseases, Osaka, Higashinari-ku, Osaka 537, Japan

[2] Department of Otorhinolaryngology, The Center for Adult Diseases, Osaka, Higashinari-ku, Osaka 537, Japan

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Serological follow-up studies for up to 4 years on the levels of IgG antibody to EBV-determined nuclear antigen (EBNA) were carried out on 36 patients with nasopharyngeal carcinoma (NPC). The serum levels of IgA antibody specific to EBV capsid antigen (VCA) were also measured in some of the patients. The titers of EBNA antibody were measured by enzyme-linked immunosorbent assay (ELISA) and those of IgA antibody to VCA were measured by the indirect immunofluorescence method. The EBNA antibody titers in most sera from the patients before radiation therapy were found to be at least 4 times the mean values in the sera of healthy control adults. Within 2 to 8 months after completion of therapy by 4-MV liniac X-ray irradiation with total doses of 60 to 80 Gy, the titers of EBNA antibody in the sera of 6 patients had returned to normal levels, and low levels of EBNA antibody were maintained for a long time after therapy. These serological data were associated with a good clinical prognosis without recurrence or metastases. But in 6 patients, the patterns of change in the EBNA antibody levels were different: the levels remained high after therapy or first decreased to the normal level and then rose to at least 4 times this level. These 6 patients showed recurrence or metastases. The patterns of change in the EBNA antibody levels were well correlated with those of change in the levels of IgA antibody specific to VCA. These changes in the levels of EBV specific antibody could be detected long before clinical symptoms of recurrence or metastasis, suggesting the usefulness

of serological follow-up studies on EBV-specific antibody, for early prediction of the prognosis of patients with NPC after radiation therapy.

INTRODUCTION

An association of Epstein-Barr virus (EBV) with nasopharyngeal carcinoma (NPC) was first suggested by Old et al. (1966), and subsequently demonstrated by Henle et al. (1977), and Zeng et al. (1979, 1982) who carried out seroepidemiological studies on EBV-specific antibodies in sera from NPC patients and normal individuals in China. Furthermore, the detection of EBV-specific DNA in NPC tissues (Desgranges et al., 1982) and of reactivation of latent EBV in the nasopharynx at the onset of NPC (de Thé, 1982) strongly support the association of EBV with NPC. EBV-determined nuclear antigen (EBNA) is an early virus-specific antigen appearing in cells infected with EBV. Serum antibody to EBNA was found at higher levels in patients with NPC and Burkitt's lymphoma than in those with other cancers (Vonka and Hirsch, 1982). EBNA is thought to be important in carcinogenesis by EBV (Klein and Klein, 1984). Serum antibody to EBNA can be detected by indirect anti-complement immunofluorescence (ACIF) using acetone-fixed EBNA-positive Raji cells as antigen.

Recently, we developed an enzyme-linked immunosorbent assay (ELISA) for detection of EBNA antibody using a nuclear extract of Raji cells as soluble antigen, and reported that this method was more useful than ACIF (Shimakage et al. 1986). This communication reports the use of this ELISA method in serological follow-up studies on NPC patients.

MATERIALS AND METHODS

1. *Subjects*

Thirty-six patients with NPC who visited the Center for Adult Diseases, Osaka from 1983 to 1987 were studied. All patients were diagnosed as having undifferentiated or poorly differentiated squamous cell carcinoma of the nasopharynx by histological examination of biopsy tissue. The patients were at stage III or IV, according to the definition of stages of NPC. All patients had received 4-MV liniac X-ray irradiation with total doses of 60 to 80 Gy. Their ages at their first visit to the Center ranged from 17 to 77 (mean age, 52.7 years). The sex ratio of men to women was 26: 10. Sera were collected from the patients before and at various times after the start of radiation therapy and were stored at −40 C.

2. *Cell Culture*

Raji, an EBV-positive African Burkitt lymphoma cell line, and BJAB, an EBV-negative lymphoma cell line, were grown in RPMI 1640 medium supplemented with 8% fetal calf serum.

3. *ELISA*

For preparation of soluble antigen, nuclei were prepared from Raji cells by fractionation of nuclei in hypotonic buffer [10 m*M* Tris-HCl (pH 7.0), 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* PMSF (phenylmethylsulfonylfluoride)] and centrifugation. The nuclei were solubilized in disruption buffer [20 m*M* Tris-HCl (pH 8.0), 1 m*M* 2-mercaptoethanol, 0.5 m*M* PMSF, 1 m*M* EDTA, 0.4 *M* NaCl] and centrifuged for 15 min at $10{,}000 \times g$. The supernatant was collected and used as antigen. Each well of a 96-well microplate was coated with antigen (5 μg protein) and was blocked by 1% bovine serum albumin for 3 h at 37 C. The test serum was diluted serially 2-fold, and 25 μl of each dilution was added to wells. After incubation for 8 h at 4 C, unbound antibodies were washed off, and peroxidase-conjugated goat anti-human IgG (γ-chain specific, Tago Inc.) was added to each well. After incubation for 90 min at room temperature, the wells were washed, and 0.4% o-phenylenediamine and 0.01% H_2O_2 were added to each well as substrate for peroxidase. The reaction was stopped by addition of 4 *N* H_2SO_4, and the absorbance was

TABLE 1. *Geometric mean titers of antibody to EBNA in sera from NPC patients and healthy adults determined by ELISA*

	Group	Number of cases	Geometric mean titers of EBNA antibody by ELISA		Mean age	Sex ratio M : F
			Pretherapy	Posttherapy		
NPC patients	A	8	14473	3891	53.2 (17–74)[a]	8 : 0
	B	11	14860	10894[b]	51.8 (30–77)	8 : 3
	C	11	13133	NT[c]	53.5 (39–75)	6 : 5
		6	NT[c]	9082	52.5 (46–63)	4 : 2
	Total	36	14080[d]	6751[d]	52.7[d] (17–77)	26 : 10
Healthy adults		23	3064[d]		(20–50)	

[a] ( ): range of age.
[b] At the time of recurrence or metastasis.
[c] Sera were not available for tests.
[d] Mean values for all patients.

measured at 495 nm. For determination of positive ELISA, a 0.05 higher absorbance value than the value in the control (without test serum and without antigen) was taken as the cut off value. The antibody titer to EBNA was expressed as the reciprocal of the maximum dilution of serum giving a positive reaction.

4. *Hemagglutination Inhibition (HI) Test*

The titers of antibodies to measles and rubella were tested by HI according to the instructions in the manual standardized by the National Institute of Health (Tokyo, Japan).

5. *Indirect Immunofluorescence Staining*

For titration of IgA antibody specific to EBV capsid antigen (VCA), VCA slides were obtained commercially from Wako Pure Chemical Industries, Osaka. Acetone-fixed P3HR-1 cells smeared on wells of VCA slides were incubated with 10 μl volumes of serially diluted sera for 30 min at 37 C, washed twice with PBS, and incubated for 30 min at 37 C with diluted (1 : 30) fluorescein isothiocyanate conjugated goat anti-human IgA (α-chain specific, Bionetics). The P3HR-1 cells in the wells of the slide were washed and examined by fluorescence microscopy.

RESULTS

Of 36 NPC patients, 19 patients were followed up for 4 months to 8 years after completion of liniac X-ray radiation therapy with total doses of 60 to 80 Gy, and sera were obtained from them before therapy and at various intervals of 2 to 39 months after therapy. The sera from the other 17 patients were obtained at various times before or after therapy. As controls, sera were obtained from 23 healthy adults. The 19 NPC patients were divided into two groups, A (good prognosis) and B (poor prognosis), based on clinical evaluation in 1987, and the other 17 NPC patients were grouped into C. Table 1 shows the geometric mean titers of EBNA antibody measured by ELISA. Before radiation therapy, the mean titers of EBNA antibody were apparently higher in the NPC patients

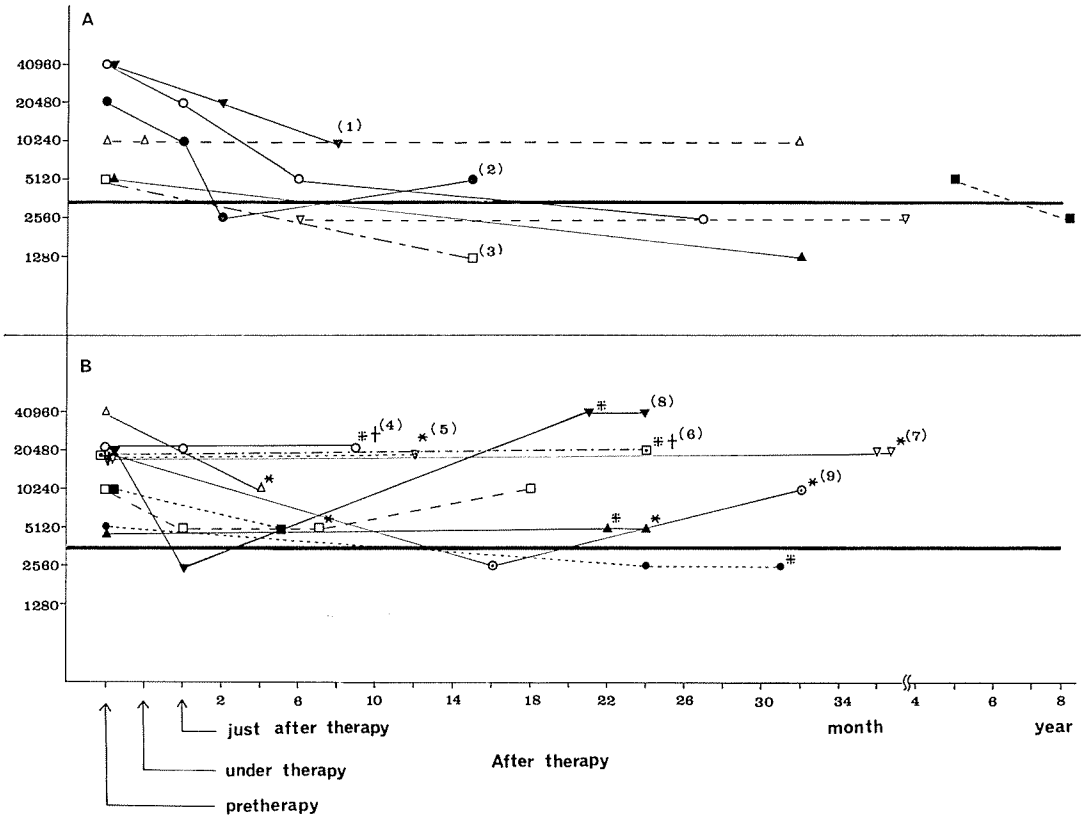

FIGURE 1. Changes in EBNA-specific IgG antibody titers of individual NPC patients before and after radiation therapy. The thick horizontal line in each panel shows the geometric mean titer of EBNA antibody of 23 sera from healthy controls cited from Table 1.

Panel A: EBNA antibody levels of individual cases in group A, whose prognosis was evaluated as good with no recurrence or metastasis up to the time of the latest clinical examination.

Panel B: EBNA antibody levels of individual cases in group B, whose prognosis was poor, with evidence of recurrence (*), or metastasis (⁂) or death (+).

than in healthy adults. There was no significant difference in the mean antibody titers in the sera of the two groups of patients, A and B before therapy, whereas a significant difference in the mean titers of EBNA antibody in the sera of the two groups was seen after therapy ($p<0.05$).

Fig. 1 shows the titers of EBNA antibody in the individual NPC patients classified in groups A and B in Table 1 and followed up for various periods. The mean titers of EBNA antibody in sera of healthy adults are shown by thick solid lines parallel with the abscissa in Fig. 1A and B. The titers of EBNA antibody in the sera of individual patients during the periods of therapy or immediately after completion of therapy were not essentially different from those before therapy. None of the patients in group A showed recurrence or metastasis. The three cases numbered (1), (2) and (3) have been followed up for 16 months, and have shown, no clinical signs of recurrence or metastasis, but their prognosis is unknown, because most

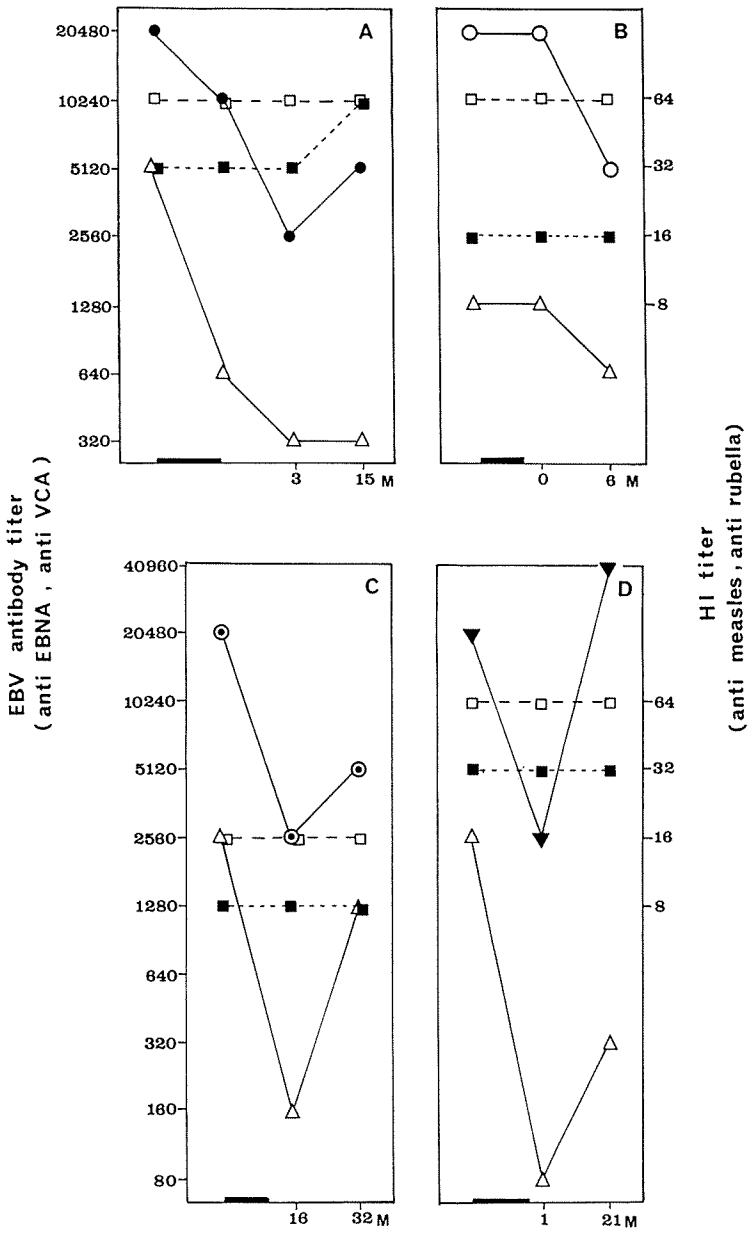

FIGURE 2. Correlation of changes in titers of EBNA-specific IgG antibody with those of VCA-specific IgA antibody in four NPC patients, in comparison with changes in the titers of HI antibodies specific to measles and rubella viruses. The thick line on the abscissa indicates the period of irradiation. The intervals of time on the abscissa are arbitrary, for simplified demonstration of patterns. Panels A and B show values for individual patients evaluated as having a good prognosis, and those in panels C and D are values for patients judged to have a poor prognosis.

Panel A: Case (2) shown by ●—● in Fig. 1A. (●—●), EBNA/IgG antibody; (△—△), VCA/IgA antibody; (□---□), HI antibody to rubella virus (■---■), HI antibody to measles virus. →

recurrences and metastases were seen within 24 months after radiation therapy (Fig. 1B) and Sato and Miyahara reported 46.7% survival three years after therapy (1977). The antibody titers of 6 patients in group A decreased progressively to the levels of normal healthy adults within 2 to 8 months after the completion of therapy (Fig. 1A). On the other hand, the patients in group B who maintained high titers (20,480) of EBNA antibody showed recurrence of NPC in the pharynx or metastasis (cases 4, 5, 6 and 7), and the two patients (cases 8 and 9) whose EBNA antibody levels first decreased to normal levels and at later increased 4-fold, also showed recurrence or metastasis (Fig. 1B).

The patterns of EBNA antibody levels after therapy in group B, that is either persistence of a high level or first decrease followed by 4-fold increase, were not seen in any case in group A. These characteristic patterns of change in EBNA antibody levels could be detected in several cases long before the appearance of clinical symptoms of recurrence or metastasis. These results suggest that follow-up studies on EBNA antibody levels after radiation therapy are helpful for prediction of the prognosis.

For determination of whether decrease in the level of EBNA antibody after local radiation therapy was due to a general immunosuppressive effect on antibody producing B cells or to a selective inhibitory and destructive effect on EBV-carrying cells, the following two tests were made.

The antibody levels to measles and rubella in the sera of two NPC patients each in group A and B respectively, were tested before and after radiation therapy. As shown in Fig. 2, no change was seen in the HI titers of antibodies to measles and rubella in the period after therapy. Thus the changes in the antibody levels to EBNA appear to be specific responses to radiation therapy.

To confirm this possibility, we measured the levels of another antibody specific to EBV, and characteristic of NPC, that is, serum IgA antibody to EBV-capsid antigen (VCA), a late antigen synthesized after the synthesis of viral genomic DNA. The serum level of IgA antibody to VCA was measured by indirect immunofluorescence, and the results are also shown in the 4 panels in Fig. 2. The patterns of changes in the IgA antibody levels to VCA were very similar to those of the changes in the IgG antibody levels to EBNA. These results suggest that the changes in the EBV-specific antibody levels in NPC patients are correlated with the replication of EBV, and that the clinical progression of NPC, such as recurrence or metastasis is associated with replication of EBV, possibly due to its reactivation.

DISCUSSION

There are many reports of association of latent or persistent EBV-infection with the occurrence of NPC, Burkitt's lymphomas, and lymphomas under cellular immunosuppressive or immunodeficient conditions, some of which are related to organ transplantation (Cleary et al., 1985). However, there are few reports of EBV-related serological follow-up studies on NPC patients for a long time after radiation therapy. Our data are similar to those reported by Henle et al. (1977) and Tamada et al. (1984), but we found that the time required for decrease in the levels of EBNA antibody determined by ELISA was shorter than those reported by these investigators. Our data showed that maintenance of nearly

Panel B: The case shown by ○—○ in Fig. 1A. (○—○), EBNA/IgG; (△—△), VCA/IgA.
Panel C: Case (9) shown by ⊙—⊙ in Fig. 1B. (⊙—⊙), EBNA/IgG; (△—△), VCA/IgA.
Panel D: Case (8) shown by ▼—▼ in Fig. 1B. (▼—▼), EBNA/IgG; (△—△).
□---□ and ■---■ in panels B, C and D represent HI antibodies to rubella and measles viruses, respectively, as described in panel A.

normal EBNA antibody levels for at least three years after radiation therapy was a good indication of a good prognosis of NPC patients without recurrence or metastasis, and that radiation therapy may controll replication of EBV. In about 55% of the patients in group B, the antibody levels to EBNA and VCA were not maintained at nearly normal levels, and these patients showed a poor clinical course with recurrence or metastasis. In these patients, replication and reactivation of EBV appeared to be one factor involved in the poor prognosis after radiation therapy. But, the other 45% of the patients in group B also showed a poor clinical course although their titers of EBNA antibody were low. In these patients, the recurrence and metastasis must have been caused by other factors than EBV, such as low cellular immunity, as indicated by Sawaki (1987).

Various factors are probably related to the prognosis of NPC. Our data indicate that one of these is EBV since it is related not only to the onset of NPC, but also to the progression of this disease during the period after therapy.

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