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Site-specific Localization of Epstein-Barr Virus in Pharyngeal Carcinomas

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In this study, the correlations of factors with Epstein-Barr virus (EBV)-association were investigated in 50 patients with nasopharyngeal carcinoma (NPC), 61 with oropharyngeal carcinoma (OPC), and 55 with hypopharyngeal carcinoma (HPC) in Okinawa and Osaka prefectures in Japan. The incidence of pharyngeal carcinoma in Okinawa was previously found to be higher than that in Osaka; the incidence of OPC was approximately 6 times higher and that of HPC was two times higher. The EBV genome was detected in the tumor cells of the present patients; 83% of the Okinawa and 92% of the Osaka NPC patients. The EBV genome was not detected in OPC or HPC. A univariate analysis showed that sex, the location of the tumor, histology, and the degree of lymphocytic infiltration correlated with the EBV-positive rate. A multivariate analysis revealed that only the location of the tumor was independently correlated with the EBV-positive rate. Histology and tumor size were factors affecting the prognosis of the patients with NPC. The NPC of poorly differentiated type frequently showed the EBV genome, and NPC with lymphocytic infiltration showed a more favorable prognosis compared to the other NPC types. These findings suggest that latent genes of EBV expressed in cancer cells might trigger a cytotoxic T cell reaction against the cancer.

Key words: Epstein-Barr virus — Pharyngeal carcinoma — Epidemiology

Epstein-Barr virus (EBV), a herpesvirus which can infect lymphocytes, was originally discovered in cultured lymphoblasts from the African type of Burkitt's lymphoma in 1964.[1)] An oncogenic role of EBV in the development of B cell lymphoma and nasopharyngeal carcinoma (NPC) was subsequently suggested.[2)] Recent studies have shown the presence of EBV sequences in carcinoma cells of thymic,[3)] gastric,[4)] salivary gland,[5, 6)] and lung cancers.[7)]

The pharyngeal space is divided into three parts; the nasopharynx, oropharynx, and hypopharynx. The nasopharynx is covered with pseudostratified respiratory epithelium, and the oropharynx and hypopharynx are covered with stratified squamous epithelium. The naso- and oropharynxes have well-developed lymphoid tissue, i.e., Waldeyer's ring, whereas the hypopharynx is devoid of lymphoid tissue. The association of EBV with NPC is well-known, and it has been reported that EBV-associated NPC usually have poorly differentiated morphology accompanied by severe lymphoid cell infiltration, forming a lymphoepitheliomatous lesion. It is not yet evident whether oropharyngeal and hypopharyngeal carcinomas (OPC and HPC), especially those of the poorly differentiated type, are EBV-associated.

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We recently reported a much higher frequency of sinonasal lymphoma in Okinawa prefecture compared to Osaka in Japan, and we showed that the majority of these lymphomas were associated with EBV.[8)] The incidence of pharyngeal carcinoma in Okinawa was also found to be higher than that in Osaka; the incidence of OPC is approximately 6 times higher and that of HPC is two times higher in Okinawa.[9)] In the present study, we investigated whether the higher frequencies of OPC and HPC in Okinawa are correlated with EBV infection. We also analyzed factors correlating with EBV positivity in pharyngeal carcinoma patients and the prognostic effect of EBV positivity among these patients.

PATIENTS AND METHODS

Fifty patients with NPC (38 from Okinawa and 12 from Osaka), 61 patients with OPC (48 from Okinawa and 13 from Osaka), and 55 patients with HPC (41 from Okinawa and 14 from Osaka) were selected for the present study; these patients were admitted to hospitals during the period from 1975 to 1995. Histologic specimens obtained by biopsy from the primary tumor were fixed in 10% formalin and routinely processed for paraffin-embedding. Histologic sections, cut at 6 μm, were stained with hematoxylin and eosin, and were reviewed by one of the authors (K. A.) for histological diagnosis. All of the

tumors were squamous cell carcinomas (SCC). The tumors with a pavement pattern of proliferation without any evidence of differentiation of squamous cells, those with small areas of squamous cell differentiation, and those showing distinct pearl formation were classified as poorly differentiated, moderately differentiated, and well-differentiated SCC, respectively. The degree of lymphocytic infiltration in the tumor tissues was categorized as severe, moderate, or slight. Of the NPCs, the tumors localized within one subsite of the nasopharynx were categorized as T1; those invading more than one subsite of the nasopharynx as T2; those invading the nasal cavity and/or oropharynx as T3, and those invading the skull and/or cranial nerve(s) as T4.

Extraction of DNA DNA was extracted from the formalin-fixed, paraffin-embedded tissue using a chelating resin method,[10] with some modifications. Paraffin blocks without any samples were used as negative controls throughout the procedures. DNA extracted from a formalin-fixed, paraffin-embedded EBV-positive Burkitt lymphoma cell line (Raji) (a gift from Dr. H. Mizusawa, National Institute of Hygiene, Tokyo) was used as the positive control of EBV DNA.

PCR amplification of β-globin and EBV genome The amplification of β-globin and the EBV genome was carried out by using polymerase chain reaction (PCR). Briefly, 10 μl of DNA sample was diluted into 25 μl of PCR solution (Promega, Madison, WI). For the amplification of the β-globin gene, 35 PCR cycles of 94°C-60°C-72°C were performed with primers designed to amplify a 123-bp segment in the exon 7–8 region (exon 7, 5′-CTTCTGACACAACTGTGTTCACTAGC-3′; exon 8, 5′-TCACCACCAACTTCATCCACGTTCAC-3′).[11] For the amplification of the EBV genome, 35 PCR cycles of 94°C-58°C-72°C were performed with primers designed to amplify a 129-bp segment in the *Bam*HI-W region of the EBV genome (5′-CCAGACAGCAGCCAATTGTC-3′, 5′-GGTAGAAGACCCCCTCTTAC-3′).[12]

Southern blot analysis of amplified samples The EBV PCR products were electrophoresed and transferred to Hybond N+ membranes (Amersham, Buckinghamshire, England). Oligonucleotide probes which hybridize to each of the intervening sequences between the two primers of the EBV genome (5′-CCCTGGTATAAAGTGGTCCTG-CAGCTATTTCTGGTCGCAT-3′) were labeled with fluorescein-deoxyuridine triphosphate using 3′-oligolabeling and detection systems (Amersham). The subsequent hybridization and development were performed with the detection system following the procedures outlined by the manufacturer.

RNA-ISH EBV RNA *in situ* hybridization (RISH) was performed as previously described with some modifications.[13] Briefly, 30-base oligonucleotide probes which were sense and antisense for a portion of the EBV early RNA 1 (*EBER-1*) gene, a region of the EBV genome that is actively transcribed in latently infected cells,[14] were synthesized using a DNA synthesizer. As a positive control, the Raji cell line was used. As negative controls, the hybridizing mixture was used with (a) sense probe and (b) antisense probe after RNAse treatment.

Immunohistochemical study An immunohistochemical study (alkaline phosphatase antialkaline phosphatase method)[15] was carried out using paraffin-embedded biopsy sections. The monoclonal antibody used was EBV CS1-4 (Dakopatts, Glostrup, Denmark diluted at 1:50), which recognizes EBV-encoded latent membrane protein 1 (LMP-1). Paraffin sections were treated with microwaves for 5 min in 0.01% citrate buffer before incubation with the antibody.

Statistical analysis The correlation of EBV positivity with several factors was analyzed in terms of Pearson's correlation coefficient and one-way analysis of variance using two statistics programs designed for use with a personal computer (STAX98, Tokyo University; SD-BASE2, MPC, Tokyo). The follow-up time from the end of the initial therapy ranged from 12 to 60 months (median 49 months) for the 16 survivors among the 50 NPC patients. Actuarial survival curves were plotted using the method of Kaplan and Meier.[16] The significance of the differences was evaluated by means of the log-rank test. Probability values of less than 5% were accepted as significant.

RESULTS

EBV positivity We classified cases as EBV-positive only when positive signals in RISH were found in the nucleus of cancer cells (Fig. 1). The histologic classification of cancers and the EBV-positivity data are summarized in Table I. A large number of the NPC cases were of poorly

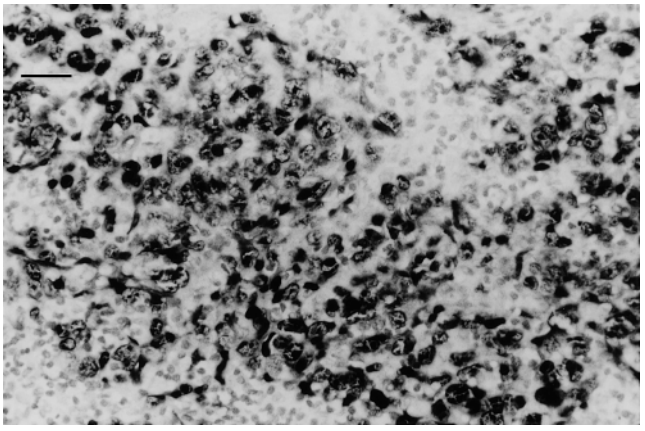

Fig. 1. Positive signals are seen in the nuclei of almost all of the cancer cells in RISH analysis. Scale bar, 25 μm.

Table I. Histologic Classification and EBV-positive Ratio in Patients with Pharyngeal Carcinoma in Okinawa and Osaka, Japan

	Total number of cases (M/F)	Lymphocyte infiltration			EBV-positive rate (positive/analyzed)	
		slight	moderate	severe	male	female
Osaka						
Nasopharyngeal cancer	12(6/6)	3	5	4	6/6	5/6
well-differentiated	1(1/0)	1	0	0	1/1	0/0
moderately diff.	2(0/2)	1	1	0	0/0	2/2
poorly diff.	9(5/4)	1	4	4	5/5	3/4
Oropharyngeal cancer	13(10/3)	7	5	1	0/8	0/3
well-differentiated	6(5/1)	4	2	0	0/5	0/1
moderately diff.	2(1/1)	1	1	0	0/1	0/1
poorly diff.	3(2/1)	2	2	1	0/0	0/1
Hypopharyngeal cancer	14(12/2)	12	1	1	0/12	0/2
well-differentiated	11(10/1)	9	1	1	0/10	0/1
moderately diff.	3(2/1)	3	0	0	0/2	0/1
poorly diff.	0				0	
Okinawa						
Nasopharyngeal cancer	38(31/7)	12	13	12	23/28	6/7
well-differentiated	6(6/0)	3	2	0	3/6	0/0
moderately diff.	4(3/1)	1	1	2	2/3	1/1
poorly diff.	28(22/6)	8	10	10	18/19	5/6
Oropharyngeal cancer	48(44/4)	19	24	5	0/36	0/3
well-differentiated	25(23/2)	10	14	1	0/19	0/2
moderately diff.	17(15/2)	6	9	2	0/12	0/1
poorly diff.	6(6/0)	3	1	2	0/5	0/0
Hypopharyngeal cancer	41(37/4)	26	12	1	0/24	0/3
well-differentiated	19(17/2)	12	5	1	0/9	0/1
moderately diff.	15(13/2)	10	5	0	0/8	0/2
poorly diff.	7(7/0)	4	2	0	0/7	0/0

M/F, male/female.

differentiated type, whereas most of the OPC and HPC cases were of moderately to well-differentiated type in both Okinawa and Osaka. The presence of EBV genome was examined in 35 NPC, 39 OPC, and 27 HPC cases from Okinawa and 12, 11 and 14 cases from Osaka, respectively. The EBV genome was amplified by PCR in 29 NPC and 2 HPC cases from Okinawa and in 11 NPC cases from Osaka. The EBV genome was detected by ISH in 29 (83%) and 11 (92%) of the NPC patients from Okinawa and Osaka, respectively. None of the cases with OPC and HPC were positive for EBV genome in tumor cells by ISH.

EBV positivity and correlated factors The correlations of various factors with EBV positivity are shown in Table II. The EBV-positive rates of the Osaka and Okinawa patient populations were almost the same (32% and 29%, respectively). A univariate analysis showed that the factors of sex (female), the location of the tumor (nasopharynx), the degree of tumor differentiation, and lymphocytic infiltration were each significantly correlated with the EBV positivity rate. A multivariate analysis revealed that only the location of the tumor (nasopharynx) was an independent factor which correlated with EBV positivity.

Immunohistochemistry The immunohistochemistry revealed that tumor cells in 18 (64%) of 28 NPC cases shown to be positive for EBV genome by PCR and RISH expressed LMP-1 (Table III). The frequency of LMP-1 expression correlated with the degree of lymphocytic infiltration; 82% for severe, 63% for moderate, and 44% for slight lymphocytic infiltration. The differences among these groups were not significant, however.

Prognostic factors for nasopharyngeal carcinomas The prognostic significance of clinical and histologic factors including EBV positivity was evaluated (Table IV). Tumor size and the degree of differentiation of the cancers were significant factors for survival. The EBV-positive NPC patients, although their carcinomas were usually of poorly differentiated type, showed a favorable prognosis compared to the EBV-negative patients (P=0.05). The female patients, the patients with no lymphnode

Table II. EBV Positivity and Various Factors in Patients with Pharyngeal Carcinoma

	Total number of cases	EBV-positive cases (%)	*P*-value
Districts			
Osaka	34	11 (32)	NS
Okinawa	101	29 (29)	
Location of tumor			
nasopharynx	47	40 (85)	<0.001
oropharynx	50	0 (0)	
hypopharynx	38	0 (0)	
Tumor differentiation			
well-diff.	53	4 (8)	
moderately diff.	33	5 (15)	<0.001
poorly diff.	49	31 (63)	
Lymphocyte infiltration			
severe	18	14 (78)	
moderate	50	13 (26)	<0.001
slight	59	12 (20)	
Sex			
male	112	29 (26)	<0.05
female	23	11 (48)	

NS, not significant.

Table III. Expression of LMP-1 of Nasopharyngeal Carcinoma Cells and Lymphocyte Infiltration

Lymphocyte infiltration	LMP-1	
	positive (n=18)	negative (n=10)
Severe	9	2
Moderate	5	3
Slight	4	5

LMP-1, latent membrane protein 1.

Table IV. Univariate Analysis of Overall Survival of NPC Patients: Histologic and Clinical Factors

Factors	Number of patients	Number of deaths	5-year survival rate, %	*P*-value
Age				
≥50 yrs	12	7	37	NS
≤49 yrs	32	12	42	
Sex				
male	29	17	34	NS
female	6	2	67	
Tumor size				
T1	8	5	33	
T2	9	3	63	
T3	7	0	100	<0.001
T4	11	11	0	
Lymph nodes				
N0	11	5	55	
N1	10	7	30	NS
N2	10	5	41	
N3	4	2	33	
Histologic differentiation				
well-diff.	5	5	0	
moderately diff.	3	1	50	<0.01
poorly diff.	25	5	51	
Lymphoid cell infiltration				
severe	10	4	51	
moderate	12	7	39	NS
slight	10	6	38	
Epstein-Barr virus				
positive	24	11	50	
negative	5	4	20	=0.05

NS, not significant.

metastases, and those with severe lymphoid cell infiltration tended to survive longer, although the differences in survival were not significant.

DISCUSSION

The EBV genome was not detected in OPC or HPC in Okinawa and Osaka, although the incidences of these diseases were much higher in Okinawa than in Osaka. Thus, a role of EBV in the development of OPC and HPC seems unlikely. EBV usually invades the body through the nose or mouth, and may thus affect the pharynx as an oncogenic agent. Although it is clinically divided into three parts, the pharynx is a continuous organ only 15 cm long. EBV is only detected in nasopharyngeal carcinoma. Based on the site specificity of EBV detection, site-specific susceptibility to EBV in the nasopharynx and a mechanism of negation of the EBV infection in the pharynx may exist.

The present univariate analysis showed that sex, the location of the tumor, histology, and the degree of lymphocytic infiltration correlated with the EBV-positive rate: female sex, nasopharyngeal location, poorly differentiated morphology, and severe lymphocytic infiltration were associated with high EBV positivity. The multivariate analysis revealed that only the location of the tumor was independently correlated with the EBV-positive rate. Poorly differentiated morphology with severe lymphocytic infiltration, the well-known histology of lymphoepithelioma, proved to be not correlated independently with the EBV-positive rate.

The question remains as to why only the nasopharynx is a site for the development of EBV-associated carcinoma. The pharynx is comprised of the upper respiratory tract covered with pseudostratified respiratory epithelium, with an underlying lymphoid apparatus in the nasophar-

ynx, and the oro- and hypopharynxes, covered with stratified squamous epithelium. The presence of the EBV genome in the differentiated forms of NPC was recently reported.[17, 18] We confirmed this in the present study; i.e., the EBV genome was also found in the tumor cells of moderately and well-differentiated SCC, though much less frequently than in the poorly differentiated form. The stable maintenance of EBV in epithelial cells of undifferentiated condition was reported by Knox *et al*.[19] This might be one of the reasons for the high EBV-association in NPC. Young *et al*. identified a cell surface protein sharing an epitope with the C3d/EBV receptor molecule, CD21, on the nasopharyngeal epithelial cells of humans.[20, 21] The replication of EBV in oropharyngeal epithelial cells was reported by Sixbey *et al*.[22] These findings may indicate that some factors assist the development of carcinomas from EBV-infected epithelial cells in the nasopharynx.

The types of EBV latent gene expression in NPC cells are Latency II; EBNA1$^+$, EBNA2$^-$, and LMP-1$^+$.[23] In the present study, 64% of the patients with the EBV genome expressed LMP-1 at the protein level. Previous studies showed that LMP-1 could serve as a target molecule for cytotoxic T-cells,[24] and thus that NPC expressing LMP-1 could induce lymphocytic infiltration.[25, 26] Indeed, the degree of lymphocytic infiltration correlated with EBV positivity in the present patients; i.e., there was a high EBV-positive rate among the patients with severe lymphocytic infiltration. NPC are frequently poorly differentiated, contain the EBV genome, and are accompanied by severe lymphocytic infiltration. The EBV genome was also detected, though infrequently, in the moderately and well-differentiated NPC, in which the expression of LMP-1 was weak and lymphocytic infiltration was mild to moderate.

Tumor size is a well-known, definite factor in the prognosis of patients with NPC,[27] and this was confirmed in the present study. The histologic type was another factor affecting the prognosis of our NPC patients. Unlike other types of cancer, however, patients with poorly differentiated or undifferentiated NPC have a favorable prognosis compared to those with moderately or well-differentiated NPCs in previous studies[28, 29] and our present NPC series. This is an interesting finding. In addition, the presence of the EBV genome in the cancer cells with poorly differentiated morphology and accompanying lymphocytic infiltration was prognostically favorable in the present patients. It was reported that fresh NPC cells from several patients with typical undifferentiated NPC containing the EBV genome produced an interleukin-1α-related factor which could stimulate T-cell migration and activation within the tumor.[30] It was also reported that NPC regularly contained a subset of activated T cells.[31] These findings suggest that latent genes of EBV expressed in the cancer cells might trigger a cytotoxic T cell reaction against the cancer, i.e., anti-tumor immunity, resulting in a favorable prognosis. Indeed, Hu *et al*.[32] reported a better prognosis of NPC patients expressing LMP-1 compared to patients without such expression.

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