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Genotypic Analysis of Epstein-Barr Virus Associated with Nasopharyngeal Carcinoma of Japanese Patients

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Types and certain genetic markers were studied for Epstein-Barr virus present in 10 specimens of nasopharyngeal carcinoma (NPC) from Japanese patients. The type 1 virus was predominant in our NPC specimens, as in the general Japanese population, and the type 2 virus was found only in one NPC specimen. The type C variant, which lacks the *Bam*HI site between the *Bam*HI-W1* and -I1* regions, appeared to be common among Japanese strains as in those in Southern China. The type f variant which has an extra *Bam*HI site in the *Bam*HI-F region and has been shown to be strongly associated with NPC in Southern China was found in only one NPC specimen. This virus strain was also the only type 2 virus among our specimens. The present study, therefore, does not show any specific association of the type f variant with NPC in Japan.

Key words: EBV — Nasopharyngeal carcinoma — RFLP

Epstein-Barr virus (EBV) is a ubiquitous herpes virus latently infecting the majority of all populations. Primary infection mostly occurs in early childhood without noticeable symptoms. Acute infection in young adults, however, causes a lymphoproliferative disease, infectious mononucleosis. EBV is also intimately associated with the pathogenesis of certain human malignancies. Two classical examples are endemic Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), both of which have characteristic geographical distributions.[1)]

It is now known that there are two major types of EBV. Type 1 (or A) corresponds to the prototype of EBV upon which our current understanding of the biology and molecular structure of EBV is mostly based.[2,3)] Type 2 (or B) was recently discovered in some Burkitt's lymphomas of Central Africa and New Guinea through a striking difference in the region encoding EBNA-2.[4,5)] Recent studies have further extended the sequence divergence to other genetic regions.[6,7)] It has been shown that type 2 virus is much less efficient in B cell-transformation than type 1 virus.[8,9)] Differences between the two types in terms of geographical distribution or disease-association are yet to be fully determined.[10–13)] EBV also has various genetic polymorphisms due to repetitive sequences and allelic sequence divergences, resulting in extensive restriction fragment lenght polymorphisms (RFLPs).[14,15)] Recently, Lung *et al.*[16,17)] reported a strong correlation of a certain genotypic variant called "f" with occurrence of NPC in Southern China. To our knowledge, they have been the first to demonstrate such a close association of a particular EBV genotype with any EBV-related diseases. We, therefore, carried out a similar analysis on EBV strains associated with NPC in Japan.

MATERIALS AND METHODS

Specimens All patients were seen at the hospital of Wakayama Medical College. Tissue specimens from primary tumors and/or metastatic cervical lymph nodes were obtained before initiation of chemotherapy or radiation therapy. A portion of each tissue specimen was snap-frozen in OCT compound (Miles, Elkhart, IN) and kept at −80°C until histological and immunological examinations. The remaining portion of each tissue was stored at −80°C for later DNA analysis.

Serological tests Serum class-specific antibody titers to EBV antigens (VCA and EA-DR) and anti-EBNA titers were determined for each patient by the standard indirect immunofluorescent staining and anti-complement immunofluorescent staining, respectively.[18,19)]

EBV typing by PCR EBV typing was carried out for each tumor sample by polymerase chain reaction (PCR) using consensus primers as described previously.[13)] The sequences of the sense and antisense primers were 5′-TTTCACCAATACATGAACC-3′ and 5′-TGGCAAA-GTGCTGAGAGCAA-3′, respectively. Each type was identified from the size of amplification products.

Southern blot analysis For the analysis of RFLP, 8 μg of DNA extracted from each specimen was completely digested with *Bam*HI, separated by electrophoresis on a 0.6% agarose gel, transferred to a nylon filter (Hybond-N, Amersham) and hybridized with ^{32}P-labeled probes following the standard methods.[20)] After washing, hybridized bands were detected by autoradiography. The

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RESULTS AND DISCUSSION

Tumor specimens from 12 Japanese patients with NPC were collected. From histological examinations, these included three squamous cell carcinomas (SCC, WHO Type I), four non-keratinizing carcinomas (NKC, WHO Type II) and five undifferentiated carcinomas (UC, WHO Type III) (Table I). Association with EBV was studied by immunological staining of EBNA in tumor tissue sections by the anti-complement immunofluorescent method[18] and Southern hybridization detection of EBV-DNA in DNA samples from tumor tissues.[20] Ten NPC tumors were demonstrated as EBV-positive from typical bright and granular staining of EBNA in nuclei of tumor cells (but not in cells from surrounding normal tissues) and detection of the highly repetitive *Bam*HI-W region of EBV-DNA in DNA samples prepared from tumor specimens (data not shown)(Table I). As reported previously, all poorly differentiated types of NPC (UC and NKC) were positive for EBV.[1] As for the well-differentiated type of NPC (SCC), which is mostly negative for EBV, one tumor (NPC case 3) was found to contain EBV-DNA. Such examples were also reported by Raab-Traub and Flynn.[21] The sera of most of the patients with EBV-positive NPC contained high titers of IgG anti-VCA together with characteristic IgA anti-VCA and IgG anti-EA (Table I) as reported previously.[19] The subsequent genotypic analysis of EBV was carried out for the ten EBV-positive NPC specimens.

Previously, we carried out typing of EBV excreted in saliva samples from healthy donors and patients with various types of tonsillitis by the PCR method using consensus as well as type-specific primers synthesized from the EBNA-2 region and found that the type 1 virus was highly dominant (>90%) in Japan.[13] It was, therefore, of interest to see whether type 1 virus was also dominant in EBV strains associated with NPC. As shown in Fig. 1, the only EBV from NPC case 7 was type 2, whereas all others were type 1. The results were further confirmed by PCR using type-specific primers[13] (data not

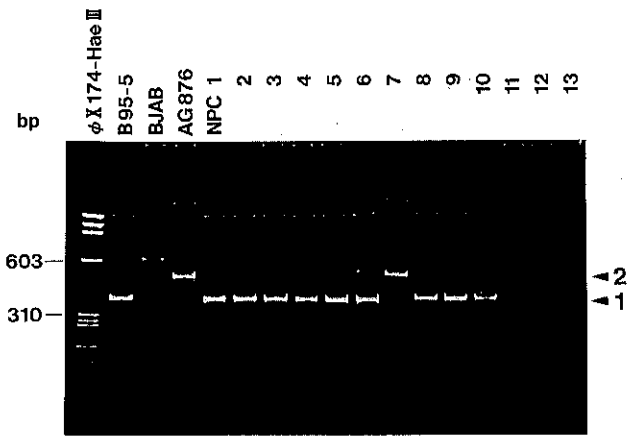

Fig. 1. PCR typing of EBV genomes associated with NPC. DNA samples prepared from NPC specimens were subjected to PCR amplification using EBV consensus primers.[13] Products were fractionated by gel electrophoresis and detected by ethidium bromide staining. DNAs from B95-8 (type 1), AG876 (type 2) and BJAB (EBV-negative) were used as controls. A faint band of about 600 bp in the lane of BJAB was nonspecific.

Table I. Summary of Histological Diagnosis, Serum Anti-EBV Titers and Tissue Detection of EBV of 12 Japanese NPC Patients

NPC case	Age	Sex	Diagnosis (stage)	Anti-VCA		Anti-EA		Anti-EBNA	Tissue EBNA	EBV DNA
				IgG	IgA	IgG	IgA			
1 T.K.	49	F	UC (IV)	5120	<10	160	40	80	+	+
2 H.K.	67	M	UC (IV)	640	<10	40	<10	640	+	+
3 K.N.	70	M	SCC (IV)	1280	40	160	<10	1280	+	+
4 S.N.	32	M	NKC (IV)	640	160	320	<10	80	+	+
5 T.N.	59	M	UC (III)	5120	320	320	<10	320	+	+
6 M.W.	50	M	UC (IV)	1280	160	40	<10	160	+	+
7 F.Y.	63	F	NKC (IV)	2560	320	160	<10	640	+	+
8 H.T.	61	M	NKC (IV)	ND	ND	ND	ND	ND	+	+
9 K.H.	48	M	NKC (IV)	320	40	80	<10	160	+	+
10 E.Y.	58	M	UC (IV)	ND	ND	ND	ND	ND	+	+
11 Y.N.	74	M	SCC (IV)	640	80	<10	<10	20	−	−
12 N.S.	43	M	SCC (IV)	40	<10	<10	<10	80	−	−

Abbreviations are as follows: UC, undifferentiated carcinoma; SCC, squamous cell carcinoma; NKC, non-keratinizing carcinoma; ND, not determined.

snown). Type 1 virus was thus predominant in our NPC specimens (9/10), as in the general Japanese population.[13] Therefore, a preferential association of either type of EBV with Japanese NPC seems unlikely. Recently, Chen *et al.*[22] also carried out typing of EBV in 25 NPC specimens derived from Southern China. Type 1 virus was predominant and type 2 virus was found only in one sample. It is probable that type 1 virus is also predominant in the general population of Southern China, as in that of Japan.

The previous prototype EBV genome of B95-8 has a large deletion in the *Bam*HI-I region.[23] In other isolates of EBV, this region is divided into three *Bam*HI fragments, *Bam*HI-B1*, -W1* and -I1*. Viral isolates with fused *Bam*HI-W1* and -I1*, i.e., no *Bam*HI site between them, were designated as type C variant, while those having the *Bam*HI site between W1* and I1*, as type D variant.[15] Lung *et al.*[15] reported a high prevalence of type C variant in Southern China; type C variant was seen only in 21% of EBV strains harbored in lymphoblastoid cell lines (LCLs) established in California, whereas 85% of EBV strains harbored in LCLs established in Southern China and Hong Kong were type C variant. Furthermore, EBV strains associated with NPC biopsies from Southern China were found to be 100% type C.[16] Hybridization of *Bam*HI-digested tumor DNA samples with the *Bam*HI-I probe from B95-8 DNA is shown in Fig. 2A. Seven samples were type C, whereas three were type D. Even though the prevalence of type C variant in the general Japanese population remains to be evaluated, it is likely that the type C variant is also a dominant type in Japan.

Another variant of particular importance is called "f," from an extra *Bam*HI site in the *Bam*HI-F region. Lung *et al.*[16] reported that 24 out of 28 (86%) NPC biopsies from Southern China contained the f variant in spite of the fact that this variant was seen in none of 39 LCLs from USA and only one of 13 LCLs from Southern China and Hong Kong. Such a strongly biased association of a particular EBV genotype with NPC or other EBV-related tumors has never previously been reported.[14] Lung *et al.*[17] further demonstrated that the f variant was detected at a much higher frequency not only in overt NPC patients but also in individuals at a high risk for developing NPC than in healthy individuals or in former NPC patients in remission for over 3 years. As shown in Fig. 2B, only NPC case 7 was found to have the f variant. The present data, therefore, do not show any special association of this genotype with NPC in Japan. Recently, Lung and Chang[24] also reported that EBV associated with NPC from Caucasian patients was exclusively the F prototype (10/10). Furthermore, Hu *et al.*[25] reported that a *Xho*I restriction site in exon 1 of BNLF-1 gene for LMP-1 was missing in most EBV strains in

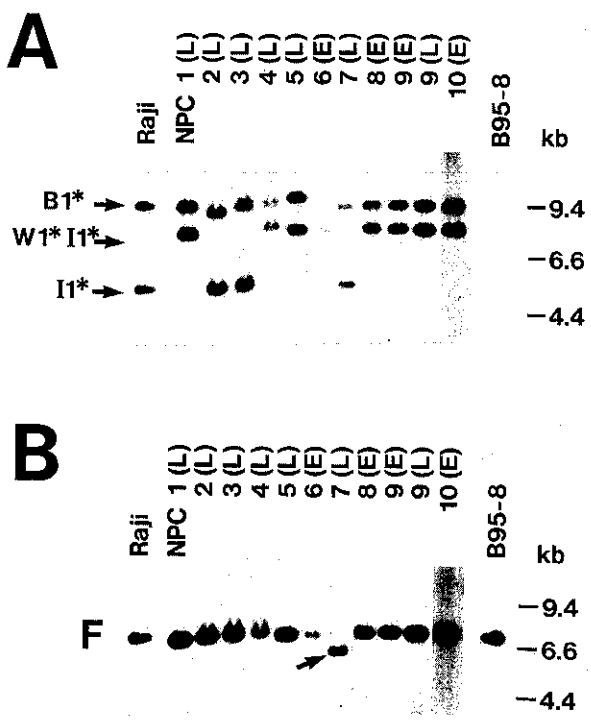

Fig. 2. RFLP analysis of EBV strains associated with NPC. NPC tissues were obtained from epipharynx (E) and/or metastatic lymphnodes (L). DNAs from NPC specimens were digested with *Bam*HI, size-fractionated by electrophoresis on a 0.6% agarose gel, blotted onto a filter and hybridized with EBV *Bam*HI probes. The probes used were *Bam*HI-I (A) and *Bam*HI-F (B) from B95-8 DNA. DNA samples from Raji, B95-8 and BJAB were used as positive and negative controls. Only positive controls are shown. Size markers are shown at the left. In the panel A, W1* was not hybridized because this region was completely missing in the *Bam*HI-I DNA from B95-8 used as the probe.

Chinese NPC biopsies but retained in most EBV strains in African NPC biopsies. Collectively, EBV strains associated with endemic NPC in Southern China may have important differences in some genetic structures and biologic functions from those associated with NPC from other parts of the world, including Japan.

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