

Detection of Epstein-Barr Virus in Oral Papilloma

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Fifty-one cases of malignant and non-malignant oral diseases were investigated for Epstein-Barr virus (EBV). EBV DNA was detected by polymerase chain reaction analysis in 2 of 4 papillomas, but not in other tissues including 36 squamous cell carcinomas and 4 leukoplakias. The copy numbers of EBV DNA in the two positive samples were estimated to be 120 and 36 per cell, respectively. Intense EBV DNA signals were detected on papilloma cells by *in situ* hybridization. DNAs for the benign and malignant types of human papilloma virus were not detected in papilloma tissues. The present results suggest that EBV is a causative agent of oral papilloma.

Key words: Epstein-Barr virus — Oral papilloma — Polymerase chain reaction — DNA *in situ* hybridization

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus, which infects the majority of the human population. EBV is commonly transmitted by saliva, and following replication in epithelial cells of the oropharynx, infects B lymphocytes, in which it persists in a latent state for the lifetime of the host.¹⁾ Therefore, oral tissues have a high likelihood of EBV infection. Oral hairy leukoplakia, which frequently occurs in AIDS patients, has been proved to be an epithelial focus of EBV replication.²⁾ However, the association of EBV with other oral diseases has not been well studied.

In the present study, we searched for the presence of EBV genomes and their products in oral diseases including squamous cell carcinoma, leukoplakia, and papilloma. We found that 2 of 4 papilloma cases we examined were associated with EBV.

MATERIALS AND METHODS

Patients and tissue specimens Formalin-fixed, paraffin-embedded samples were obtained from patients who visited the Department of Oral and Maxillofacial Surgery, Yamaguchi University Hospital. The patients comprised 34 males and 17 females ranging in age from 6 to 90 (average 62.8) years. These samples were diagnosed histologically according to the WHO international classification, and included 36 squamous cell carcinomas, 1 mucoepidermoid carcinoma, 1 undifferentiated carcinoma, 1 malignant melanoma, 4 papillomas, 1 epulis fibroma, 1 granuloma, 4 leukoplakias, and 2 hyperplasias.

Polymerase chain reaction (PCR) One microgram of DNA from each oral tissue sample was subjected to 25

cycles of PCR to amplify the 125-bp *Bam*HI W region as described previously.³⁾ The human β -globin gene was also amplified by 15 cycles of PCR in each DNA sample to assess the adequacy of each specimen. To avoid false-positive PCR results, strict physical separation was instituted between DNA extraction and each PCR step.

The PCR products were electrophoresed in 2% agarose gel, transferred to a nylon membrane, subjected to Southern blot hybridization as described previously³⁾ and exposed to a molecular imager ("Molecular analyst," Bio-Rad, Hercules, CA). According to the manufacturer's protocol, the density of the positive band was measured and the corresponding EBV DNA copy number was calculated by reference to a standard curve obtained using serial dilutions of Raji cell DNA (50 EBV DNA copies per cell⁴⁾), which was used as a positive control for semiquantitative estimation of positive bands. The EBV DNA copy number of each specimen was normalized by reference to the human β -globin gene that had been subjected to PCR. DNA from EBV-free BJAB cells⁵⁾ was used as a negative control.

An attempt was also made to detect human papilloma virus (HPV) DNA in EBV-positive tissues. DNA preparation and PCR were performed in a manner similar to that described above. DNA was subjected to 40 cycles of PCR amplification for detection of HPV 6, 11, 16, 18, 31, 33, 35, 52b and 58 according to the manufacturer's instructions (Takara PCR human papilloma virus typing set, Takara Biomedicals, Ohtsu).

DNA *in situ* hybridization As described previously,⁶⁾ 5- μ m sections from formalin-fixed, paraffin-embedded tissues were digested for 10 min with pronase (Sigma, St. Louis, MO) at a concentration of 1 mg/ml, followed by dehydration through a graded ethanol series and air-dry-

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ing. The slides were then denatured on a hot plate at 95°C for 8 min and hybridized at 42°C overnight. For the EBV probe, the *Pst*I subfragment of the *Bam*HI W fragment of EBV DNA (nucleotides 16,287 to 16,535) was subcloned into the *Pst*I site of the pBSII KS+ vector (Stratagene, La Jolla, CA). The digoxigenin-labeled RNA probe was generated by *in vitro* transcription with T7 RNA polymerase using a DIG RNA labeling kit (SP6/7; Boehringer Mannheim, Indianapolis, IN). *In situ* hybridization (ISH) was detected using an anti-digoxigenin antibody-alkaline phosphatase conjugate (DIG nucleic acid detection kit; Boehringer Mannheim). Digoxigenin-labeled neo RNA was used as a negative probe. Akata cells,^{7,8)} which were derived from an EBV-positive Burkitt's lymphoma, were treated with an antibody to surface immunoglobulin G and used as a positive control.

RNA ISH The EBV-encoded small RNA-1 (EBER1) was detected with a digoxigenin-labeled oligonucleotide probe complementary to the EBER1 sequence, 5'-AGACACCGTCCTACCACCCGGGACTTGTA-3', using the procedure described previously.⁹⁾

Immunohistochemistry Immunohistochemical analysis was performed using anti-latent membrane proteins (LMP) 1 (S12), anti-BZLF 1 (BZ1, Dako, Carpinteria, CA) and anti-gp350/220 monoclonal antibodies by a standard immunoperoxidase staining procedure as described previously.¹⁰⁾

RESULTS

EBV DNA was detected by PCR in 2 of 4 samples of papilloma tissue (cases 1 and 2, Fig. 1A). The positive bands corresponded to 120 and 36 EBV DNA copies per cell by reference to Raji cell DNA (Fig. 1C). The clinical and pathological data for the papilloma cases are listed in Table I.

The results of DNA ISH analysis were consistent with those of PCR. The positive signals recognized in papilloma cells were distributed diffusely in case 1 (data not shown) and in the intermediate and lower epithelial layers in case 2 (Fig. 2A). EBV DNA signals were also detected in Akata cells that had been treated with an antibody to surface immunoglobulin G to induce EBV replication. Akata cells contain about 20 copies of EBV plasmid per

Table I. Profile of Patients

Case no.	Age	Gender	Site	Pathology
1	81	Female	Palate	Fibroepithelial papilloma
2	52	Male	Palate	Squamous cell papilloma
3	10	Male	Tongue	Papilloma
4	51	Male	Gingiva	Squamous cell papilloma

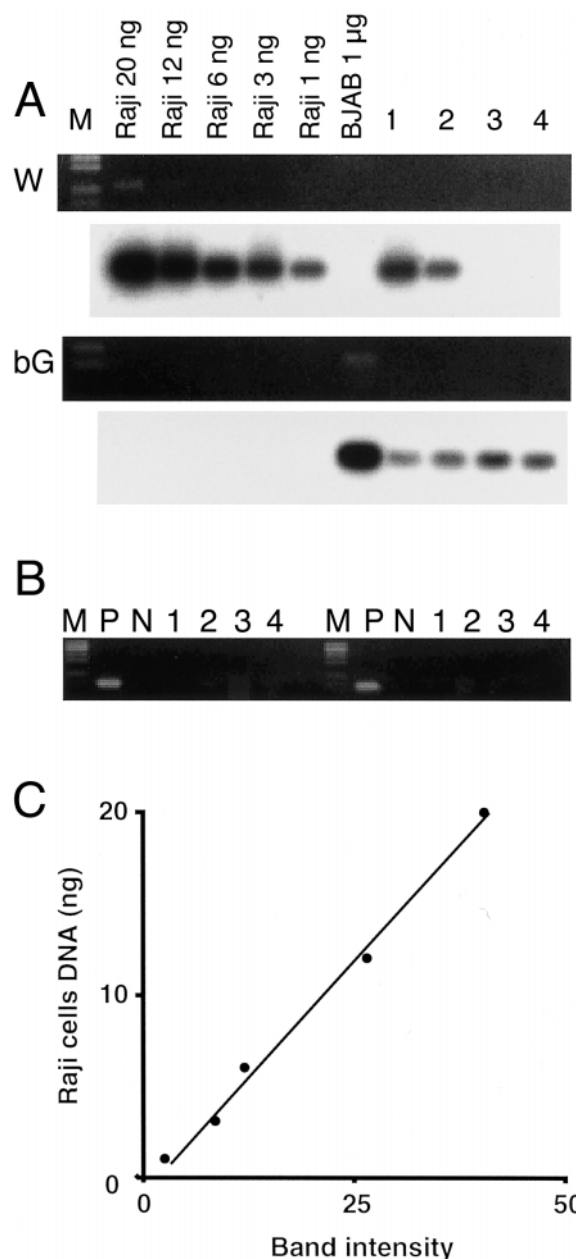


Fig. 1. Southern blot hybridization of serial dilutions of DNA extracted from Raji cells and papilloma tissues (A). M, size marker (*Hae*III digest); W, *Bam*HI W segment of EBV DNA; bG, β-globin. The relationship between band intensity and EBV DNA copy number is expressed as a standard curve (C). The weight of Raji cell DNA applied (y) can be calculated with the following equation when the band intensity (x) ranges between 2.7 to 40.7: $y=0.5x-6.0$. The results of polymerase chain reaction for malignant (16, 18, 31, 33, 35, 52b, 58, left side) and benign (6, 11, right side) types of human papilloma virus are also shown (B). P, positive control; N, water as negative control. The number on each lane indicates the corresponding number of the patient from which the DNA was extracted.

cell. We could not detect positive signals on Akata cells in which EBV replication had not been induced. Therefore, the present method for DNA ISH can detect DNA

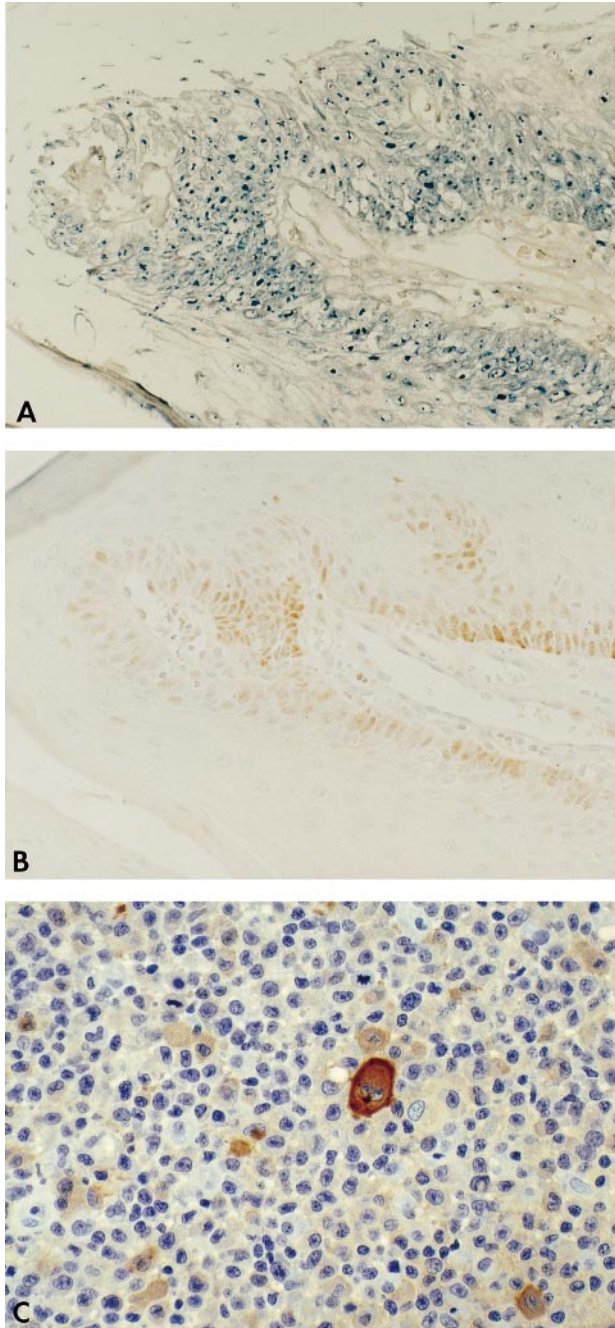


Fig. 2. *In situ* hybridization for EBV DNA with methyl green counterstaining (A) and immunohistochemical staining for LMP1 in the tissue of papilloma (B). LMP 1 staining of an opportunistic lymphoma case which was complicated in a recipient of bone marrow transplantation as a positive control (C). Original magnification, $\times 400$ for A and B, $\times 1000$ for C.

signals in lytic infection only. In contrast, RNA ISH failed to detect positive signals in any of the samples, although positive signals were demonstrated in EBV-positive gastric carcinoma.

Immunohistochemical analysis revealed that some of these EBV DNA-positive cells were also positive for LMP 1 (Fig. 2B). However, they were negative for BZLF 1 or gp350/220.

We then investigated if papilloma samples were infected with HPV. The results of PCR analysis of DNA for malignant (16, 18, 31, 33, 35, 52b, 58, left side) and benign (6, 11, right side) types of HPV are shown in Fig. 1B. Neither type of HPV was detected in any of the papilloma tissues.

DISCUSSION

In this study, we detected EBV DNA and its products in tissues from 2 of 4 papilloma cases. The results of PCR studies were consistent with those of DNA ISH, and no positive signals were seen in other tissues, including squamous cell carcinoma and leukoplakia.

Although EBER1 ISH is widely used for detecting and localizing EBV in EBV-associated diseases,⁹ the present papilloma cases were completely negative for EBER1. The intensity of EBV signals on the papilloma cells detected by DNA ISH was similar to that of the signals detected in EBV-replicating Akata cells. Since it is well established that EBER1 is not expressed in the tissue of hairy leukoplakia, which represents a lytic state of EBV infection,¹⁰ our results suggest that papilloma tissues are in a state of lytic EBV infection. It is known that LMP 1 is highly expressed in lytically infected cells.^{11, 12} The absence of other replicative antigens in this study is not necessarily incompatible with papilloma tissues being in a lytic state, since antigens in paraffin-embedded tissues are often undetectable.¹¹

It has been reported that HPV is often detected in papilloma tissues.¹³⁻¹⁵ However, our papilloma cases were completely negative for both benign and malignant HPVs. With regard to the association between EBV and papilloma, Macdonald and colleagues¹⁶ reported that EBV was detected in 13 of 20 inverted Schneiderian papillomas by PCR. On the other hand Gaffey and associates¹⁷ reported that the association was less frequent; 1 of 12 cases. The present results indicate that some cases of non-inverted-type papilloma in the oral cavity are associated with EBV.

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