J Tokyo Wom Med Univ
71 (2) 99～106 (2001)

Detection of Epstein-Barr Virus in Warthin's Tumor

Yasubumi MARUOKA, Sanzhe CUI, Tomohiro ANDO, Yoichiro KATO*, Noriyuki SHIBATA*, Makio KOBAYASHI*, and Hideki OGIUCHI

Department of Oral and Maxillofacial Surgery
(Director: Prof. Hideki OGIUCHI),
*Department of Pathology (Director: Prof. Makio KOBAYASHI),
Tokyo Women's Medical University, School of Medicine

(Received Oct. 17, 2000)

Epstein-Barr virus (EBV) is known to be associated with Burkitt's lymphoma, and undifferentiated nasopharyngeal carcinoma of the major salivary glands has also been found to harbor EBV. The virus can also be recovered from the saliva of healthy seropositive individuals. To determine the pathogenic role of EBV infection in salivary gland diseases, formalin-fixed, paraffin embedded specimens from 18 cases were used to study EBV by nested PCR, PCR-Southern blot hybridization, and *in situ* PCR for EBNA-1 (EBV nuclear antigen-1) and by immunohistochemistry (IHC) for LMP-1 (latent membrane protein-1). Ten cases of Warthin's tumor (WT) (100%) were positive by PCR and *in situ* PCR, and EBV DNA was detected in the nuclei of the epithelial cells and the surrounding lymphocytes. All ten cases of WT were positive for LMP-1 by IHC, and all the other lesions were negative. The positive signals were found in the cytoplasm of the epithelial cells. These results suggest that EBV infection may be involved in the pathogenesis of WT.

Introduction

Epstein-Barr virus (EBV) was discovered in a lymphoma cells line in 1964, was implicated as a cause of endemic Burkitt's lymphoma[1] and undifferentiated nasopharyngeal carcinoma[2], because the virus genome is present in the tumors and high levels of EBV antibody titers are found in patient's serum. Involvement EBV in disease processes has recently been extended to gastric carcinoma[3], lymphoepithelioma of the thymus[4][5], and T-cell lymphoma[6].

Warthin's tumor (WT) is a benign neoplasm that represents 10% of all epithelial tumors of the major salivary glands. WT is mainly observed in male patients and is almost exclusively limited to parotid gland, where it may manifest itself bilaterally and as multiple lesions. Histologically, WT consists of oncocytic epithelium that lines cystic structures and forms papillary projections in conjunction with a benign lymphoid component.

EBV is a member of the human herpes virus family[7], and after primary infection it persists in the host for life. Circumstantial evidence indicates that EBV is primarily spread by intimate oral contact[8]. The major salivary glands have been found to harbor EBV, and the virus can be recovered from the saliva of healthy seropositive individuals[9][10]. The latent EBV genomes express the viral genes encoding six EBV-determined nuclear antigens (EBNA; EBNA-1, EBNA-2, EBNA-

3a, EBNA-3b, EBNA-3c, and EBNA-LP); three latent membrane proteins (LMP; LMP-1, LMP-2A, LMP-2B), and two EBV encoded small RNAs (EBER; EBER-1, EBER-2)[10]. The detection of EBV in salivary glands has been well documented[11)~18)]. Most previous studies employed PCR or *in situ* hybridization (ISH) to detect EBV DNA or EBER, which is a good marker for latent EBV infection. PCR is an extremely sensitive technique that is capable of amplifying rare or single copy gene sequences to high levels, making them easy to detect by gel electrophoresis and Southern blot hybridization.

However, nucleic acid extraction and cell destruction are required before the PCR amplification, and thus the cellular source of the positive signals has remained unknown. While ISH is useful for detecting EBV DNA, it is occasionally limited by its low sensitivity. ISH for EBER is often used to investigate EBV-infected cells because of their high copy number (106 copy per cell)[19], but as in hairy leukoplakia[20], in which the EBV replicating cells do not express EBER, EBER-negative cells are not always uninfected cells. In addition, apparently contradictory results of ISH for EBV DNA and ISH for EBER have been reported in WT[13)15)]. ISH for EBV DNA detect EBV in the cytoplasm of neoplastic WT cells, whereas the neoplastic epithelial cells of all tumors are negative by ISH for EBER, and only the stromal lymphocytes are positive. Therefore, the association between EBV and human tumors must be clarified by *in situ* detection of EBV DNA by using a more sensitive method. In this study, we used IHC for LMP-1 and, PCR and *in situ* PCR for EBNA-1 to determine the role of EBV in salivary gland tumors.

Materials and Methods

1. Patients and tissue preparations

Salivary gland tumors from 18 cases were selected from the files of the Department of Oral and Maxillofacial Surgery of Tokyo Women's Medical University. The specimens used in this study were formalin-fixed, paraffin-embedded tissue from 10 cases of WT, 4 cases of pleomorphic adenoma, 2 cases of adenoid cystic carcinoma, and 2 cases of mucoepidermoid carcinoma.

2. Nested PCR and PCR-Southern blot hybridization

Two-steps nested PCR and PCR-Southern-blot hybridization[21] were performed. Briefly, DNA was extracted from sections of paraffin-embedded specimens. Two primer sets, which amplify a 297 base pair (bp) and a 209 bp fragment of the EBNA-1 gene, respectively were used for the nested PCR, and consistent results of DNA amplification were obtained in two successive trials (Table 1). Amplification products were tested for the presence of a band corresponding to 209 bp DNA fragment with an 8% agarose gel containing ethidium bromide. The PCR products were Southern blotted on a nylon membrane and hy-

Table 1 Sequences of primers and internal probe used in nested PCR and Southern blot hybridization

Primers/Probe	Nucleotide sequences	Base location	Amplified products
Outer: EB3	5'-AAGGAGGGTGGTTTGGAAAG-3'	109332–109351	297 bp
Outer: EB4	5'-AGACAATGGACTCCCTTAGC-3'	109609–109628	
Inner: EB1	5'-ATCGTGGTCAAGGAGGTTCC-3'	109353–109372	209 bp
Inner: EB2	5'-ACTCAATGGTGTAAGACGAC-3'	109542–109561	
Probe: EBP5	5'-GTTTAAGAGCTCTCCTGGCTA GGAGTCACGTAGAAAGGA-3'	109400–109439	

bridized with an EBP-5 probe (Table 1).

3. *In situ* PCR

After deparaffinizing 4 μm-thick sections on glass slides in xylene, dehydrating them, and digesting them with proteinase K for 30 min at 37 ℃, the sections were treated with 4% paraformaldelye for 5 min at room temperature, washed three times in PBS, washed three times in deionized destilled water (dH_2O), and finally dehydrated with pure ethanol. The total reaction volume was 100 μl and contained 10 μl of Taq buffer, 20 μl of dNTP mixture for first round or a 20 μl digoxigenin-labeled dNTP mixture for the second round, 5 units of Taq DNA polymerase (Takara, Otsu, Japan), and 1.6 μl of each of the EB-3 and EB-4 primers for the first round and 3.2 μl of each of the EB-1 and EB-2 primers for the second round. Prior to *in situ* PCR, the slides were heated to 82 ℃ for 7 min (hot start), the tissue on the glass slides was sealed, the reaction solution was added, and the sections were covered with a coverglass and sealed off with mineral oil to prevent evaporation. Amplification was performed in two successive rounds of 20 cycles of DNA denaturation (1 min at 95 ℃), annealing (1 min at 55 ℃ for the first round and 60 ℃ for the second round), and elongation (1 min at 72 ℃) in an automated *in situ* PCR thermal cycler (ATTO, Tokyo, Japan).

After the first amplification with the outer primers, the coverglass was removed, and the slide were washed once in PBS and once in dH_2O, and then dehydrated in pure ethanol. The second round of amplification was also performed beginning with a hot start. After PCR amplification, the slides were washed three times in PBS, three times in dH_2O, and three times in a 0.1 M Tris HCl (pH 7.5) /0.15 M NaCl solution for 5 min at room-temperature. The slides then blocked with 2% blocking reagent containing 0.3% Triton X and 10% sheep serum 30 min at room-temperature, and incubated with anti-DIG antibody labeled with alkaline phosphatase diluted 500 fold (Boehringer, Mannheim, Germany) at 37 ℃ for 30 min, and then rinsed in a 0.1 M Tris HCl (pH 9.5)/0.2 M NaCl solution containing 50 mM $MgCl_2$. Visualization was achieved by staining with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate disodium salt in the dark and counterstaining with methyl green.

4. Immunohistochemical staining for LMP-1 protein

After deparaffinizing 4-μm-thick sections, hydrating them, and incubating them with 1 : 300 monoclonal anti-LMP-1 antibody (Novacastra, Newcastle, United Kingdom), detection was performed by the Avidin Biotin Complex technique.

Results

Immunostaining of salivary gland tumors revealed expression of EBV protein in the cytoplasm of the epithelial cells in all ten cases of WT, but no evidence of EBV expression in 4 cases of pleomorphic adenoma, 2 cases of adenoid cystic carcinoma, and 2 cases of mucoepidermoid carcinoma (Fig. 3A, B). Electrophoresis of the PCR amplification product yielded a band at the 209 bp position, and positive results were thus obtained in all 10 cases of WT, 2 of the 4 cases of

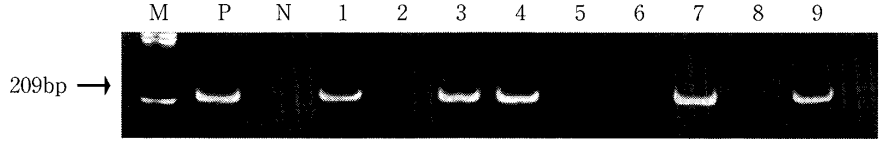

Fig. 1 Gel electrophoresis of nested PCR products for EBV DNA
M: marker, P: positive control, N: negative control, 1: Warthin's tumor, 2~5: pleomorphic adenoma, 6, 7: adenoid cystic carcinoma, 8, 9: mucoepidermoid carcinoma.

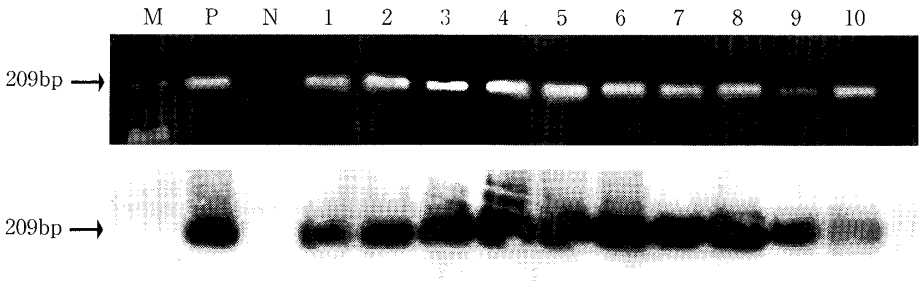

Fig. 2 PCR and PCR-Southern blot hybridization analysis of EBV DNA in Warthin's tumor
M: marker, P: positive control, N: negative control, 1～10: Warthin's tumor.

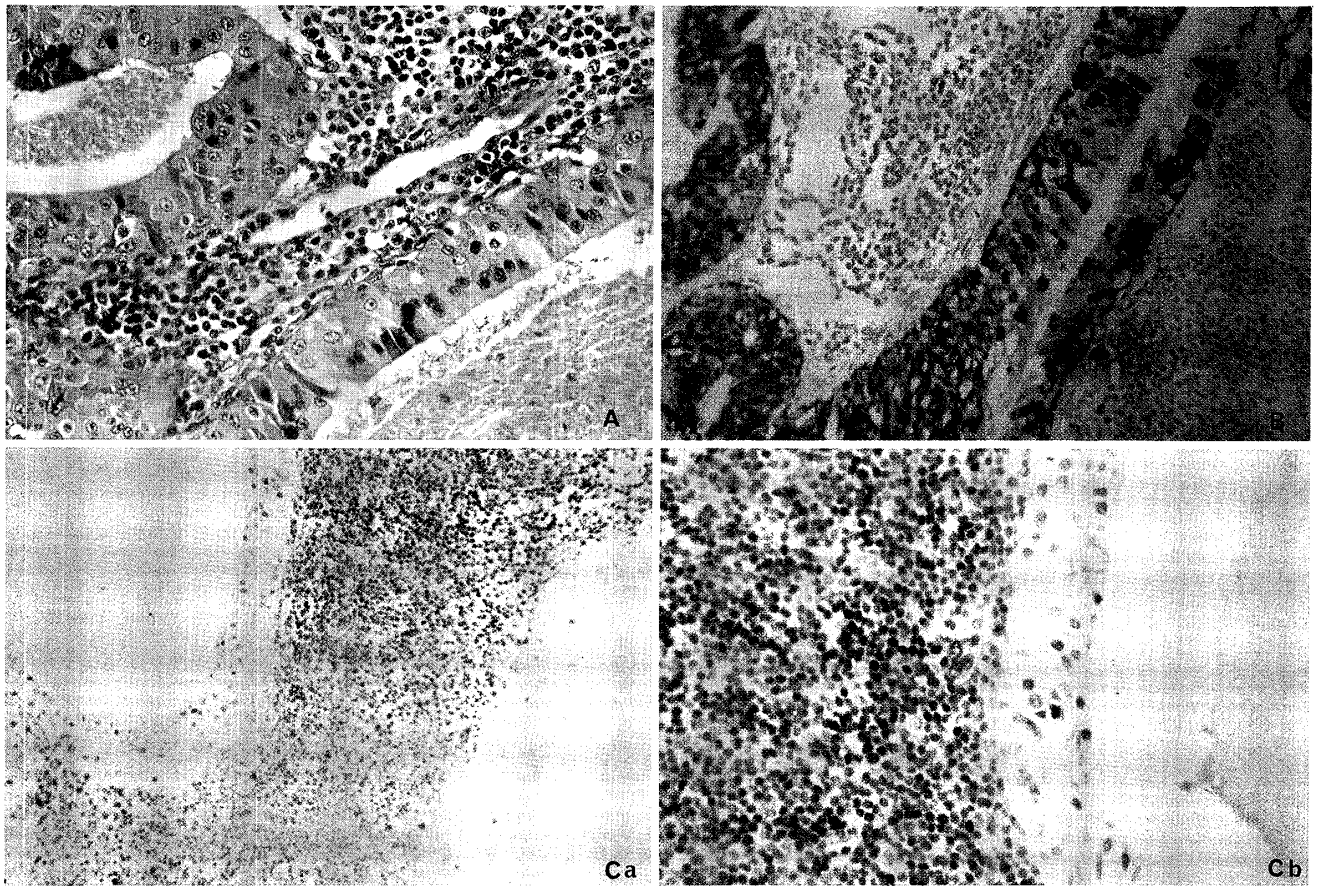

Fig. 3 A: Hematoxylin-eosin stain of Warthin's tumor, original magnification × 200
B: LMP-1 expressed in Warthin's tumor at the cytoplasm of the epithelial cells, original magnification × 200
C: EBV DNA detected in the nuclei of the epithelial cells and the surrounding lymphocytes in Warthin's tumor as examined by *in situ* PCR.
(a) original magnification × 100, (b) original magnification × 200.

pleomorphic adenoma, one of the 2 cases of adenoid cystic carcinoma, and one of the 2 cases of mucoepidermoid carcinoma (Fig. 1, 2). *In situ* PCR revealed a high rate of signals corresponding to the neoplastic cells of the salivary gland and lymphocyte nuclei in the WT patients that was consistent with the results obtained by PCR (Table 2). The positive signals were found in the

Table 2 Expression of EBV genome in salivary gland tumor

Histological types	No. of cases	LMP-1 positive	EBNA-1 positive
Warthin's tumor	10	10	10
Pleomorphic adenoma	4	0	2
Adenoid cystic carcinoma	2	0	1
Mucoepidermoid carcinoma	2	0	1

lymphocytes of the patients with the other diseases, but not in their epithelial cells (Figs 3Ca, b).

Discussion

The 12 EBV proteins that are expressed in latently EBV-infected cells consist of six EBV-determined nuclear antigens (EEBNA-1, -2, -3a, -3b, -3c, -LP), three latent membrane proteins (LMP-1, -2A, -2B), poly-A-negative small RNAs (EBER-1, -2), and a gene product of the BamHIA region (BARF0)[10]. EBNA-1 plays an important role in the replication and preservation of EBV plasmids. LMP-1 is thought to be involved in the inhibition of apoptosis through *bcl-2*, revealing oncogenic activity[21]. Various methods of detecting EBV are available. In this study, we used the protein LMP-1 for immunohistochemical examination. For molecular biological examination by PCR, we used EBNA-1, which is essential for preservation of the intracellular virus episome of the BamHIK region, which exhibits greater conservation with less variation among mutant strains. However, because only a single copy of BamHIK is present per genome, Southern blot hybridization was performed following nested PCR to improve detection sensitivity. Localization of the EBV genome was also investigated by *in situ* PCR assay[22][23], a technique that allows identification of viral infection and gene expression in the target gene structure by highly sensitive detection of PCR in specimens on glass slides. Detection is performed either by the immunohistochemical method following direct incorporation with biotin or digoxigenin as a nucleotide substrate, that is, by the direct method, or by ISH with a labeled probe of the PCR product, the indirect method. In this study, we chose the direct method[23] to detect the EBV genome (EBNA-1).

In an earlier study on the association between salivary gland tumors and EBV, Stephen et al[12], detected EBV DNA signals in the nuclei of carcinoma cells by ISH in all 11 cases of undifferentiated salivary gland carcinoma in Greenland Eskimos. There are many different kinds of salivary gland tumors[12][16]~[18]. It has been pointed out that there is an association between lymphoepithelial carcinoma and EBV in cases of salivary gland tumors. Racial differences have been reported[18] as well: EBV has been found to be correlated with lymphoepithelial carcinoma in Eskimos[12], Chinese, Taiwanese[17] and Japanese[16]. Santucci et al[15], observed EBV DNA signals in the cytoplasm of adenoma cells by ISH in 13/15 cases of bilateral or multiple WT. In Japan, Taira et al[13], detected EBV DNA by PCR in salivary gland tumors, including 7/7 cases of WT, 2/26 cases of pleomorphic adenoma, and 3/5 cases of mucoepidermoid carcinoma. However, in Finland, Vesa et al[14], reported findings no EBV DNA signals by ISH in 219 cases of salivary gland tumor and 17 cases of WT were further studied by PCR, but none of these tumors contained EBV. Discrepancy of these results may be due to the high sensitivity of PCR, which can detect EBV DNA in latently infected lymphocytes. Furthermore, probes and primers detect specific parts of viral genome, which does not exclude the presence of other parts of the genome.

In this study, EBV protein expression was identified in the cytoplasm of the epithelial cells of WT by LMP immunostaining, however, no expression was detected in other salivary gland tumors. PCR detected the band in all specimens of WT, 2/4 of the pleomorphic adenoma specimens, 1/2 of the adenoid cystic carcinoma specimens,

and 1/2 of the mucoepidermoid carcinoma specimens. *In situ* PCR revealed a high rate of signals corresponding to the neoplastic cells of the salivary gland and lymphocyte nuclei in the WT patients. The positive signals were found in the lymphocytes of the patients with the other diseases. EBV DNA (EBNA-1) was detected in every case of WT, suggesting a correlation between WT and EBV. WT is a benign tumor of the parotid gland characterized by columnar epithelial cells and stroma rich in lymph follicles.

Since carcinomas of the nasopharynx is strongly suspected of being related to EBV and is associated with infiltration by numerous lymphocytes, it seems to be common with WT, despite one being benign and the other malignant. The EBV genome in carcinoma of the nasopharynx is found in the epithelial tumor, but it is unknown whether the EBV genome in WT is located in the epithelial tumor or the lymphocytes. *In situ* PCR was performed to clarify the localization of the EBV genome. In WT, a high rate of signals was simultaneously observed in both the lymphocytes from lymph follicles and the nuclei of the epithelial cell portion. However, positive cells were also recognized among the lymphocytes in pleomorphic adenoma, adenoid cystic carcinoma, and mucoepidermoid carcinoma, suggesting that latently EBV-infected lymphocytes are detectable by PCR in cases of salivary gland tumors other than WT. EBV is a possible factor involved in the development of WT. There is a report that since T- and B-lymphocytes and plasmacytes infiltrate directly under the epithelial tissue of WT, an immune reaction may occur in the area between neoplasm and lymphatic tissue. This seems to be attributable to the fact that lymphocytic tissue proliferates and infiltrates as a result of the virus antigen that induces tumors. Further investigation is necessary to elucidate the mechanism by which EBV is involved in the development of WT.

Acknowledgments

We wish to thank professor T. Sawada for the valuable advises. We also thank Mr F. Muramatsu Mrs N. Sakayori and Mr H. Takeiri for their technical assistance.

References

1) **Epstein MA, Achong BG**: The relationship of the virus to Burkitt's lymphoma. The Epstein-Barr Virus (Epstein MA et al eds). pp321–337, Springer-Verlag, Berlin (1964)
2) **Liebowitz D**: Nasopharyngeal carcinoma: the Epstein-Barr virus association. Semin Oncol **21**: 376–381, 1994
3) **Shibata D, Weiss LM**: Epstein-Barr virus associated gastric adenocarcinoma. Am J Pathol **140**: 769–774, 1992
4) **Leyvraz S**: Association of Epstein-Barr virus with thymic carcinoma. N Engl J Med **312**: 1296–1299, 1985
5) **Julia CL, Michael JG, Lawrence MW et al**: The role of Epstein-Barr virus in lymphoepithelioma-like carcinomas. Am J Clin Pathol **103**: 308–315, 1996
6) **Hamilton-Dutoit S, Pallesen G**: A survey of Epstein-Barr virus gene expression in sporadic non-Hodgkin's lymphomas. Detection of Epstein-Barr virus in a subset of peripheral T-cell lymphomas. Am J Pathol **140**: 1315–1325, 1992
7) **Liebowitz D, Kieff E**: Epstein-Barr virus. *In* The Human Herpes Viruses (Roizuman B, Whithley RJ eds) pp107–172, Raven Press, New York (1993)
8) **Gerber P, Nonoyama M, Lucus S et al**: Oral expression of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis. Lancet **2**: 988–989, 1972
9) **Sixbey JW, Davis DS, Young LS et al**: Epstein-Barr virus replication in oropharyngeal epithelial cells. J Gen Virol **68**: 805–811, 1987
10) **Oikawa O**: Studies on tissue distribution and expression of Epstein-Barr virus using polymerase chain reaction. Hokkaido J Med Sci **70**: 729–742, 1995 (in Japanese)
11) **Boulter A, Johnson NW, Birnbaum W et al**: Epstein-Barr virus (EBV) associated lesions of the head and neck. Oral Diseases **2**: 117–124, 1996
12) **Stephen J, Hamilton D, Mrcp A et al**: Undifferential carcinoma of the salivary gland in Greenland Eskimos. Human Pathol **22**: 811–815, 1991
13) **Taira H, Baba K**: The relationship between

Warthin's tumor and Epstein-Barr virus. Practia Otologica **70**: 30–35, 1994

14) **Vesa K, Kari S, Stina S et al**: No Epstein-Barr and cytomegalovirus DNA found in salivary gland tumors. ORL **59**: 97–99, 1997

15) **Santucci M, Gallo O, Calzolari A**: Detection of Epstein-Barr viral genome in tumor cells of Warthin's tumor of parotid gland. Am J Clin Pathol **100**: 662–665, 1993

16) **Nagao H, Ishida K, Sugano I et al**: Epstein-Barr virus associated undifferentiated carcinoma with lymphoid stroma of the salivary gland in Japanese patients. Cancer **78**: 695–703, 1996

17) **Tsai CC, Chen CL, Hsu HC**: Expression of Epstein-Barr virus in carcinomas of major salivary glands: a strong association with lymphoepithelioma-like carcinoma. Hum Pathol **27**: 258–262, 1996

18) **John KC, Timothy TC, William YW et al**: Specific association of Epstein-Barr virus with lymphoepithelial carcinoma among tumors and tumors like lesions of salivary gland. Arch Pathol Lab Med **118**: 994–997, 1994

19) **Chang KL, Chen YY, Shibata D et al**: Description of an *in situ* hybridization methodology for detection of Epstein-Barr virus RNA in paraffin-embedded tissue, with a survey of normal and neoplastic tissue. Diagn Mol Pathol **1**: 246–255, 1992

20) **Thomas JA, Felix DH, Wray D et al**: Epstein-Barr virus gene expression and epithelial cells differentiation in oral hairy leukoplakia. Am J Pathol **139**: 1369–1380, 1991

21) **Vera-Sempere FJ, Burgos JS, Botella MS et al**: Immunohistochemical expression of Epstein-Barr virus-encoded latent membrane protein (LMP-1) in paraffin section of EBV-associated nasopharyngeal carcinoma in Spanish patients. Oral Oncol **32 B**: 163–168, 1996.

22) **Novo G, Gallery F**: An improved technique for the *in situ* detection of DNA after polymerase chain reaction amplification. Am J Pathol **139**: 1239–1244, 1991

23) **Takeuchi H, Kobayashi R, Hasegawa M et al**: Detection of Epstein-Barr virus (EBV) DNA in paraffin section of nasopharyngeal carcinomas expressing no EBV-encoded small RNAs using *in situ* PCR. Arch Virol **142**: 1743–1756, 1997

Warthin 腫瘍における Epstein-Barr ウイルスの発現

東京女子医科大学　医学部　歯科口腔外科学（主任：扇内秀樹教授）
*同　第一病理学（主任：小林槇雄教授）
丸岡（マルオカ）　靖史（ヤスフミ）・崔（サイ）　三哲（サンテツ）・安藤（アンドウ）　智博（トモヒロ）・加藤陽一郎（カトウヨウイチロウ）*
柴田（シバタ）　亮行（ノリユキ）*・小林（コバヤシ）　槇雄（マキオ）*・扇内（オギウチ）　秀樹（ヒデキ）

Epstein-Barr virus（EBV）は Burkitt リンパ腫，上咽頭癌の発生に関連しているだけでなく，最近では胃癌をはじめとし EBV 関連悪性腫瘍が次々と明らかにされてきた．EBV は唾液を介して伝播し，既感染者の唾液中にも EBV がかなりの率で排泄されることから，唾液腺が持続感染の器官として考えられている．今回 EBV 潜伏感染と唾液腺腫瘍との関連を明らかにする目的で，唾液腺腫瘍組織中における EBV の発現を免疫組織化学的，ならびに PCR 法，*in situ* PCR 法を用いて EBV ゲノムの検出を行い検討した．対象は唾液腺腫瘍 18 例を対象とし，その内訳は Warthin 腫瘍 10 例，多形性腺腫 4 例，腺様嚢胞癌 2 例，粘表皮癌 2 例でいずれも 10% ホルマリン固定，パラフィン包埋組織を用いて検討を行った．免疫組織化学的には LMP-1 を 1 次抗体として使用し ABC 法で免疫染色を行った．PCR では，EBV ゲノムの *Bam*HIK 領域中の 209bp を増幅する primer を使用し，nested PCR を行った．さらに EBNA-1 を組織切片上での同定を行うため *in situ* PCR で増幅，検出した結果，唾液腺腫瘍において免疫染色では，Warthin 腫瘍全例の腺腫部分の細胞質に EBV 蛋白の発現が認められたが，その他の唾液腺腫瘍においては認められなかった．PCR では Warthin 腫瘍全例，多形性腺腫（2/4），腺様嚢胞癌（1/2），粘表皮癌（1/2）が陽性であり，*in situ* PCR では，Warthin 腫瘍において腺腫部分およびリンパ球の核に一致して高頻度にシグナルが認められた．しかしその他の唾液腺腫瘍においてはリンパ球に陽性細胞を認めるが，上皮部分は陰性であった．Warthin 腫瘍においては EBV ゲノムが高頻度に認められ，EBV が Warthin 腫瘍の発生に関与している可能性が示唆された．