Abstract: Background. The purpose of this study was to investigate the frequency of Epstein-Barr virus (EBV) latent membrane protein-1 (LMP-1) in tonsils and non-neoplastic nasopharynxes in Taiwan.

Methods. Nest-polymerase chain reaction (nest-PCR) was used to examine the presence of LMP-1 gene in lymphoid hyperplasia from non-neoplastic tonsillar and nasopharyngeal tissues and in tonsillar cancers.

Results. In 152 cases, 64 biopsy tissues were obtained from lymphoid hyperplasia of nasopharynxes, 57 from tonsillectomy of non-neoplastic tonsils, and 31 from tonsillar cancers. LMP-1 was detected in 43.4% of the study group. Nineteen (29.7%) and 29 (50.9%) lymphoid hyperplasias from normal nasopharynxes and tonsils, respectively, and 18 (58.1%) biopsies from tonsillar cancers had positive LMP-1. The 30-base pair (bp) deleted variant was detected in 89.5% and 82.8% of normal nasopharynxes and tonsils, respectively, and in 66.7% of biopsies from tonsillar cancers (p = .198).

Conclusion. This study found that the 30-bp variant was the predominant type of LMP-1 from a healthy population in Taiwan.

Keywords: Epstein-Barr virus (EBV); LMP-1; nest-polymerase chain reaction (nest-PCR); tonsil; nasopharynx

Epstein-Barr virus (EBV) is a common herpesvirus present in all human populations, and it has been associated with the development of several malignancies of epithelial or lymphoid origin: undifferentiated nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma, Hodgkin’s disease, lymphomas in immunocompromised patients, and peripheral T-cell lymphomas. Investigations in Spain and Denmark found a high frequency of deletion of the latent membrane protein-1 gene (del-LMP-1) in Hodgkin’s disease cases with HIV infection, in contrast to ordinary Hodgkin’s disease cases. An Italian study found that the prevalence of del-LMP-1 in the patients with HIV-associated Hodgkin’s disease was significantly higher than that...
detected in peripheral blood cells from other HIV-seropositive patients. These findings suggested that infections with del-LMP-1 variants appear to increase the risk of developing Hodgkin’s disease in HIV-infected patients.3

The fact that EBV infection occurs extensively globally but NPC incidence differs among geographic regions indicates that a subtype of EBV is involved in NPC. LMP-1 of EBV is the most likely candidate virus gene to be involved in epithelium transformation and tumor development. Consequently, significant efforts were undertaken to analyze the 30-bp deletion in LMP-1 for NPC. On the basis of past experience, del-LMP-1 occurred in more than 75% of NPC patients in southern China.4,5 Meanwhile, in Taiwan, del-LMP-1 was found in more than 90% of infected NPC.6,7 Furthermore, del-LMP-1 has less immunogenicity than does the non-del variant, which might cause tumors to develop in immunocompetent hosts by escaping immunosurveillance.8 This phenomenon also might explain why the 30-bp-deleted variant is so frequent in fully immunocompetent NPC-bearing patients. On the contrary, the detection rate of del-LMP-1 in NPC or other EBV-related malignancies in Caucasians was less frequent than in the Chinese group above.2,9–11 Currently, detection of LMP-1 in biopsy specimens or nasopharyngeal swabs from patients carrying NPC has reasonable sensitivity and specificity and is recommended as a reliable method for screening primary NPC or predicting local recurrence for patients treated with definite radiotherapy.12 However, few studies of high endemic areas of NPC have focused on the general population in these areas.

Whether the association of del-LMP-1 with the EBV-related malignancies in endemic regions, such as NPC in south China and Taiwan, results from predominance of del-LMP-1, which has more tumorigenic ability in vitro, remains unclear.13 Such a cause-and-effect relationship may not exist, because predominance of del-LMP-1 also exists in normal populations in areas with a high incidence of these EBV-related diseases. Tsang et al14 found that LMP-1 was not detected by nest-polymerase chain reaction (PCR) in lymphoid hyperplasia in nasopharynxes and tonsils in Taiwan. However, investigations demonstrated that in Hong Kong or Guangzhou, the deleted variant was more prevalent in normal populations with positive LMP-1.

This study detected the prevalence of deleted late membrane protein-1 del-LMP-1 in lymphoid tissues from non-neoplastic nasopharynxes and tonsils to analyze the EBV strains carrying the del-LMP-1 in the general population in Taiwan.

**MATERIALS AND METHODS**

**Tissue Samples.** Patients in the present study were enrolled in a research protocol (the Departments of Radiation Oncology and Otolaryngology–Head and Neck Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan) to analyze the EBV strains that carried the deleted variant in reactive lymphoid tissues of non-neoplastic nasopharynxes from cases that were proven pathologically not to be associated with nasopharyngeal carcinoma, tonsillectomy specimens from patients with chronic hyperplastic tonsillitis, and biopsies from patients with tonsillar cancers. All patients provided written informed consent for participation in the study.

**Tissue Processing.** The tissue samples were obtained from archival paraffin-embedded tissue blocks by cutting 10-μm-thick sections, with a fresh microtome blade being used for each specimen to reduce the risk of contamination.

**DNA Extraction and Purification.** Two 10-μm-thick tissue were placed in a sterile 1.5-ml Eppendorf tube filled with 400 μl of lysis buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl2; 0.45% Tween 20); the sample was then placed to a heat block (Thermolyne dry-bath) to incubate at 70°C for 1 h and then was centrifuged at 12,000 rounds-per-minute (RPM) for 5 min at room temperature. The paraffin solidified on the top of the buffer and was removed from the tube using a sterile pipette tip. The sample tubes were incubated at 60°C for 2 h after 20 μl proteinase K (20mg/ml) (Sigma Chemical Co., St. Louis, MO) was added. Another 10 μl of proteinase K was added to the solutions for more complete tissue digestion, and then the tissue pellets were suspended a vortex and the samples were allowed to digest at 55°C for 2 h. To inactivate proteinase K after digestion, the samples were boiled for 10 min and then centrifuged at 12,000 RPM for 5 min; the supernatants containing DNA were pipetted into a new sterile Microcon microconcentrator (Millipore Corp., Billerica, MA), and DNA purification was performed in accordance with the operating instructions of the manufacturer. The final volume of each specimen was adjusted to approximately 100 μl.
Polymerase Chain Reaction Amplication and Gel Electrophoresis. The PCR was performed in a total volume of 50 μl, containing 5 μl of extracted DNA, sense and antisense primers (50 pmol), 200 μmol/l dNTP, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl₂, and 2 units of (Taq) DNA polymerase (Promega Corp, Madison, WI). The prepared sample was amplified using the following procedures: denaturation at 94°C for 40 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 90 seconds in a programmable thermal controller (MJ Research Co., PTC-100, MA) without the overlay of mineral oil. The products were then examined on 1.5% agarose gel electrophoresis in 1× Tris-Boric acid-EDTA (TBE) solution and stained with ethidium bromide to verify the presence of PCR products for the (LMP-1) gene.

Detection of the Presence of the LMP-1 Gene in the Specimens. Duplex PCR was performed to detect EBV LMP-1 using two sets of primers. The first amplification regions in the EBV LMP-1 gene amplified for identification of viral DNA were as follows: sense, 5’-CCG CTG CCT CAT AGC CCT A-3’; antisense (3’), 5’-TGA TTA GCT AAG GCA TTC CCA-3’. One microliter of first amplified PCR product was then taken for second amplification. The second amplification primers were sense, 5’-AGC GAC TCT GCT GGA AAT GAT-3’; and antisense, 5’-CAA GCC TAT GAC ATG GTA ATG-3’. Amplification of a genomic region in the hemoglobin gene (HEMH-1, 5’-CGT CTC CTT TCC GGA-3’, or HEMH-2, 5’-CAC AGT GAC CTT CCC ATC-3’) served as a marker of the presence of intact genomic DNA. Negative control samples containing water are always processed in parallel to the patient samples. Moreover, DNA from the B95.8 cell line is employed as the EBV positive control.

Auto Sequencing. All specimens in this investigation were sent for direct sequencing. ABI PRISM (Applied Biosystems, Rockville, MD) dRhodamine terminator cycle sequencing ready-reaction kit coupled with the purified DNA was used for amplification. The amplicon was purified by the ethanol/sodium acetate precipitation method and then submitted to a DNA sequencer (ABI 377 sequencer, Applied Biosystems, Foster City, CA) for direct sequencing.

Statistical Analysis. The chi-square test was used to analyze the association of the presence of the LMP-1 and deleted variant with various pathologies and tissue locations. Data were processed with the SPSS program for Windows, version 9.0 (SPSS Inc., Chicago, IL).

RESULTS

Of 152 tissue samples, 64 biopsy tissues were obtained from lymphoid hyperplasia of nasopharynges, 57 from tonsillectomy of non-neoplastic tonsils, and 31 from tonsillar cancers. The samples originated from 55 female and 97 male subjects, with a median age of 36.6 years (range, 2.0–3.6 years).

Detection of LMP-1 gene. The overall frequency of the LMP-1 gene was 43.4% (66 in 152 tissues) for the entire sample group. Nineteen (29.7%), 29 (50.9%), and 18 (58.1%) paraffin-embedded tissue samples from lymphoid hyperplasia of nasopharynges, tonsils, and malignant tonsillar cancers, respectively, were detected to be harboring EBV. No statistically significant differences in the frequency of the LMP-1 gene were observed between normal tonsillar tissues and tonsillar cancers (p = .519). However, both normal and malignant tonsillar tissues displayed higher frequencies of the LMP-1 gene than did lymphoid tissues from non-

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**Table 1.** Frequency of LMP-1 and del-LMP-1 variant in tissues from non-neoplastic nasopharynges and tonsils and from tonsillar cancers.

<table>
<thead>
<tr>
<th>Group</th>
<th>Specimen, no.</th>
<th>Positive LMP-1 no. (%)</th>
<th>Non-del only, no. (%)</th>
<th>Del only</th>
<th>Mixed del and non-del</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-neoplastic nasopharynges</td>
<td>64</td>
<td>19 (29.7%)</td>
<td>1 (5.3%)</td>
<td>17 (89.4%)</td>
<td></td>
</tr>
<tr>
<td>Non-neoplastic tonsils</td>
<td>57</td>
<td>29 (58.1%)</td>
<td>5 (17.2%)</td>
<td>24 (82.8%)</td>
<td></td>
</tr>
<tr>
<td>Tonsillar cancers</td>
<td>31</td>
<td>18 (50.9%)</td>
<td>4 (22.2%)</td>
<td>12 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>152</td>
<td>66 (43.4%)</td>
<td>13 (19.7%)</td>
<td>53 (80.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: del, deleted variant of LMP-1.
neoplastic nasopharynxes ($p = .017$ and .008) (Table 1).

**Detection of del-LMP.** Of 66 tissue samples that were positive for the LMP-1 gene, 53 (80.3%) displayed the existence of del-LMP-1. The detection rate of LMP-1 in tissues with positive LMP-1 gene was 80.3% in the entire group. Moreover, the prevalence of del-LMP-1 by pathologic group was 89.5%, 82.8%, and 66.7% ($p = .198$) in tissues from non-neoplastic nasopharynxes, tonsils, and tonsillar cancers, respectively. The frequencies of del-LMP-1 did not differ significantly among these three groups. Three of these groups showed coexistence of EBV with non-deleted variant and del-LMP-1, and two of these tissues were obtained from tonsillar cancers and one was from lymphoid hyperplasia of the nasopharynx.

**DISCUSSION**

The tonsils, as part of the oropharyngeal epithelial, are organized structures and immunologically are considered to be a site of initial viral approach and viral persistence and replication. Infection and hypertrophy are part of the immunologic reaction of tonsils. Some studies have demonstrated a close relationship between certain infection and tonsillitis, and some have found that EBV can colonize the tonsils of children and that the virus may be involved in the development of recurrent tonsillitis and tonsillar hypertrophy. Because EBV transcripts, EBV genome, and lytic proteins had been detected in tonsillar lymphocytes from healthy donors, EBV-infected cells may confront immunosurveillance and then survive persistently. Thus, EBV replication may occur in tonsillar lymphocytes, and tonsillar lymphoid tissues may be important in maintaining EBV in vivo. In the present investigation, the prevalence of del-LMP-1 in tonsils was higher than that in normal nasopharynxes, supporting the significant role of the tonsils in reservoiring EBV. Normal and malignant tonsils do not differ in the prevalence of LMP-1 and deleted variant.

EBV contains 172,000 base pairs of DNA, including a 25-amino acid N-terminal intracellular region, a 161-amino acid transmembrane region, and a 200-amino acid C-terminal cytoplasmic region. EBV infects up to 95% of adults between 35 and 40 years of age in all populations worldwide and typically persists for the remainder of the life of the host without obvious symptoms. EBV currently is known to be associated with various human lymphoproliferative and neoplastic diseases of epithelial or lymphoid origin, and generally is identified in patients with NPC, B- and T-cell lymphoma, and Hodgkin’s disease.

The LMP-1 with a 30-bp deletion at its C-terminal exhibits transformation activity and induces tumorigenic change; in contrast, this condition was not observed with the B95.8–LMP-1 strain. Conflicting opinions exist regarding whether the del-LMP-1 possesses more tumorigenic potential than the non-deleted variant does. Additionally, a Russian investigation of NPC demonstrated that del-LMP-1 did not correlate with the presence of NPC, and it was concluded that the deleted variant was not a predisposing factor for the development of NPC. Studies of Hodgkin’s disease and nasal lymphoma showed lower prevalence of del-LMP-1 for the lymphoma group than for the reactive lymphoid tissue group. On the other hand, investigations found a high prevalence of del-LMP-1 in Hodgkin’s disease patients with HIV infection, in contrast to ordinary Hodgkin’s disease cases. In south China and Taiwan, the endemic regions of NPC, a predominance of the 30-bp deleted variant was found. Studies on lymphomas and various EBV-related malignancies found a high prevalence of del-LMP-1 compared with that of reactive lymphoid tissue, supporting the tumorigenic role of del-LMP-1 in developing neoplasms. Furthermore, the prevalence of del-LMP-1 in normal populations is not well established. In endemic NPC areas, few studies have investigated the association between del-LMP-1 and normal healthy populations. Unfortunately, most of these studies involved limited case numbers. These studies found that the del-LMP-1 variant was more predominant in normal populations than the non-del-LMP-1, corresponding to the relative prevalence of these variants in NPC-bearing patients or other EBV-associated neoplasms, whereas the reverse occurred in Western EBV-associated malignancies and healthy donors, in which cases non-del-LMP-1 variants were more prominent than del-LMP-1 variants. Zhang et al. reported the prevalence of del-LMP-1 using nest-PCR in endemic and nonendemic regions of China. Zhang et al found no significant difference in the frequency of del-LMP-1 in throat washing specimens from endemic and nonendemic China. Therefore, they concluded that del-LMP-1 exhibits a geographic or ethnic-correlated polymorphism rather than an NPC phenotype.
correlated polymorphism. However, the results of the studies mentioned above may vary because of different sampling methods, deparaffinized management, PCR methods, and the number of PCR amplification cycles.38–37

In our previous study, LMP-1 was not detected using PCR application in biopsies from non-neoplastic tonsils and nasopharynxes and most head and neck cancers. Nevertheless, in the present study, LMP-1 could be detected and analyzed using nest-PCR, which successfully amplifies a few copies of the LMP-1 gene in these normal lymphoid tissues and malignant tonsils.

This investigation detected a predominant prevalence of del-LMP-1 in normal lymphoid tissues in Taiwan. The high prevalence of del-LMP-1 in NPC or other EBV-related malignancies in endemic southern China may exist because of a corresponding frequency of del-LMP-1 exists in the general population. Presently, the mechanism of the development of NPC remains unclear which may be more complicated if it interacts with geographic, ethnic factors, and human immunogenetics. It remains possible that the del-LMP-1, through its transforming activity, contributes to the high incidence of NPC or other EBV-associated malignancies in this geographic region. However, other tumorigenic strains of EBV, such as BamHI variant, which is more frequent in NPC patients in southern China than in healthy Chinese individuals, or XhoI-loss (the loss of XhoI restriction site), which was found in almost 100% of Chinese NPC and in throat washings from 30% to 40% of healthy Chinese, also may influence the development of EBV-related carcinomas or lymphomas.38,39

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