Detection of Epstein-Barr Virus and Human Papillomavirus in Head and Neck Tumors

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Epstein-Barr virus (EBV) is a human herpesvirus that causes widespread infection. It is found to be the causative agent of infectious mononucleosis and is closely associated with Burkitt's lymphoma (5), nasopharyngeal carcinoma (7), and EBV-induced disorders in immunodeficient patients (9). However, only a handful of other epithelial-cell malignancies of the head and neck have been linked to EBV infection (2, 6, 14).

Papillomaviruses, on the other hand, are members of the family Papovaviridae. To date, over 60 types of human papillomaviruses (HPV) have been identified. Like EBV, HPV infects squamous epithelia of the skin and mucosa. In addition to their involvement in benign neoplasms, HPV are implicated in several human cancers, particularly in tumors of the cervix, the anogenital region, the skin (18), and the head and neck (1). Recently, it was reported that both EBV and HPV were detected in oral epithelial tissues of AIDS patients (17). Since both EBV and HPV can infect cells of epithelial origin and are closely associated with carcinomas, it was interesting to evaluate the infections caused by these two viruses in tumor cells.

In the present study, we describe our application of the polymerase chain reaction (PCR) method (15) and DNA sequencing analysis (16) to examine the presence of HPV and EBV in various head and neck tumors.

MATERIALS AND METHODS

Thirty nasopharyngeal-carcinoma biopsy samples and 44 tissue biopsy samples of other head and neck carcinomas (Table 1) were collected from the Departments of Otolaryngology of both Chang-Gung Memorial Hospital and Veterans General Hospital and stored at −70°C for DNA extraction. All of the nasopharyngeal-carcinoma tissue samples examined in this study were diagnosed as poorly differentiated or undifferentiated carcinomas. The other head and neck tumors were all considered squamous cell carcinomas by the pathologists. Also analyzed in this study were 11 normal nasopharynx and oral-cavity tissue specimens. EBV-containing cell lines, B95-8 (ATCC CRL 1612) and Jijoye (ATCC CCL 87), as well as EBV-negative cell lines, CA46 (ATCC CRL 1648) and Ramos (ATCC CRL 1596), were obtained from the American Type Culture Collection and used as controls for the PCR study. Recombinant plasmids containing various types of HPV were used as the HPV type controls (10–12).

DNAs were obtained from the samples as described before (3). Briefly, the homogenized tissues were treated with lysis buffer (0.5% sodium dodecyl sulfate, 10 mM Tris [pH 8.0], 10 mM EDTA) and 1 mg of proteinase K per ml, then purified with phenol-chloroform extraction, and finally precipitated with ethanol.

For PCR, primer set X (5'-AGAAGACAGCGGTATTACT CTA-3' and 5'-GGGTGCTGGGCAAGGCTG-3'), corresponding to the EBV region of strain B95-8 bracketed by nucleotides 169,081 and 169,577 (covering part of the BnLF 1 open reading frame [obtained from EMBL data library]), was used to detect EBV DNA. Nested primer set 5R/SL (5'-GAACGTCAATGTTCAGGACC-3' and 5'-CGTTTCTGATGATCTGC-3') and 5R/SLN (5'-CAATGGTCTT GAACCACAGG-3' and 5'-GCAAAGACATACATCG ACCG-3'), corresponding to regions bracketed by nucleotides 95 and 540 and 102 and 523 of HPV type 16 DNA, respectively (within the E6 open reading frame [obtained from EMBL data library]), were utilized to amplify HPV type 16 DNA. Other primer sets specific for the amplification of HPV types 6, 11, 18, and 33 were the same as those reported before (12). PCR amplifications of EBV and HPV were done separately; each reaction was done with fresh DNA template, corresponding primers, and other PCR reaction components. One hundred microliters of PCR reaction mixture for EBV DNA amplification contained 1 μg of template DNA, 100 pmol of each primer, 1.25 mM each of four deoxyribonucleoside triphosphates (dATP, dCTP,
TABLE 1. Prevalence of EBV and HPV in head and neck tumors

<table>
<thead>
<tr>
<th>Type of carcinoma</th>
<th>No. of samples</th>
<th>EBV</th>
<th>HPV</th>
<th>EBV and HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>6</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Nasopharyngeal</td>
<td>30</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypopharyngeal</td>
<td>12</td>
<td>8</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Laryngeal</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>1*</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neck (metastatic)</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Paranasal sinus</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Laryngeal-carcinoma biopsy sample L-41 contained both HPV type 16 and HPV type 18 DNAs. This particular sample was EBV negative.

Because of the amplification power of the PCR procedures, special steps were taken to minimize the possibility of sample-to-sample contamination and PCR product carry-over. These precautions included UV irradiation of the utensils before and after use and aliquoting of all reagents, including sample DNA.

RESULTS

Primer set X and PCR were used to examine the presence of EBV in tumor biopsy specimens and in normal tissues. A 497-bp band appeared after PCR, indicating the presence of EBV DNA in tumor tissues (Fig. 1A, lanes 2 to 6). EBV DNA was found in all of the nasopharyngeal-carcinoma samples and in 30 of 44 various head and neck carcinoma biopsy samples. The latter 30 EBV-positive specimens included 8 of 12 hypopharyngeal carcinoma samples, 6 of 10 laryngeal carcinoma samples, 4 of 5 metastatic neck carcinoma samples, 4 of 9 oral-cavity carcinoma samples, and all of the samples collected from salivary-gland and paranasal-sinus carcinoma tissues (Table 1). The same result was obtained in all cases when the samples were reexamined a year later.

To evaluate the prevalence of HPV in this group of cancers, the PCR method and type-specific primers for HPV were used. For detection of HPV type 16 DNA in these samples with the first set (5R/SL) of the nested primers, the PCR products show a band of 445 bp on the 2% agarose gel after ethidium bromide staining. Subsequently, these first
products are served as the DNA template for the second set of primers, giving a band of 422 bp. Indeed, a 422-bp band was detected in the PCR products of five head and neck tumor samples as shown in Fig. 1B, lanes 2 to 6. For the presence of HPV types 6, 11, 18, and 33, the PCR products show bands of 263, 144, 351, and 422 bp, respectively (Table 1). Among the samples examined, only two laryngeal-carcinoma samples contained HPV type 11 (sample L-30) and HPV type 18 (sample 41). None of the samples examined contained HPV type 6 or 33 DNA. The DNA fragments with corresponding HPV sequences in these two laryngeal-carcinoma samples were confirmed by digestion of the PCR products with restriction enzymes Nde1 (for HPV type 11) and Xba1 (for HPV type 18). The resulting fragments are 97 and 47 bp for HPV type 11 and 226 and 125 bp for HPV type 18 (12) individually (Fig. 2). By using the methods described above, 14 of 30 nasopharyngeal-carcinoma samples were positive for HPV, and of the 44 other head and neck carcinoma biopsy specimens, 12 were found to contain HPV DNA positive. Interestingly, laryngeal-carcinoma sample L-41 was found to contain both HPV type 16 and HPV type 18. Among the 11 normal tissue samples, only 1 contained EBV and 1 contained HPV type 16 (Table 1).

The PCR products were subcloned into pGEM3, and their sequences were determined to confirm EBV or HPV type 16 positivity in our samples. A total of 10 of the EBV-positive samples and 10 of the HPV type 16-positive samples were further confirmed by direct sequencing of the PCR products. The data indicated that all EBV- or HPV-positive samples contained the corresponding DNA sequences from the EBV and HPV type 16 viral genomes compared with the sequences obtained from the EMBL data base and from our positive controls. Figure 3A shows the sequence data of samples positive for EBV, and Fig. 3B demonstrates the

**DISCUSSION**

Nasopharyngeal carcinoma is a common cancer in South-eastern Asia and has been considered 1 of the top 10 malignant tumors in Taiwan. The data that we present indicate that EBV is closely associated with nasopharyngeal carcinoma as previously reported (3). In addition, of the 30 nasopharyngeal-carcinoma samples studied, 14 were shown to contain HPV DNA sequence (Table 1; Fig. 1). Coinfection of EBV and HPV was detected in other head and neck carcinomas in addition to its nasopharyngeal carcinoma. Three hypopharyngeal-carcinoma samples were positive for both EBV and HPV (Fig. 1A and B). Although the correlation of the presence of EBV with hypopharyngeal carcinoma is not as strong as that with nasopharyngeal carcinoma, it suggests that the virus is possibly associated with this tumor. Similarly, the correlation of HPV with either nasopharyngeal or hypopharyngeal carcinoma implies potential association of these tumors with HPV. EBV and HPV positivity in normal tissues from the oropharynx is low (<10% for EBV and HPV) compared with EBV and HPV positivity for nasopharyngeal carcinoma (100% for EBV and 47% for HPV) and hypopharyngeal carcinoma (67% for EBV and 58% for HPV) tissue samples. A total of 60% (6 of 10) of laryngeal-carcinoma samples contained EBV, but only 20% (2) that were positive for HPV with one sample contained both HPV type 16 and HPV type 18 (L-41). This indicated that our finding ruled out the possibility of detecting the viruses that are normally shed from the oropharynx. Since both EBV and HPV can infect epithelial cells, it is therefore possible that they remain latent in these cells. However, the significance of coinfection by EBV and HPV in the same tumor tissue has not yet been determined.

EBV DNA sequence was also detected in tumors that...
arose from salivary-gland, paranasal-sinus, and neck tissues (Table 1). Similar results were also reported previously (6, 18). Therefore, we cannot rule out the possibility that EBV is involved in the pathogenesis of at least some of these tumors.

In contrast, HPV DNA sequence was not found in most of the tissues examined. Recently, molecular epidemiological evidence indicated that more than 80% of HPV-positive patients contain HPV type 16 and that another 10% of infections are due to HPV type 18 infection among genitally HPV-infected patients in Taiwan (12). In the present study, a similar result for the prevalence of HPV infection in the head and neck cancer group was obtained. In other words, we also found that the majority of HPV infection is due to HPV type 16. The sequence of the E6 open reading frame of HPV type 16 was used to design the nested primers. Since this open reading frame encodes one of the virus-transforming genes, its sequence was maintained in HPV-containing cell lines and tumor samples (4). Use of the nested primers further ensured the sensitivity and specificity of the PCR products (11, 13). Among the samples examined, higher percentages of only nasopharyngeal-carcinoma and hypopharyngeal-carcinoma tissue samples were positive for HPV infection. Although our study showed a low rate of HPV infection in other tissues, it is possible that other HPV types are present since we chose only five different HPV types (types 6, 11, 16, 18, and 33), precluding the detection of HPV types.

The technologies used in this study are by far the most sensitive and specific methods for the detection of viruses in a small amount of tissue sample such as nasopharyngeal-carcinoma tissues (3). The combination of PCR and sequence-specific and nested primers, in conjunction with the confirmation of DNA sequencing analysis or restriction digestions, allows us detect viral-DNA sequence in tumor biopsy samples which usually contained various proportions of both the tumor cells and normal tissues.

In summary, by using sequence-specific primers and the sensitive PCR method (i.e., single copy) for detection of viral DNA in tissues (3, 11), we examined 30 nasopharyngeal-carcinoma samples and 44 biopsy tissues of other head and neck tumors for the presence of EBV and HPV. Our data revealed that at least in some of the tumors, such as nasopharyngeal carcinoma and hypopharyngeal carcinoma, the viral infections are considered significantly frequent. Our data also indicated coinfection of EBV and HPV in samples of nasopharyngeal and hypopharyngeal carcinomas examined. This may suggest a possible role of synergistic effect or interaction of both viruses in the pathogenesis of such tumors. Further detailed studies at both molecular and cellular levels will be needed to confirm this relationship in oncogenesis.

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