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

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The presence of Epstein-Barr virus (EBV) DNA and human papillomavirus (HPV) DNA in 74 head and neck tumor tissues was examined by the polymerase chain reaction and DNA sequencing analysis. EBV DNA sequence was detected in all 30 nasopharyngeal-carcinoma tissue samples and in 30 of 44 other head and neck tumor samples. HPV DNA sequence was detected in 14 of 30 nasopharyngeal-carcinoma tissue samples and in 11 of 44 other tumor samples. Coinfection of both viruses was observed in 14 nasopharyngeal-carcinoma tissue samples but only in 5 other head and neck tumor samples including 3 hypopharyngeal-carcinoma tissue samples. Our data indicate that EBV is closely associated with nasopharyngeal carcinoma and may also be related to hypopharyngeal carcinoma. In addition, a relatively high percentage of EBV-positive nasopharyngeal- and hypopharyngeal-carcinoma tissue specimens contained HPV sequence. The significance of the coexistence of EBV and HPV in these tumor tissues requires further study.

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 mucosae. 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′-AGAAACACGCGTTACTC TGA-3′ and 5′-GGGTGTGGGGCAAAGGGTG-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 sets 5R/5L (5′-GAACTGCAATGTTTCAGGACC-3′ and 5′-CGTGTT CTTGATGATCTGC-3′) and 5RN/5LN (5′-CAATGTTTCA GGACCCACAGG-3′ and 5′-GCAACAAGACATACATCG 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,

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TABLE 1. Prevalence of EBV and HPV in head and neck tumors

Type of carcinoma	No. of samples							
	Total	EBV	HPV					EBV and HPV
			6	11	16	18	33	
Nasopharyngeal	30	30	0	0	14	0	0	14
Hypopharyngeal	12	8	0	0	7	0	0	3
Laryngeal	10	6	0	1	1[a]	1[a]	0	0
Salivary gland	5	5	0	0	0	0	0	0
Neck (metastatic)	5	4	0	0	1	0	0	1
Paranasal sinus	3	3	0	0	1	0	0	1
Oral cavity	9	4	0	0	1	0	0	0
Normal tissue	11	1	0	0	1	0	0	0

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

dTTP, and dCTP), *Taq* buffer, and 2.5 U of *Taq* polymerase (Promega Corporation, Madison, Wis.). The amplification was carried out as previously described (3) with a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction conditions for HPV types 6, 11, 18, and 33 were the same as those used for EBV amplification, with the exception that HPV types 18 and 11 were coamplified within one reaction mixture. DNA amplification of HPV type 16 was done by using the nested primers. The first run of PCR was carried out with the primer set 5R/5L. Five microliters of the PCR products of the first run was then subjected to a second amplification with primer set 5RN/5LN. Each PCR reaction mixture included EBV strains B95-8 and Jijoye or the corresponding plasmids containing various types of HPV as positive controls and CA46, Ramos, pBR322 (plasmid vector), and reaction components (*Taq* polymerase buffer, primers, and deoxyribonucleoside triphosphates) as negative controls. The amplification reaction mixtures for the positive and negative controls were concurrently processed and interspersed among the tumor samples. The PCR products were then analyzed by subjecting 10 μl of the mixture to electrophoresis with a 2% agarose gel. After ethidium bromide staining, the gel was examined under UV light for the presence of target DNA bands. DNA samples that were positive for HPV and EBV were reextracted from the original frozen biopsy tissues and retested by the methods described above 1 year later to rule out the false-positive results.

PCR products positive for the presence of the EBV and HPV type 16 DNAs were treated with the Klenow fragment of DNA polymerase I (Stratagene, La Jolla, Calif.) at room temperature for 20 min and then by phenol extraction and ethanol precipitation. Samples were resuspended in TE (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]) buffer, and the suspensions were ligated overnight with *Sma*I-digested pGEM3 plasmid vector with DNA ligase (Promega Corporation). The DNA inserts were sequenced by the dideoxy chain termination method (16) with T7 DNA polymerase (Promega Corporation) and oligonucleotide primers complementary to the SP6 and T7 promoter sites of the pGEM3 vector. A total of 10 samples positive for EBV and 10 samples positive for HPV type 16 were further confirmed by direct sequencing of the PCR products (8). The samples positive for HPV type 11 and HPV type 18 infections were checked by digestion of the PCR products with restriction enzymes *Nde*I (for HPV type 11) and *Xba*I (for HPV type 18), respectively.

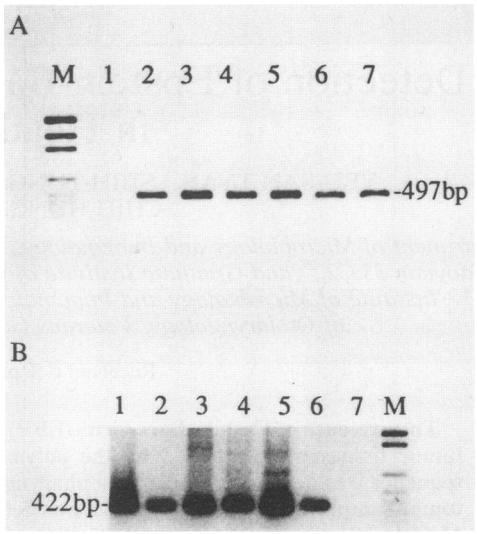

FIG. 1. PCR products of biopsy specimens. (A) PCR products of tumor DNA with primer set X of EBV after staining with ethidium bromide and photography under UV illumination. A DNA band of 497 bp indicated the presence of EBV DNA. Lanes: 1, the component control; 2, B95-8 (EBV-containing cell line) as the positive control; 3 to 7, NPC-133, NPC-125, hypopharyngeal-carcinoma tissues L-7 and L-8, and laryngeal-carcinoma tissue L-9, respectively; M, molecular weight markers (2,645, 1,605, 1,198, 676, 517, 460, 396, 350, and 222 bp, respectively). (B) PCR products of tumor DNA with nested primer sets of HPV. A DNA band of 422 bp for HPV-containing DNA after ethidium bromide staining was observed. Lanes: 1, pHPV-16 as the positive control; 2 to 6, the same order as lanes 3 to 7 described for panel A; 7, the component control; M, molecular weight markers (1,605, 1,198, 676, 517, 460, 396, and 350 bp, respectively).

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 carryover. 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 (set 5R/5L) 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

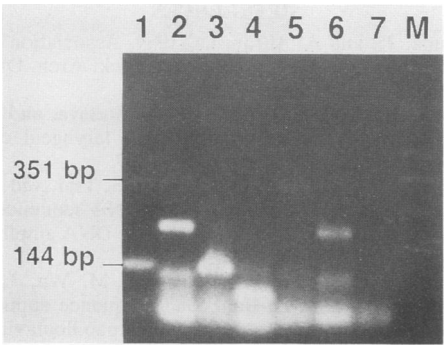

FIG. 2. PCR products of samples containing HPV types 11 and 18. Lane 1, PCR products of coamplification with primers specific for HPV types 11 and 18 with positive-control plasmid DNA. Two bands (351 bp for HPV type 18 and 144 bp for HPV type 11) indicated the presence of the viral-DNA fragments. Lane 2, DNA fragments digested with *Nde*I and *Xba*I, resulting in four fragments with sizes of 226, 125, 97, and 47 bp; lane 3, PCR product of laryngeal-carcinoma sample (L-30) which contained HPV type 11; lane 4, the two DNA fragments (97 and 47 bp) resulting from the digestion of the PCR product of lane 3 with *Nde*I; lane 5, a band of 351-bp (HPV type 18) PCR products of sample L-41 after ethidium bromide staining; lane 6, the *Xba*I-digested DNA fragments (226 and 125 bp, respectively) of the product shown in lane 5. The 47-bp fragment comigrated with the unincorporated primers in lanes 2 and 4. Lane 7, negative control, showing the unincorporated primers only. M, molecular weight markers (1,605, 1,198, 676, 517, 460, 396, and 350 bp, respectively).

A EBV SEQUENCE

AGAAACACGC GTTACTCTGA CGTAGCCGCC CTACATAAGC CTCTCACACT GCTCTGCCCC
CTTCTTTCCT CAACTGCCTT GCTCCTGACA CACTGCCCTG AGGATGGAAC ACGACCTTGA
GAGGGGCCCA CCGGGCCCGC GACGGCCCCC TCGAGGACCC CCCCTCTCCT CTTCCCTAGG
CCTTGCTCTC CTTCTCCTCC TCTTGGCGCT ACTGTTTTGG CTGTACATCG TTATGAGTGA
CTGGACTGGA GGAGCCCTCC TTGTCCTCTA TTCCTTTGCT CTCATGCTTA TAATTATAAT
TTTGATCATC TTTATCTTCA GAAGAGACCT TCTCTGTCCA CTTGGAGCCC TTTGTATACT
CCTACTGATG AGTAAGTATT ACACCCTTTG CCCCACACCC CCTTTCCCTT ACTCTTCCTT
CTCTAACGCA CTTTCTCCTC TTTCCCCAGT CACCCTCCTG CTCATCGCTC TCTGGAATTT
GCACGGACAG GCATTGT

B HPV SEQUENCE

CAATGTTTCA GGACCCACAG GAGCGACCCA GAAAGTTACC ACAGTTATG ACAGAGCTGC
AAACAACTAT ACATGATATA ATATTAGAAT GTGTGTACTG CAAGCAACAG TTACTGCGAC
GTGAGGTATA TGACTTTGCT TTTCGGGATT TATGCATAGT ATATAGAGAT GGGAATCCAT
ATGCTGTATG TGATAAATGT TTAAAGTTTT ATTCTAAAAT TAGTGAGTAT AGACATTATT
GTTATAGTTT GTATGGAACA ACATTAGAAC AGCAATACAA CAAACCGTTG TGTGATTTGT
TAATTAGGTG TATTAACTGT CAAAAGCCAC TGTGTCCTGA AGAAAAGCAA AGACATCTGG
ACAAAAAGCA AAGATTCCAT AATATAAGGG GTCGGTGGAC *CGGTCGATGT ATGTCTTGTT*
GC

FIG. 3. Nucleotide sequences of the PCR products derived from the samples containing EBV and HPV. The PCR products were cloned into the *Sma*I site of pGEM3 and then sequenced by the Sanger dideoxy method (16) or sequenced directly by PCR (8). (A) EBV sequence from the 497-bp PCR fragments from nasopharyngeal-carcinoma biopsy samples compared with the EBV sequence from the EMBL data base. Similar sequences were obtained with the other head and neck tumor samples containing EBV. (B) HPV sequence from the 422-bp PCR products. All of the samples containing HPV type 16 had more than 99% sequence homology and also had homology to the HPV type 16 sequence registered in the EMBL data base. The primers used in the PCR reactions are in italics.

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 421 bp, respectively, on the 2% agarose gel. 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 *Nde*I (for HPV type 11) and *Xba*I (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 be 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 HPV type 16-corresponding sequences from these samples. Therefore, the PCR products indeed represent the presence of the virus sequences in the samples.

DISCUSSION

Nasopharyngeal carcinoma is a common cancer in Southeastern 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 with EBV and HPV was detected in other head and neck carcinomas in addition to in 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 those 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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