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# Human papillomavirus type 16 DNA detected by the polymerase chain reaction in non-cancer tissues of the head and neck

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**Abstract** Cancer-free tissues from various anatomical subsites in the head and neck were examined by the polymerase chain reaction (PCR) for the incidence of human papillomavirus (HPV) types 16 and 18. We detected HPV-16 DNA in 9 of 103 samples (8.7%), including specimens from the paranasal sinuses, tonsil, hypopharynx and larynx. However, no HPV-16/18 DNA was detected by Southern hybridization in these 9 samples. The significance of the presence of HPV-16 DNA in non-cancer tissues is still unknown, but PCR detection only of high-risk HPV DNA in head and neck cancer should be evaluated cautiously because of its ubiquity in this region.

**Key words** Human papillomavirus · Head and neck cancer · Polymerase chain reaction · Southern blot hybridization

## Introduction

Several types of human papillomaviruses (HPV) are risk factors for human neoplasms [22]. In particular, HPV DNAs of types 16 and 18 have been closely associated with squamous cell carinoma of the mucoepithelium. These "high-risk" HPV DNAs have been detected in head and neck cancers [1–5, 9, 10, 12, 16, 18, 19], and we also reported the detection of HPV types 16 and 18 DNA in 12 out of 77 cancers (15.6%) in the head and neck in Japan, inlcuding specimens from nasal/paranasal, tonsillar, hypopharyngeal and laryngeal squamous cell carcinomas [14]. Based on these data, a role of HPV 16/18 DNA in the carcinogenesis of head and neck cancer was suspected.

For further understanding of the role of HPV in head and neck cancer, the incidence of HPV in cancer tissue should be compared with that in non-cancer tissue which is anatomically matched from the same region. The presence of these high-risk HPVs in non-cancer tissue has been reported in the anogenital region [11, 21]. However, relatively little has been known up to now about the incidence of HPV in non-cancer tissue in the head and neck. HPV-16/18 DNA has been detected only in samples obtained from cheek or tongue mucoepithelia [6–8, 20], and the detection rate of high-risk HPV in non-cancer tissue of the head and neck, except for the oral cavity, has been examined insufficiently. In this study, we examined the presence of HPV 16/18 DNA in non-cancer samples from various areas of the head and neck.

## Materials and methods

### Tissue specimens

Specimen samples comprised 103 cancer-free tissues from patients aged 1–82 years treated at the Department of Otolaryngoloy, Okayama University Medical School. Full-depth epithelial specimens included basal layers and were surgically removed from sites within the upper aerodigestive tract (58 from the pharynx, 40 from the larynx and 8 from the nasal cavities and paranasal sinuses) (Table 1). Portions of samples were fixed with formalin and embedded into paraffin blocks for routine histological examination with hematoxylin-eosin staining. The remaining tissues were stored at –80°C until DNA extraction. Some "normal" samples (30 cases) were histologically normal mucoepithelia from the cancer patients that were located at a distance from the cancerous area. All cancer samples were previously proven not to contain HPV-16 or 18 DNA by polymerase chain reaction (PCR) and Southern hybridization [14]. Sixty-six other cases were diagnosed clinically and histologically as non-proliferative mucoepithelium showing simple inflammation or lymphoid hyperplasia. Seven cases of leukoplakia were also obtained as non-cancerous benign proliferative tissues from the oral cavity and larynx.

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**Table 1** Detection of HPV 16/18 DNA in non-cancer tissues from the head and neck

| Site of biopsy | Diagnosis | HPV-positive rate[a] | |
|---|---|---|---|
| *Pharynx* | | | |
| Adenoids | Adenoid hyperplasia | 0/ 8 | |
| Tonsils | Chronic tonsillitis | 5/ 38 | (13.2%) |
| Oral cavity | Normal | 0/ 4 | |
| | Leukoplakia | 0/ 2 | |
| Hypopharynx | Normal | 1/ 3 | (33.3%) |
| *Larynx* | | | |
| | Normal | 2/ 21 | (9.5%) |
| | Hyperkeratosis | 0/ 1 | |
| | Polyp | 0/ 9 | |
| | Granuloma | 0/ 1 | |
| | Leukoplakia | 0/ 5 | |
| | Chronic inflammation | 0/ 3 | |
| *Nasal/paranasal cavities* | | | |
| | Granuloma | 0/ 1 | |
| | Chronic sinusitis | 1/ 3 | (33.3%) |
| | Polyp | 0/ 1 | |
| | Normal | 0/ 3 | |
| Total | | 9/103 | (8.7%) |

[a] Number of HPV-positive cases/total cases in each anatomical site

DNA extraction

To extract DNA, tissue specimens were homogenized and incubated at 37°C overnight in 10 m*M* TRIS-HCl (pH 8.0) and 1 m*M* ethylenediamine tetraacetate (TE buffer) containing 1% sodium dodecyl sulfate and 250 µg/ml proteinase K. DNA was isolated from lysates by phenol-chloroform extraction followed by RNase digestion [13]. Purified DNAs were suspended in TE buffer and stored at –4°C until use.

Polymerase chain reaction

Oligomer primers and probes were designed to amplify and detect the E6 ORF of HPV 16/18 DNA. The sequences were the same as those reported by Shimada et al. [15]: 5´-AAGGGCGTAACC-GAAATCGGT-3´ was the universal sense primer for both HPV 16 and 18; 5´-GTTTGCAGCTCTGTGCATA-3´ was the antisense primer for HPV 16; 5´-CATTTTATGCACCAAAAGAGAACTG-CAATG-3´ was the probe for HPV 16; 5´-GTGTTCAGTTCCGT-GCACA-3´ was the antisense primer for HPV 18 and 5´-TGAG-AAACACACCACAATACTATGGCGCGC-3´ was the probe for HPV 18. These primers and probes were synthesized on an Applied Biosystems DNA synthesizer. PCR mixtures containing 0.5 µg template DNA, 0.2 µ*M* of each primer, 200 µ*M* each of adenosine triphosphate, guanosine triphosphate, cytosine triphosphate, thymidine triphosphate, and 1.25 unit of *Taq* polymerase were prepared in 50 µl reaction volumes using the GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus, Norwalk, Conn., USA). DNA amplification by PCR was performed on a DNA thermal cycler (Astec, Fukuoka, Japan) for 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C. After amplification, 10 µl of the reaction mixture was visualized by electrophoresis on 3% agarose gels, followed by ethidium bromide staining [12].

Slot blot hybridization

PCR products were denatured with 0.2 N NaOH and blotted onto Nytran membrane (Schleicher and Shuell, Dassel, Germany) using a slot blotter (Bethesda Res. Lab., Gaithersburg, Md., USA). Oligonucleotide probes labeled by α-$^{32}$Pdd ATP (Amersham, Tokyo, Japan) with a terminal transferase kit (Boehringer, Mannheim, Germany) were used for hybridization with blotted PCR products under stringent conditions [14].

The presence of hybrids was determined by exposing Nytran membranes to X-ray films for 3–5 h at –70°C.

Southern hybridization

The sample DNAs proven to contain HPV 16 or 18 DNA by PCR were examined further by Southern blot hybridization. HPV 16 and 18 DNAs were obtained from Dr. H. zur Hausen, Heidelberg, Germany, and were labeled with digoxigenin-deoxyuridine triphosphate, using a kit from Boehringer, Mannheim, Germany. Sample DNAs were digested with *Bam*HI and electrophoresed in 0.8% agarose gels. They were then transferred to Nytran membranes by Southern blotting. After hybridizing the sample DNAs with the probes under stringent conditions, hybrid bands were detected by methods described previously [13, 14].

## Results

### Detection of amplified DNA by PCR

Amplified HPV 16/18 DNA was confirmed by the presence of a band at the position of 140 base pairs in 3% agarose gels after ethidium bromide staining (Fig. 1). Reliability of PCR efficiency was determined by amplification of cloned HPV 16 and 18 DNA. No amplified DNA band was observed with the DNA extracted from human embryonic cells or with DNA-free distilled water.

In 9 out of 103 PCR products (8.7%), a single band corresponding to 140 base pairs was observed. Five samples were from tonsillar tissues, 2 from a false cord, 1 from the hypopharyngeal mucosa and 1 from the maxillary mucoepithelium. The incidence of each anatomical subsite was 6 of 58 (10.3%) in the pharynx, 2 of 40 (5%) in the larynx and 1 of 8 (12.5%) in the paranasal sinus, respectively (Table 1). Among these high-risk HPV-positive cases, 2 laryngeal and 1 hypopharyngeal normal samples were obtained from the patients with laryngeal cancers

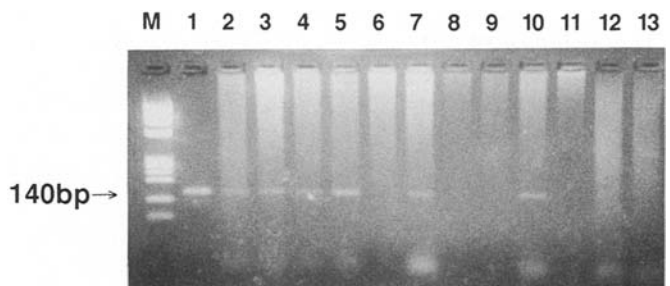


**Fig. 1** Detection of HPV-16 DNA amplified by polymerase chain reaction (PCR). PCR products (10 µl) were electrophoresed through a 3% agarose gel and bands corresponding to 140 base pairs were visualized by ethidium bromide staining. *Lanes: M*, phix DNA digested by *Hae*III as size marker; *1*, HPV 16 DNA as positive control; *2–12*, DNA samples from non-cancer tissues. *Lanes 2–5, 7 and 10* show positive bands; *13*, HEC DNA as negative control

**Table 2** HPV-positive non-cancer cases

| Region | Diagnosis | Age | Sex | HPV type |
|---|---|---|---|---|
| Oropharynx | Chronic tonsillitis | 41 | M | 16 |
| | Chronic tonsillitis | 4 | M | 16 |
| | Chronic tonsillitis | 19 | F | 16 |
| | Chronic tonsillitis | 24 | M | 16 |
| | Chronic tonsillitis | 22 | M | 16 |
| Hypopharynx | Normal | 65 | M | 16 |
| Larynx | Normal | 56 | M | 16 |
| | Normal | 82 | M | 16 |
| Nasal/paranasal cavities | Chronic sinusitis | 59 | F | 16 |

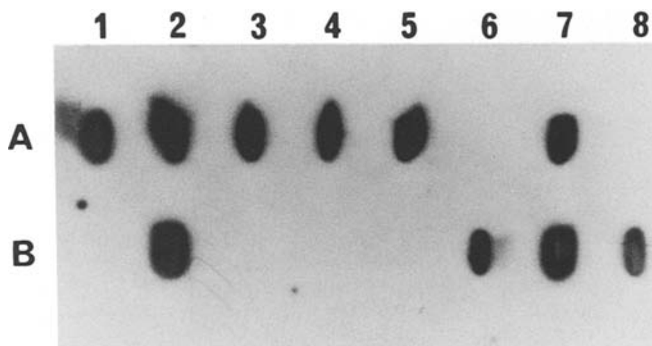


**Fig. 2** Slot blot hybridization of PCR products with the $^{32}$P-labeled synthetic HPV-16 probe. *A1*, HPV-16 DNA as positive control; *B1*, HEC DNA as negative control; *A2–A8* and *B2-B8* were DNA samples from non-cancer tissues. *A2–A5, A7, B2, B6–B8* showed positive signals

which were proved not to contain the HPV-16/18 DNA. The rest of the 6 samples were obtained from chronic inflammatory tissues from tosillitis and chronic sinusitis (Table 2). No HPV involvement was identified in 7 cases of oral or laryngeal hyperkeratosis or leukoplakia.

All HPV-positive PCR products hybridized with a synthesized HPV 16-specific probe by slot hybridization (Fig. 2), but not with that of HPV 18 (data now shown).

### Southern hybridization

The HPV-positive cellular DNAs demonstrated by the PCR were hybridized after Southern blotting with whole cloned HPV 16 and 18 DNA probes However, no hybridization signals were detected in any of these samples.

## Discussion

In the present study, HPV-16 DNA was detected in non-cancer tissues from various anatomical sites in the head and neck. The overall incidence of HPV-16 in non-cancer tissues was 9 out of 103 samples (8.7%). We previously detected DNAs of HPV types 16 and 18 in 12 out of 77 head and neck cancers (15.6%) by PCR [14]. This incidence of HPV in non-cancer tissues detected by PCR is not negligible in comparison with that in cancer tissues. However, certain risks exist in relating HPV to carcinogenesis if it is only based on the detection of these viral sequences by PCR.

As far as we could determine, the available references concerning the frequency of HPV DNA in clinically normal tissue were limited almost completely to the oral cavity. Four different laboratories have reported this occurrence [6–8, 20]. According to these reports, 8–23% samples positive by PCR or 6.9–41.7% positive by Southern blot hybridization of normal oral samples contained the high-risk HPV DNA. However, we were unable to detect HPV-16/18 DNA in the samples obtained from the oral cavity with similar methods. This difference may have resulted from the following factors: (1) sample numbers, (2) technical problems, (3) geographical difference. It is still possible to detect high-risk HPVs if more oral samples are examined. Nonetheless, various methodological modifications may cause different sensitivities, specificities, and therefore different incidences. At least, the detectable limit of HPV-16 DNA in our study was 0.01 pg by PCR, and 10 pg by Southern blot hybridization [14]. Ours is the first report about Japanese latent or asymptomatic infection of HPV in non-cancer tissues of the head and neck. According to studies on the prevalence of HPV DNA in the normal cervix of Japanese women using PCR [11, 21], a relatively low incidence of HPV was noted compared with that found in the United States or United Kingdom. In general, the prevalence of HPV in Japan seems to be less frequent than that in Western countries.

No HPV DNA was detectable by Southern hybridization even in HPV DNA-positive samples by PCR in our present study. This difference reflects the sensitivity of both methods, as mentioned above. Although the presence of HPV in non-cancer tissue seems to imply latent or asymptomatic infection, the role of a small quantity of high-risk HPVs is still uncertain. However, the presence of a limited copy number of HPV DNA itself cannot exclude the possibility that HPV is a risk factor for future carcinogenesis. It has been reported that stimulation from chronic irritation can activate latent papillomavirus genes [17]. If such is true, it should be realized that the epithelium in the head and neck is frequently exposed to numerous dietary or inhalant carcinogens and these entities may interact with high-risk HPV genomes in malignant transformation.

At the least interpretation of the prevalence of HPV DNA in cancers should be cautious because a variety of non-cancer samples from the head and neck commonly contain "high-risk" HPVs.

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## References


1. Brandsma JL, Abramson AI (1989) Association of papillomavirus with cancers of the head and neck. Arch Otolaryngol Head Neck Surg 115:621–625
2. De Villiers EM (1989) Papilloma viruses in cancers and papillomas of the aerodigestive tract. Biomed Pharmacother 43:31–36
3. Dickens P, Srivastava G, Liu YT (1992) Human papillomavirus 16/18 and nasopharyngeal carcinoma. J Clin Pathol 45:81–82
4. Hoshikawa T, Nakajima T, Uhara H, Gotoh M, Shimosato Y, Tsutsumi K, Ono I, Ebihara S (1990) Detection of human papillomavirus DNA in laryngeal squamous cell carcinomas by polymerase chain reaction. Laryngoscope 100:647–650
5. Ishibashi T, Matsushima S, Tsunokawa Y, Asai M, Nomura Y, Sugimura T, Terada M (1990) Human papillomavirus DNA in squamous cell carcinoma of the upper aerodigestive tract. Arch Otolaryngol Head Neck Surg 116:294–298
6. Jenison SA, Xiu-ping Yu, Valentine JM, Koutsky LA, Christiansen AE, Beckmann AM, Galloway DA (1990) Evidence of prevalent genital type human papillomavirus infection in adults and children. J Infect Dis 162:60–69
7. Kashima H, Kutcher M, Kessis T, Levin LS, Villiers EM de, Shah K (1990) Human papillomavirus in squamous cell carcinoma, leukoplakia, lichen planus and clinically normal epithelium of the oral cavity. Ann Otol Rhinol Laryngol 99:55–61
8. Maitland NJ, Cox MF, Lynas C, Prime SS, Scully C (1987) Detection of human papillomavirus DNA in biopsies of human oral tissue. Br J Cancer 56:245–250
9. Morgan DW, Abdullah V, Quiney R Myint S (1991) Human papillomavirus and carcinoma of the laryngopharynx. J Laryngol Otol 105:288–290
10. Niedobitek G, Pitteroff S, Herbst H, Shepherd P, Finn T, Anagnostopoulos I, Stein H (1990) Detection of human papillomavirus type 16 DNA in carcinomas of the palatine tonsil. J Clin Pathol 43:918–921
11. Nishikawa A, Fukushima M, Shimada M, Yamakawa Y, Shimano S, Kato I, Fujinaga K (1991) Relatively low prevalence of human papillomavirus 16, 18 and 33 DNA in the normal cervices of Japanese women shown by polymerase chain reaction. Jpn J Cancer Res 82:532–538
12. Ogura H, Watanabe S, Fukushima K, Masuda Y, Fujiwara T, Yabe Y (1991) Presence of human papillomavirus type 18 DNA in a pharyngeal and a laryngeal carcinoma. Jpn J Cancer Res 82:1184–1186
13. Ogura H, Kawakami T, Fujiwara T, Sakai A, Saito R, Watanabe S, Masuda Y, Yabe Y (1992) Detection of human papillomavirus type 6f genome in nasal papillomatosis. Acta Otolaryngol (Stockh) 112:115–118
14. Ogura H, Watanabe S, Fukushima K, Masuda Y, Fujiwara T, Yabe Y (1993) Human papillomavirus DNA in squamous cell carcinomas of respiratory and upper digestive tracts. Jpn J Clin Oncol 23:221–225
15. Shimada M, Fukushima M, Mukai H, Kato I, Nishikawa A, Fujinaga K (1990) Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. Jpn J Cancer Res 81:1–5
16. Shindoh M, Sawada Y, Kohgo T, Amemiya A, Fujinaga K (1992) Detection of human papillomavirus DNA sequence in tongue squamous cell carcinoma utilizing the polymerase chain reaction method. Int J Cancer 50:167–171
17. Siegsmund M, Wayss K, Amtmann E (1991) Activation of latent papillomavirus genomes by chronic mechanical irritation. J Gen Virol 72:2787–2789
18. Steinberg BM (1990) Human papillomaviruses and upper airway oncogenesis. Am J Otolaryngol 11:370–374
19. Watts SL, Brewer EE, Fry TL, Hill C (1991) Human papillomavirus DNA types in squamous cell carcinomas of the head and neck. Oral Surg Oral Med Oral Pathol 71:701–707
20. Yeudall WA, Campo MS (1991) Human papillomavirus DNA in biopsies of oral tissue. J Gen Virol 72:173–176
21. Yokota H, Yoshikawa H, Shiromizu K, Kawana T, Mizuno M (1990) Detection of human papillomavirus types 6/11, 16 and 18 in exfoliated cells from the uterine cervices of Japanese women with and without lesion. Jpn J Cancer Res 81:896–901
22. Zur Hausen H, Schneider A (1987) The papillomaviruses. In: Howley PM, Salzman NP (eds) The papillomaviridae, vol 2. Plenum Press, New York, pp 245–263