HUMAN PAPILLOMAVIRUS TYPE 6 AND 11 IN LARYNGEAL PAPILLOMAS

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Introduction

More than sixty different types of the human papillomaviruses (HPV) have been found so far⁷. It has been proved that some of them are specifically and closely associated with certain benign or malignant tumors of dermatological and gynecological origin. Recently, data showing the presence of HPV genome in head and neck tumors are accumulating⁴-⁸.

Papillomas are the most frequent laryngeal tumor and it is well known that multiple laryngeal papillomas are recurrent and resist treatment. We attempted to confirm the etiological role of HPV in laryngeal papilloma, using the techniques of histopathology, immunohistochemistry, dot blot and Southern blot hybridization. Laryngeal polyps were also examined as a control. Among these HPV detection methods, Southern blot hybridization is the most reliable technique, and furthermore, classification of HPV is also possible. The identification was homology with molecular cloned probes under stringent conditions.

Materials and Methods

Papilloma and polyp samples

The present series consists of 5 patients of laryngeal papilloma and 5 patients of laryngeal polyp operatively treated at the Department of Otolaryngology, Okayama University Medical School, during the years 1981 to 1990. A part of each papilloma was fixed and processed for light microscopic examination and the rest of the unfixed samples was kept frozen at −70°C until this study. The diagnosis of

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every tumor was confirmed by histological examination. Age, sex and clinical features of the patients are summarized in Table 1.

**Histopathology**

Each sample stained with hematoxylin-eosin was examined for the histomorphological change characteristic of HPV infection, or koilocytosis, under light microscope.

**Immunohistochemical staining**

Sections prepared from paraffin-embedded specimens were dewaxed with xylene and ethanol. Endogenous peroxidase activity was eliminated by 0.3% H2O2 treatment for 30 min at room temperature. After the procedure, each section was treated with 0.1% trypsin at 37°C and washed with 0.1%NaN-containing fetal calf serum. The papillomavirus genus specific antigen (pgs-antigen) was detected by commercially available antibody from DAKO Company (Denmark). The antibody was diluted 500 times and reacted with each section for 60 min at room temperature. After being washed by phosphate buffered saline with detergent, these sections were reacted with horseradish peroxidase-labeled, anti-rabbit IgG, goat IgG for 30 min at room temperature. After the treatments, sections were stained with buffer containing H2O2 with diaminobenzidin and hematoxylin for morphological study. As a positive control, a case of verruca vulgaris positive for pgs-antigen was included in this test.

**Dot blot hybridization**

The dot blot hybridization kit, vira probe HPV, was obtained from Life Technologies Inc., (U.S.A.) through Toray-Fuji Bionics Inc., (Japan). The RNA cocktail probe of HPV types 6, 11, 16, 18, 31, 33 and 35 was labeled with 32P.

Tumor material was thawed, cut into small pieces with scissors, ground in a precooled mortar with quartz and incubated at 37°C for 6 hrs in a 3% lysis solution containing 250 μg/ml proteinase K (Sigma, W. Germany), 1% sodium dodecyl sulfate in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM ethylenediamine tetra-acetate). Two hundred and fifty microliters of the homogenate solution was tested by dot blot hybridization after alkaline denaturation as indicated by the manufacturer.

**Extraction and digestion of DNA**

Extraction and purification of DNA from tissue homogenate were performed by phenol extraction and RNase treatment. Digestion of DNA with endonucleases BamHI (Boehringer Mannheim, W.Germany), Hind II (Takara, Kyoto, Japan) was performed according to the methods indicated by the manufacturer.

**Nonradioactive DNA labeling and Southern blot hybridization**

The HPV type 11 DNA was cloned in pBR322 in this laboratory. The HPV type 16 and 18 DNA in pBR322 were the generous gift of Dr. E.-M.deVilliers, Heiderberg, W.Germany. The DNA probes of HPV type 11, 16 and 18 were separated from vector pBR322 and labeled with digoxigenin-deoxyuridine triphosphate using the kit prepared by Boehringer Mannheim, W.Germany.

Electrophoresis of each endonuclease-digested DNA was done in 0.8% agarose gel in TEA-NaCl buffer (50 mM Tris-HCl, pH8.0, containing 20 mM sodium acetate, 2 mM Na2EDTA and 18 mM NaCl). After gel electrophoresis, DNA was transferred to Nytran membrane (Schneider and schuell, Passel, W.Germany) by Southern blotting. After hybridization under stringent conditions, the detection of hybrids to the target DNA by enzyme-linked immunosassay using an antibody-conjugate and subsequent enzyme catalyzed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt was performed according to the manual prepared by the manufacturer.

**Results**

**Histopathology**

Koilocytosis, defined as large epithelial cells with vacuolated cytoplasm and an eccentric pyknotic nucleus, were identified in 4 of 5 laryngeal papillomas (Fig 1). These 4 samples with koilocyte were all multiple type (Table 1).

In laryngeal polyps, no koilocyte was identified. Five laryngeal papillomas and 5 laryngeal polyps were investigated immunohistochometrically for pgs-antigen. No cell with a pgs-antigen-positive nucleus was detected in any of these lesions. Many pgs-antigen-positive cells, however, were detected in the superficial layers and in the intermediate layers (not shown) in the specimens of verruca vulgaris used as positive control.

**HPV detection by dot blot hybridization**

Fig 2. shows an autoradiogram obtained after dot blot hybridization using 32P-labeled vira probe HPV. Hela cell DNA containing HPV 18 DNA hybridized with vira probe HPV (Fig 2.A) while HTB-31 cell DNA containing no HPV DNA (Fig 2. B) and
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Fig. 1 Histopathology of laryngeal papilloma. Note the koilocytosis.

Table 1 Search for HPV in Laryngeal Papillomas and Polyps

<table>
<thead>
<tr>
<th>Papilloma Case</th>
<th>Sex</th>
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<th>D.B.</th>
<th>S.B.</th>
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M: male  F: female  D.B.: Dot blot hybridization  S.B.: Southern blot hybridization
pBR322 DNA (100ng) (Fig 2,C) did not. One of laryngeal papilloma DNA hybridized with viral probe HPV showing black spot (Fig 2.D). Four cases out of 5 laryngeal papillomas were HPV-DNA positive by this method (Table 1). These 4 cases were all multiple type and koilocytosis-positive (Table 1). Two cases out of 5 laryngeal polyps were also positive by this method (Table 1).

**HPV detection of Southern blot hybridization**

Endonuclease Bam H1 digested DNA isolated from laryngeal papillomas and laryngeal polyps was electrophoresed, Southern blotted and hybridized with digoxigenin-dUTP-labeled DNA mixture of HPV 11, 16 and 18. As is shown in Fig. 3, a band of 7.5 kb was detected in lane 1 and 5. All 4 laryngeal papillomas which were positive by dot blot hybridization were also positive and one negative sample by dot blot hybridization was negative by this method. Two HPV-positive laryngeal polyps by dot blot hybridization were negative by Southern blot hybridization (Table 1). HPV-DNA detected in laryngeal papillomas was further analysed by Hind II digestion and hybridization with HPV 11 probe. Hind II cuts HPV 6 DNA into 2 fragments, one of which is sometimes not visible because of its small size and amount, and cuts HPV 11 DNA into 4 fragments. Fig. 4 lane 2 shows HPV 6 DNA closely related with HPV 11 DNA and Fig. 5 lane 2 shows HPV 11 DNA cut by Hind II. The results are summarized in Table 1.

**Discussion**

Cutaneous and genital warts have been extensively studied virologically and the role of HPV as an etiological agent has been established.

As laryngeal papilloma resembles cutaneous and genital warts histopathologically, HPV infection as a cause of this benign tumor has been speculated...
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In this paper we examined the presence of HPV in laryngeal papillomas and in laryngeal polyps as control. Histopathology, immunohistochemistry and molecular hybridization by dot blot as well as Southern blot have been included in this study.

Koilocytosis was observed in all HPV positive papillomas but not in polyps. Ferenczy et al. reported that in condyloma of the genital tract, a presumed viral replication within the cells near the epithelial surface results in a characteristic degeneration of nuclear chromatin. Koilocyte is manifested as large epithelial cells with vacuolated cytoplasm in superficial cell layers. We agree that the presence of koilocytes provide indirect evidence of HPV infection.

We attempted to detect HPV pgs-antigen in laryngeal papilloma by the immunohistochemical method. Though this method worked well in a specimen of verruca vulgaris, no positive result was obtained in specimens of laryngeal papillomas. It has also been reported by other groups that the sensitivity of the immunohistochemical method in cases of laryngeal papilloma was low. This may be due to the low production of HPV capsid antigen in the infected cells as well as the small number of infected cells in case of laryngeal papilloma when compared with verruca vulgaris.

To prove the presence of HPV genome directly, we applied dot blot and Southern blot hybridization method. The dot blot hybridization method used in this study was a commercially developed HPV-screening kit. Tissue homogenate treated with proteinase K without DNA isolation was applied, and the result agreed completely with that of Southern blot hybridization in laryngeal papillomas. However, no HPV DNA band was detected by Southern blot hybridization in purified DNA of 2 HPV-positive laryngeal polyps by dot blot hybridization. As the Southern blot hybridization is the most reliable method, these 2 HPV-positive cases by dot blot hybridization seem to be false positives, although the possibility of the presence of HPV other type than 6, 11, 16 and 18 was not excluded. The etiology
of laryngeal polyp is thought to be local circulatory insufficiency due to overwork of vocal cords.

The fact that every multiple type of laryngeal papilloma contains HPV 6 or 11 while no laryngeal polyp does, supports the etiological role of HPV in laryngeal papilloma. In this study, confirming the reports of other groups10,11,12), HPV type 6 in 2 cases and HPV type 11 in 2 cases were detected. There were no recognizable differences in histological features, age of onset or clinical course between HPV 6 and 11 infections.

Conclusion

This work was undertaken to study the etiological role of HPV in laryngeal papilloma.

Five cases of laryngeal papilloma and 5 cases of laryngeal polyp were examined histopathologically, immunohistochemically and molecular biologically. The presence of koilocytosis correlated well with the presence of HPV. Immunohistochemical staining of HPV pgs-antigen was all negative probably due to low sensitivity. In laryngeal papilloma, the results of dot blot and Southern blot hybridization agreed completely and 4 out of 5 cases were HPV positive. HPV type 6 was detected in 2 cases and HPV type 11 also in 2 cases. These 4 HPV-positive cases were clinically all multiple type and the HPV negative 1 case was single type. Among laryngeal polyp cases, 2 positive results by dot blot hybridization seemed to be false positive.

Acknowledgments

We are grateful to Drs. H. zur Hausen and E.M. de Villiers for kindly providing us with DNAs of HPV types 16 and 18.

References