Abstract: To date, more than 120 total types of HPV have been identified, and in a recent meta-analysis, HPV was confirmed as an independent risk factor for oral carcinoma. Some investigators have reported that HPV infection is related to certain factors, including the gender, age, alcohol consumption, smoking habit, sexual behavior and denture wearing. Denture wearing can lead to denture epulis, which is a hyperplasia of fibrous connective tissue caused by denture irritation. Recently, HPV infection was detected in the hyperplastic epithelium of denture fibroma, but it still has received little study. The objective of the present investigation, therefore, was to clarify the relationship between the hyperplastic epithelium of the denture epulis and HPV infection. DNA of 118 formalin-fixed, paraffin-embedded hyperplastic epithelium biopsies of epulis specimens was extracted. Firstly, HPV infection was detected by PCR using consensus primers. Secondary, PCR using HPV type-specific primers (low risk types 6 and 11; high risk types 16, 18 and 33) was done in positive PCR samples. HPV infection was also detected by in situ hybridization and immunohistochemical techniques. Eighteen (23.1%) of the 78 cases of denture epulis were positive, and 2 (5.0%) of the 40 cases of non-denture epulis were positive. The difference was statistically significant ($p < 0.05$, using Fisher’s exact test). The most frequent type of HPV found in the 14 positive samples was HPV 16. It seems that the hyperplastic epithelium of denture epulis is easily infected with viruses because the epithelium is exposed daily to traumatic irritation from dentures. These results suggest that the hyperplastic epithelium of denture epulis might be an important reservoir for HPV infection of the oral region where later HPV-associated diseases, such as oral cancer and other oral lesions, may develop.

Key words: HPV, Denture wearing, Epulis, PCR, Oral mucosa

Introduction

Human papillomavirus (HPV) infection is the most common worldwide sexually transmitted viral infection. Over 120 types of HPVs have been identified to date, and HPVs have been divided into low risk (LR) and high risk (HR) groups. LR-HPVs cause wart-like lesions of the skin, anogenital region and oral mucosa. HR-HPVs are etiologically associated with cervical, anogenital and oral cancers. Recent studies have shown that oral infection with human papillomaviruses is associated with a significant risk of developing oropharyngeal cancer and oral potentially malignant disorders (OPMDs). Oral mucosal lesions associated with denture wearing, such as frictional keratosis, denture fibroma, denture epulis and OSCC, are sometimes observed. These lesions may be due to acute or chronic reactions to microbial denture plaque, the denture base materials, or mechanical denture injury. Some investigators have reported that HPV infection is related to certain factors, including the sexual act, gender, age, smoking habit, alcohol consumption and denture wearing.

Denture epulis has been described as denture fibroma, epulis fissuratum, denture fibrosis, denture injury tumor, or hyperplasia from denture irritation. Clinically, and histopathologically, this lesion is a product of constant trauma and inflammation resulting from pressure on the denture flange. Although HPV infection has been detected in the hyperplastic epithelium of denture fibroma, the hyperproliferation of the epithelium in this lesion has been little studied.

The objectives of the present investigation are to clarify the relationship between the hyperplastic epithelium of denture epulis and HPV infection using PCR, DNA sequencing analysis, immunohistochemical techniques, and in situ hybridization.
Materials and Methods

**Eplulis specimens**

One hundred and eighteen archival, formalin-fixed and paraffin-embedded epulis biopsy specimens were obtained from the Aichi Gakuin University Dental Hospital, Nagoya, Japan. All samples were biopsies and surgical specimens derived from the gingiva. Forty-nine specimens were derived from male patients, and 69 from female patients. The age of patients ranged from 20 to 91 years, with a mean age of 64.4 years. Seventy-eight samples (66.0 %) were derived from denture wearing patients, and the remaining 40 samples (34.0 %) were derived from non-denture wearing patients.

**Normal gingival specimens**

Twelve gingival samples were obtained from a group of healthy subjects, consisting of 3 men and 9 women. Ages ranged from 21 to 87 years, with a mean age of 64.4 years. Seventy-eight samples (66.0 %) were derived from denture wearing patients, and 40 samples (34.0 %) were derived from non-denture wearing patients.

**Experimental Procedure**

HPV genomic DNA was detected with a highly sensitive PCR assay. To avoid false-negative results due to low DNA yield or a low number of viral copies, extracted DNA quantity and purity (calculated by use of the ratio of the absorbance at 260 nm to that at 280 nm [260/280 ratio]) were measured with the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and extracted DNA integrity was confirmed by amplifying the 110 base pair ß-globin gene (Table 1) in all specimens (data not shown).

In the first step of the PCR assay, we used the degenerate L1 consensus primers GP5/6 18) and GP5+/6+ 19), which can detect the presence of DNA of 9 HR-HPV types (16, 18, 31, 33, 35, 45, 51, 56 and 58) and seven LR-HPV types (1, 6, 8, 11, 13, 30, 32 and 58) (Table 1). Secondly, the positive PCR samples in the first step of the assay were analyzed by type-specific PCR with primers designed for the detection of HPV types 6, 11, 16, 18 and 33 13) (Table 1). The HPV genotyping procedure employed in this study was confirmed by direct DNA sequence HPV.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence, 5’-3’</th>
<th>Size (bp)</th>
<th>PCR Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>beta-globin</td>
<td></td>
<td>110</td>
<td>95 °C 10 min: 94 °C 1 min, 55 °C 2 min, 72 °C 2 min (40 cycles): 72 °C 10 min</td>
</tr>
<tr>
<td>PC03</td>
<td>ACACAACTGTGTACCACCTAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC04</td>
<td>CACTTTATCGTATCAGCTGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP5/6</td>
<td></td>
<td>140-150</td>
<td>94 °C 5 min: 94 °C 1 min, 50 °C 1 min, 72 °C 1 min (40 cycles): 72 °C 5 min</td>
</tr>
<tr>
<td>GP5</td>
<td>TTCATGTGTAGTAGTAGATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP6</td>
<td>GAAAAATAAACTGTAAATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP5+/6+</td>
<td></td>
<td>140-150</td>
<td>96 °C 1 min: 94 °C 30 sec, 40 °C 30 sec, 72 °C 45 sec (40 cycles): 72 °C 5 min</td>
</tr>
<tr>
<td>GP5+</td>
<td>TTCATGTGTAGTAGTAGATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP6+</td>
<td>GAAAAATAAACTGTAAATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-6</td>
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<td>111</td>
<td></td>
</tr>
<tr>
<td>HPV-6F</td>
<td>TATACCTGCCTCCACCTCC</td>
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<td></td>
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<td>HPV-6R</td>
<td>CCACTTGTAATAAGCCGGA</td>
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<td>HPV-11</td>
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<td>HPV-11F</td>
<td>CAACACGGTGATATTGCCC</td>
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<td>HPV-11R</td>
<td>TGCCCTAGTITATTAAGCGGA</td>
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<tr>
<td>HPV-16</td>
<td></td>
<td>126</td>
<td>95 °C 5 min: 94 °C 1.5 min, 50 °C 1.5 min, 72 °C 2 min (40 cycles): 72 °C 10 min</td>
</tr>
<tr>
<td>HPV-16F</td>
<td>ACGACCTGTACATCAAGGGC</td>
<td></td>
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<td>HPV-16R</td>
<td>GCCAGTAGGGGTTTGGAC</td>
<td></td>
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<td>HPV-18</td>
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<td>HPV-18F</td>
<td>CTGTTGCAATAAGCGAGT</td>
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<td>HPV-18R</td>
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<td>HPV-33</td>
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<td>CGTTTTCGGTACCTTGGCAT</td>
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<tr>
<td>HPV-33R</td>
<td>TGTATGCAATGCAGCAAGT</td>
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</tr>
</tbody>
</table>

### Table 1. PCR Primers used in the Experiments

- **beta-globin**
  - PC03: ACACAACTGTGTACCACCTAGGC
  - PC04: CACTTTATCGTATCAGCTGACC
- **GP5/6**
  - GP5: TTCATGTGTAGTAGTAGATAC
  - GP6: GAAAAATAAACTGTAAATCA
- **GP5+/6+**
  - GP5+: TTCATGTGTAGTAGTAGATAC
  - GP6+: GAAAAATAAACTGTAAATCA
- **HPV-6**
  - HPV-6F: TATACCTGCCTCCACCTCC
  - HPV-6R: CCACTTGTAATAAGCCGGA
- **HPV-11**
  - HPV-11F: CAACACGGTGATATTGCCC
  - HPV-11R: TGCCCTAGTITATTAAGCGGA
- **HPV-16**
  - HPV-16F: ACGACCTGTACATCAAGGGC
  - HPV-16R: GCCAGTAGGGGTTTGGAC
- **HPV-18**
  - HPV-18F: CTGTTGCAATAAGCGAGT
  - HPV-18R: CCCACAAAGGCAACACCTA
- **HPV-33**
  - HPV-33F: CGTTTTCGGTACCTTGGCAT
  - HPV-33R: TGTATGCAATGCAGCAAGT

### PCR Cycle

- **beta-globin**
  - 95 °C 10 min; 94 °C 1 min, 55 °C 2 min, 72 °C 2 min (40 cycles): 72 °C 10 min
- **GP5/6**
  - 94 °C 5 min: 94 °C 1 min, 50 °C 1 min, 72 °C 1 min (40 cycles): 72 °C 5 min
- **GP5+/6+**
  - 96 °C 1 min: 94 °C 30 sec, 40 °C 30 sec, 72 °C 45 sec (40 cycles): 72 °C 5 min
Extraction of DNA

Analysis of epulis and normal specimens was performed on six adjacent 6-μm sections of each paraffin-embedded tissue, with the first section being stained with hematoxylin and eosin to visualize the hyperplastic epithelium of epulis and normal gingival epithelium. The remaining slices were collected in a sterile 1.5-ml micro centrifuge tube. DNA was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Tokyo, Japan) according to the manufacturer’s protocol. To avoid cross contamination at any step of the procedure, paraffin-embedded compounds were cut between samples and were subsequently subjected to DNA extraction and PCR analysis. For the HPV-positive controls, a CaSki cell line served as positive control for HPV 16, HeLa for HPV 18, a positive oral leukoplakia for HPV 6 and 11, and a cervical squamous cell carcinoma for HPV 33.

PCR analysis

Detection of HPV sequences was performed using two sets of consensus primers, GP5/6 and GP5+/6+ , which amplify a 150 bp region, in the highly conserved L1 HPV gene. Positive specimens were analyzed by type-specific-PCR for HPV types 6, 11, 16, 18 and 33 infection, as previously described (Table 1).

These oligonucleotide primers were synthesized by Sigma-Aldrich Japan (Tokyo, Japan). In this study we used 2×PCR Solution Premix Taq Polymerase (TaKaRa ExTaq Version 2.0, Takara Bio Inc., Tokyo, Japan). The thermal profiles used were described in Table 1. After the reaction, one-tenth of the reaction mixture was electrophoresed through 2% NuSieve GTG agarose (Takara Bio Inc.) gel containing 1 μg/ml ethidium bromide and visualized under an ultraviolet transilluminator. The gel was subsequently photographed.

Direct DNA sequencing

PCR products were sequenced with fluorescent dye-labeled deoxyribonucleotides and cycle sequencing methods utilizing the Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems, New Jersey, USA). Sequencing products were purified of unincorporated dye labeled deoxyribonucleotides by processing through Centri-Sep spin columns (PE Applied Biosystems). Sequence analysis was automatically performed on the ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems).

Histological and immunocytochemical examination, in situ hybridization

To confirm the presence of HPV protein immunohistochemically, we used monoclonal mouse anti-HPV (Clone K1H8, M3528, Dako, Carpinteria, USA). In 118 cases, only histological sections positive for HPVs by PCR methods were stained with the EnVision™/HRP method (K1392, Dako). To improve the staining pattern, antigen retrieval by heating in 10 mM citrate buffer was performed. Normal gingival specimens were also examined immunohistochemically as controls.

The localization of HPV DNA in epithelium was evaluated by in situ hybridization using Dako In-situ Hybridization System for Biotinylated Probes (BCIP/NBT) (K0601, Dako) with HPV Types Wide Spectrum Biotinylated DNA Probe (Y1404, Dako), according to the product protocol.

Statistical analysis

Statistical analysis was done using Fisher’s exact test. Differences with a p value <0.05 (*) were considered statistically significant.

Figure 1. The positive rates of epulis in denture wearers and in non-denture wearers for all specimens were 23.1% (18/78) and 5.0% (2/40), respectively. A statistically significant association was found in the positivity between epulis in denture wearers and in non-denture wearers (p<0.05).

Figure 2. Type-specific PCR assay showed that 38.9 % (7/18), 66.7% (12/18) and 5.6 % (1/18) of denture epulis samples were positive for HPV 11, 16 and 18, respectively, and two denture epulis samples showed positive for both HPV 11/16 and 16/18, respectively. In epulis of non-denture wearers, HPV 16 and 18 were detected in 1 sample each.
Results

Twenty (16.9 %) of all specimens were positive for HPV consensus primer detection. The positive rates of males and females in all specimens were 14.3% (7/49) and 18.8 % (13/69), respectively. The positive rates of epulis in denture wearers and in non-denture wearers for all specimens were 23.1 % (18/78) and 5.0 % (2/40), respectively (Fig. 1). A statistically significant association was found in the positivity between epulis in denture wearers and in non-denture wearers ($p=0.05$). In all of the control normal gingival epithelium, however, HPV infection could not be detected. Type-specific PCR assay showed that 38.9 % (7/18), 66.7 % (12/18) and 5.6 % (1/18) of denture epulis samples were positive for HPV 11, 16 and 18, respectively (Fig. 2), while two denture epulis samples showed positive for both HPV 11/16 and 16/18, respectively. In the epulis of non-denture wearers, HPV 16 and 18 were detected in 1 sample each (Fig. 2). The most frequent type of HPV of the 20 positive samples was HPV 16. Type-specific PCR products were defined as having a sequence similarity to each HPV type by direct sequencing.

Histopathologically, hyperplastic epithelium with hyperkeratosis was observed on the surface of denture epulis (Fig. 3a). Immunohistochemically, positive staining for the anti-HPV was also observed in all epulis which showed positive HPV DNA by PCR methods. Positive staining was seen in the nuclei of upper layers of the epithelium (Fig. 3b). By in situ hybridization, all epulis which showed positive HPV DNA by PCR methods showed positive staining in the nuclei of all layers of the epithelium (Fig. 3c).

Discussion

HPVs are small epitheliotropic DNA viruses that can induce hyperplastic, papillomatous and verrucous squamous cell lesions in the stratified squamous epithelia of the skin and mucosa, including the oral mucosa\textsuperscript{20-24}. Nearly 120 HPV genotypes have been identified, and among them HPV 16 and HPV 18 are considered to be strongly associated with the development of malignancy and are thought to be malignant, oncogenic or high-risk genotypes\textsuperscript{25}. Recent studies have detected HPV infection in the oral lesions of denture wearers in Japan\textsuperscript{13}. It seems that HPV infection and denture wearing may be related and may be risk factors for oral mucosal lesions, such as OSCC. The relationship between HPV infection and denture wearing, however, is not clearly understood.

In this study we demonstrated HPV infection in the hyperplastic epithelium of denture epulis. This is the first report documenting the presence of HPV type 11, 16 and 18 infection in the hyperplastic epithelium of denture epulis. To date, there has been only one other report concerning HPV infection in denture epulis; however, it did not specify the types of HPV\textsuperscript{17}. In the oral region, HPV 11 is commonly associated with oral benign lesions, such as squamous papilloma, oral condyloma, papillary hyperplasia, lichen planus and leukoplakia\textsuperscript{14-26}. In the present study, HPV 16 and 18 were also detected in the hyperplastic epithelium of denture epulis. Although HPV infection is probably not directly associated with denture epulis, HPVs may cause hyperplasia in the epithelium of denture epulis.

In this experiment, the positive rate of HPV infection was higher in denture epulis than in non-denture epulis. In all of the
Hyperplastic Epithelium of Oral Mucosal Lesions as a Potential Reservoir of Human Papillomavirus

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control normal gingival epithelium, however, HPV infection could not be detected. Furthermore, the most frequent type of HPV of the 20 positive samples was HPV 16. These results suggest that HPV-16 infection was related to denture wearing. Long-term mechanical and sometimes traumatic irritations by the dentures are generally regarded as one of the causes of fibrous hyperplasia. Moreover, Yamaguchi et al. reported that HPV was widely detected by in situ and Southern blot hybridization in the hyperplastic epithelium of oral denture fibroma. It seems that the hyperplastic epithelium of denture epulis is easily infected with viruses because the hyperplastic epithelium is exposed daily to traumatic irritation by dentures and also because this causes an increase in epithelial turnover. When the surface of the oral mucosa is injured by the mechanical irritation of dentures, HPV easily can move into the epithelial basal cells, which are the target cells of HPVs. That is, the chance of being infected with HPVs may increase when the epithelium is injured. Therefore, the physical and chemical irritations caused by dentures may lead to infection with HPV. In addition, the continuous epithelial proliferation and chronic inflammation in the epithelium of denture epulis could favor the replication of HPV and might be an important reservoir of HPVs in the oral mucosa where later HPV-associated diseases may develop.

It is proposed that HPV infection, especially high-risk HPV 16 infection caused by factors such as mechanical stress, is more important than denture stomatitis for oral carcinogenesis, because high-risk HPV 16 has the potential to transform into malignant lesions depending on E6 and E7 oncogenes. Recently, it has been revealed that E6 protein binds p53 protein and hastens its destruction, while the E7 protein binds to the retinoblastoma gene product and inactivates it. This finding indicates that HPV infection may be enhanced in the oral mucosal lesions associated with denture wearing.

The results of this study suggest that the hyperplastic epithelium of denture epulis might be the important reservoir for HPV infection of the oral region where later HPV-associated diseases, such as oral cancer and other oral lesions, may develop. Therefore, it is necessary to prevent denture-associated oral mucosal lesions in order to reduce the chance of HPV infection. In order to reduce oral mucous lesions, denture wearers should be recalled regularly for an examination of the condition of the oral cavity and dentures. Further studies are required to determine the exact roles and relationships between HPV infection and denture wearing in oral mucosal lesions.

Acknowledgments

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