INTRODUCTION

Squamous cell carcinoma (SCC) is the most frequent oral malignancy, accounting for over 90% of oral lesions. Globally, epidemiological data indicate an increasing number of deaths due to oral cancer. SCC frequently occurs in patients with a history of tobacco smoking and alcohol consumption. Recently, although the prevalence of tobacco smoking and alcohol consumption has decreased, the prevalence of SCC has increased. It has been suggested that there may be an increasing incidence of HPV related SCC of the head and neck region.

In previous reports, HPV has been associated with oropharyngeal SCC and cervical SCC. Also, some reports state that HPV is a risk factor for head and neck squamous cell carcinoma (HN-SCC). HPV is classified into high-risk and low-risk types according to oncogenic potential. High-risk HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82; low-risk types include HPV 6, 11, 42, 43 and 44. However, the range of the detection rate of HPV in HNSCC is wide (22.7 - 66.7%), and the role of HPV in malignant transformation is still controversial.

The tumor suppressor gene p53 controls the cell cycle by regulating cyclin-dependent kinase (CDK) at the G1 check point. The E6 protein of HPV 16 and 18 binds to and ubiquitinates p53. At this point, p53 loses its cell cycle control. The E6 and E7 proteins of high-risk HPV are associated with carcinogenesis in oropharyngeal SCC by inactivation of the tumor suppressor gene p53. The cyclin dependent kinase inhibitor p16INK4a is a regulator at the part of cell cycle that controls the transition at the G1 check point. Under normal conditions, p16INK4a controls retinoblastoma (Rb) expression.

However, in the presence of HPV 16 infection, the E7 protein of HPV 16 directly affects Rb, causing acceleration of the cell cycle and overexpres-
sion of Rb. Overexpression of p16INK4a is associated with high-risk HPV-positive HNSCC, and can indicate a biologically active HPV infection. Therefore, p16INK4a has been proposed as a practical marker for HPV in clinical practice. Some reports have shown that HPV infection might cause oral dysplasia and OSCC, whereas others state that HPV infection is not always associated with OSCC. In this report, we investigated the expression of HPV 16/18 E6, HPV L1, p53 and p16INK4a in oral papilloma (OP), oral dysplasia (OD), and OSCC, and evaluated the relationship between these factors.

MATERIALS AND METHODS

Specimens
We used 11 samples of OP, 14 of low grade oral dysplasia (LGD), 10 of high grade oral dysplasia (HGD), 21 of well to moderately differentiated squamous cell carcinoma (W-MSCC) and 8 of poorly differentiated squamous cell carcinoma (PSCC) obtained from Osaka Dental University Hospital. The specimens were fixed in 10% formalin solution, dehydrated in a graded ethanol series, and embedded in paraffin. The age, gender and site distribution are shown in Tables 1 and 2. This research was approved by the Ethics Committee at Osaka Dental University (Approval number 110813).

Immunohistochemistry
Sections 4 μm thick were deparaffinized in Hemede (Falma, Tokyo, Japan) and rehydrated through a graded ethanol series. Antigen retrieval to anti-human p53 mouse monoclonal antibody (DAKO, Glostrup, Denmark) was carried out by autoclaving at 121°C for 15 min in 0.01 M citrate buffer at pH 6.0. Washing in 0.01 M tris buffer containing EDTA at pH 9.0 was carried out under the same conditions for anti-human p16INK4a rabbit monoclonal antibody (Abcam), anti-human HPV 16/18 E6 mouse monoclonal antibody (Abcam) and anti-human HPV L1 mouse monoclonal antibody (Abcam). Endogenous peroxidase was blocked by 0.3% hydrogen peroxidase.

For HPV 16/18 E6 and HPV L1, in order to bind the primary antibody, specimens were incubated in 0.25% casein solution for 15 min. These antibodies were diluted with Antibody Diluent solution (p53 1 : 20, p16INK4a 1 : 200, HPV 16/18 E6 1 : 20, HPV L1 1 : 200; DakoCytomation, Carpinteria, CA, USA). The sections with p53 and p16INK4a were reacted for one night at 4°C and the sections with HPV 16/18 E6 and HPV L1 were reacted for 60 min at room temperature. For p53 and p16INK4a, the sections were incubated with peroxidase dextran polymer (Envision + ; DakoCytomation) for 30 min at room temperature. For HPV 16/18 E6 and HPV L1, the sections were incubated with biotinylated link antibody, streptavidin-biotin-peroxidase complex, biotinyl tyramide (amplification reagent), and streptavidin peroxidase, each for 15 min. The sections were visualized by 3,3′-diaminobenzidine-tetrahydrochloride (DAB, DakoCytomation) and counterstained with hematoxylin.

Table 1 Age and gender of subjects

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Age (yrs)</th>
<th>Gender</th>
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<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Range</td>
</tr>
<tr>
<td>OP</td>
<td>70</td>
<td>44-84</td>
</tr>
<tr>
<td>LGD</td>
<td>60</td>
<td>31-76</td>
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<tr>
<td>HGD</td>
<td>68</td>
<td>63-80</td>
</tr>
<tr>
<td>W-MSCC</td>
<td>66</td>
<td>48-81</td>
</tr>
<tr>
<td>PSCC</td>
<td>63</td>
<td>54-72</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>31-81</td>
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Table 2 Site of lesion

<table>
<thead>
<tr>
<th>Site</th>
<th>Cases</th>
<th>Lesion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>OP</td>
<td>LGD</td>
</tr>
<tr>
<td>Tongue</td>
<td>41</td>
<td>2</td>
</tr>
<tr>
<td>Maxillary gingiva</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Mandibular gingiva</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Hard palate</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>11</td>
</tr>
</tbody>
</table>
Evaluation of immunohistochemistry

For p53 and p16\textsuperscript{INK4a}, immunohistochemical expression was evaluated as (-) when less than 10% positive cells, and (+) when greater more than 10%. For HPV 16/18 E6 and HPV L1, immunohistochemical expression was evaluated negative or positive, with a positive reaction when more than 10% of the cells were stained, according to criteria reported previously.\textsuperscript{18, 19}

RESULTS

Immunohistochemical evaluations (Figs. 1 and 2)

**HPV L1**
HPV L1 immunoreactivities were indicated by nuclear and cytoplasm staining. Six cases of OP were positive (55%). For OD, all cases of LGD were positive (100%) and four cases of HGD were positive (40%). For SCC, thirteen cases of W-MSCC were positive (62%), while four of PSCC were positive (50%).

**HPV 16/18 E6**
HPV 16/18 E6 immunoreactivities were indicated by nuclear and cytoplasm staining. Five cases of OP were positive (45%). For OD, twelve cases of LGD were positive (86%) and seven of HGD were positive (70%). For SCC, twelve cases of W-MSCC were positive (57%) and four of PSCC were positive (50%).

**p53**
p53 immunoreactivities were indicated by nuclear staining. Five cases of OP were positive (45%). For OD, nine cases of LGD were positive (64%) and four of HGD were positive (40%). For SCC, twelve cases of W-MSCC were positive (57%) and six of PSCC were positive (75%).

**p16\textsuperscript{INK4a}**
p16\textsuperscript{INK4a} immunoreactivities were indicated by nuclear and cytoplasm staining. Eight cases of OP were positive (73%). For OD, eight cases of LGD were positive (57%) and six of HGD were positive (60%). For SCC, thirteen cases of W-MSCC were positive (62%) and five of PSCC were positive (63%).

Fig. 1 Immunohistochemical results examined by Fisher’s exact test. Less than 0.05 was considered significant.
Relation between the expression of HPV 16/18 E6 and p16INK4a, and between HPV L1 and p53 (Tables 3 and 4)

In W-MSCC, ten cases that were positive for p16INK4a were positive for HPV 16/18 E6 (p < 0.05). In OD, twelve cases that were positive for p53 were positive for HPV L1 (p < 0.05).

DISCUSSION

Cervical cancer is an HPV associated cancer. Most cases of cervical cancer are believed to be caused by HPV 16 and HPV 18, which have recently been strongly thought to be involved in pharyngeal cancer as well. Although HPV 16 and HPV 18 are also suggested to be involved in oral cancer, the rates of detection of HPV 16 and HPV 18 in oral cancer are lower than in cervical cancer or pharyngeal cancer. A previous study reported that in OSCC, the rates of detection of HPV L1 and HPV

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### Table 3 Correlation between HPV 16/18 E6 and p16INK4a in W-MSCC

<table>
<thead>
<tr>
<th>p16INK4a</th>
<th>HPV 16/18 E6</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>12</td>
<td>21</td>
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### Table 4 Correlation between HPV L1 and p53 in OD

<table>
<thead>
<tr>
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<th>HPV L1</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Positive</td>
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<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>18</td>
<td>24</td>
</tr>
</tbody>
</table>

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Fig. 2 Immunohistochemical staining showing (A) overexpression of HPV1618E6 in W-MSCC, (B) overexpression of p16INK4a in W-MSCC, (C) overexpression of HPV L1 in HGD, and (D) overexpression of p53 in HGD.
16/18 E6 were 31.5% and 39.5%, respectively.

We successfully detected HPV L1 and HPV 16/18 E6 in all cases at higher rates than in past studies. In addition, we used a CSA method, which utilizes the biotin-streptavidin interaction for extremely sensitive detection of endogenous biotin; although some non-specific reactions are enhanced, the method has a remarkably high sensitivity that enables detection of smaller amounts of antigens. This may be why our detection rates were higher than conventional rates. In our results, LGD had the highest rate of HPV L1 infections, and the results were similar for HPV 16/18 E6.

Generally, in cervical cancer, HPV starts out in an episomal form existing as circular DNA at the time of infection; however, from the cervical intraepithelial neoplasia (CIN) II stage, HPV is integrated into the host DNA. Therefore, the amount of HPV is believed to be greatest in the early stage of infection, when it propagates actively in the cytoplasm of infected cells (progression from normal cells to moderate dysplasia). In contrast, the amount of HPV is believed to be low when it is incorporated into the nuclei of infected cells (progression from moderate dysplasia to invasive cancer). The results of our study were consistent with the above, as we obtained high rates of detection for both E6 and E7.

p53 is a tumor suppression gene that regulates the cell cycle and induces apoptosis of cells. In cervical cancer, Anwar et al. and Kim et al. have reported that p53 is overexpressed in cases with HPV infection. In the head and neck region, Taylor et al. reported overexpression of p53 in HNSCC with HPV infection, while Tsuji et al. similarly reported overexpression of p53 in OSCC with HPV infection. In all of these reports of high-risk HPV infection, p53 was overexpressed in cases of HPV infection.

We found a significantly increased expression of p53 in cases of HPV infection in OD; in OSCC, although there was no significant difference in p53 expression, a similar trend was observed. Generally, in cervical cancer, expression of HPV E6 in association with HPV infection promotes the proteasome-mediated degradation mechanism through the ubiquitination of p53, thereby effectively obliterating the function of p53. We found that a similar phenomenon occurs in the oral region.

p16INK4a suppresses phosphorylation of Rb and stops the cell cycle by binding with the cyclin D-CDK 4 complex in the event of cellular senescence or damage. In oropharyngeal cancer, a strong correlation has recently been observed between HPV infection and p16INK4a overexpression, suggesting that p16INK4a can be used as a surrogate marker of HPV infection. We found a significant difference in p16INK4a expression in W-MSCC when HPV 16/18 E6 expression was also observed.

Hernandez et al. reported that p16INK4a expression is not correlated with HPV 16 infection in PSCC in laryngeal cancer. In vulvar cancer, Sznurkowski et al. have reported that alongside E7-induced functional inactivation of Rb, 25-70% of HPV 16 positive cases of PSCC demonstrate inactivation by p16INK4a. In addition, Mehrad et al. reported that p16INK4a is not expressed in some cases of high-risk HPV infection. The absence of a significant difference in p16INK4a expression in PSCC in the present study suggests that HPV infection is difficult to confirm with p16INK4a alone in PSCC in the oral region.
In contrast, p16\textsuperscript{INK4A} was found useful in W-MSCC. The significant increase in HPV detection rates obtained with a CSA method in the present study suggests not only the usefulness of such methods in IHC going forward, but also that IHC analysis of p16\textsuperscript{INK4A} expression has demonstrated its relationship with HPV infection. Future studies will need to include larger numbers of cases and examine associated factors.

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REFERENCES


