Human Papillomavirus in Oral Lichen Planus of Japanese Patients

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Abstract: Oral lichen planus (OLP) can undergo malignant transformation and become squamous cell carcinoma (SCC). Oral infection with human papillomavirus (HPV) is associated with a significant risk of developing oral cancer. Although HPV DNA is detected more often in OLP tissue than in normal oral mucosa, there is as yet no firm evidence that HPV is a causative factor of malignant transformation in OLP. The objective of the present investigation was to assess HPV-genotype distribution in OLPs of Japanese patients and additionally to clarify the relationship between malignant transformation in OLP and HPV infection using PCR, in situ hybridization, and immunohistochemistry. DNA of 200 formalin-fixed, paraffin-embedded biopsy and surgical specimens of OLP was extracted. HPV infection was first detected by PCR using consensus HPV primers. Positive PCR samples were then further analyzed by PCR using HPV type-specific primers (HPV-6, -11, -16, -18 and -33). Eighty-three samples (41.5 %) out of the total 200 OLP specimens analyzed were HPV positive. In the HPV type-specific PCR assay, the most frequent type of HPV was HPV-16 (25.5 %), which is a high-risk type of HPV that is associated with malignant disorders and is often detected in SCC. The highest HPV-16 positive rate was obtained for the erosive type of OLP (28.3 %). Positive staining for HPV DNA by in situ hybridization was observed in the nuclei of cells in all layers of the epithelium in all HPV PCR positive samples. Immunohistochemically, nuclei of cells in the upper layer of the epithelium in all HPV PCR positive samples stained positive for the anti-HPV antibody. These results indicated that HPV-16 was often present in OLP of Japanese patients, especially in the erosive type of OLP, and suggested that HPV infection is a risk factor for malignant transformation in OLP lesions.

Key words: Oral lichen planus, Human papillomavirus, Oral potentially malignant disorder, Japanese

Introduction

Oral lichen planus (OLP) is a chronic inflammatory autoimmune disorder that affects the oral mucosa as well as the skin, genital mucosa, scalp and nails. The prevalence of OLP in the population ranges between 0.2 % to 3.3 % in a selected Japanese population1-4, 2.6 % in an Indian population5, 0.4 % in a Malaysian population6, and 1.9 % in a Swedish population7. Microscopically, OLP is characterized by hyperkeratosis of the mucosal epithelium, hydropic degeneration of the epithelial basal layer, and a band-like infiltrate of inflammatory cells in the dermal-epidermal interface8,9. The histological characteristics of OLP are a dense, band-like subepithelial lymphocytic infiltrate beneath the basement membrane, an increased number of intraepithelial lymphocytes, and liquefactive degeneration of basal cells10-13. The predominance of T cells in the inflammatory infiltrate suggests the involvement of cell-mediated immunity in OLP abnormalities, but the etiology and pathogenesis of OLP is not completely understood14.

Clinically, OLP can present as many different forms: reticular, plaque-type, erosive, atrophic, papular and bullous forms15. OLP should be strictly diagnosed based on both clinical and histopathological definitions according to the World Health Organization (WHO)15,16. The risk of malignant transformation of OLP was reported to range from 1.2 % to 3.2 % in follow-up of up to 10 years13. Whether OLP is associated with an increased risk of malignant transformation was recently reviewed17. These results raise the possibility that other factors indirectly related to OLP may play a role in the malignant process and may occur in the mouths of patients who also happen to have OLP. It is generally accepted that there is an increased risk of oral cancer development in patients with OLP; hence these conditions are mostly categorized as oral potentially malignant disorders (OPMDs)18.

Human papillomavirus (HPV) infection is the most common
sexually transmitted viral infection worldwide\textsuperscript{[10]. Over 120 types of HPVs have been identified to date\textsuperscript{[11-13]}. HPV's have been divided into low-risk (LR) and high-risk (HR) groups based on the clinical behavior of the virally infected tissues. LR-HPV's cause wart-like lesions of the anogenital region, skin and oral mucosa. HR-HPV's are etiologically associated with anogenital, cervical and oral cancers such as squamous cell carcinoma (SCC)\textsuperscript{[14-23]}. Recent studies have shown that oral infection with HPVs is associated with a significant risk of developing oropharyngeal cancer and OPMD\textsuperscript{s}\textsuperscript{[24-26]}, although HPV infection has been detected in OLP tissue\textsuperscript{[25,27-29]} and HPV DNA is detected in OLP tissue more often than in normal oral mucosa\textsuperscript{[11,19]}, there is no firm evidence as yet that HPV is a causative factor of oral cancer.

The objective of the present investigation was to assess HPV-genotype distribution in OLPs of Japanese patients and also to clarify the relationship between malignant transformation in OLP and HPV infection using PCR, in situ hybridization, and immunohistochemistry.

Materials and Methods

OLP specimens

Two hundred formalin-fixed and paraffin-embedded OLP biopsy and surgical specimens were obtained from the Aichi Gakuin University Affiliated Dental Hospital, Nagoya, Japan. All cases that were confirmed as OLP positive based on histopathological evaluation were selected for DNA extraction. Seventy-seven specimens were derived from male patients (mean age 55.7 years), and 123 from female patients (mean age 58.5 years). The age of all patients ranged from 25 to 80 years, with a mean age of 57.3 years. The samples were derived from the buccal mucosa (132 cases), gingiva (30 cases), tongue (19 cases), lip (15 cases) and palate (4 cases). OLP clinical type of the samples was erosive (46 cases), reticular (43 cases), plaque-type (27 cases), atrophic (6 cases) and unidentified (78 cases).

Extraction of DNA

Six adjacent 4-μm sections of each paraffin-embedded tissue sample were analyzed. The first section was stained with hematoxylin and eosin to visualize the OLP appearance. DNA was extracted from the remaining five sections using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Tokyo, Japan) according to the manufacturer’s protocol. To avoid false-negative results due to low DNA yield or low number of viral copies, extracted DNA quantity and purity (calculated based on the ratio of the absorbance at 260 nm to that at 280 nm (260/280 ratio)) were measured using the Nanodrop 1000 spectrophotometer\textsuperscript{TM} (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Extracted DNA from all specimens was confirmed by PCR amplification using the primers for the 110 base pair β-globin gene (Table 1) (data not shown). The following cells served as the indicated HPV-positive controls: the cell line CaSkI (for HPV-16), HeLa cells (for HPV-18), a positive oral leukoplaikia (for HPV-6 and 11), and a cervical squamous cell carcinoma (for HPV-33).

PCR analysis

In the first PCR assay of HPV DNA, three sets of L1 consensus primers were used: GP5/6\textsuperscript{[30]} and GP5+/6+\textsuperscript{[27]}, each of which amplifies a 150 bp region, and MY09/11\textsuperscript{[31]}, which amplifies a 450 bp region, in the highly conserved L1 region of the HPV gene (Table 1). GP5/6 can detect the presence of DNA of 4 HR-HPV types (16, 18, 31 and 33) and 5 LR-HPV types (1, 6, 11, 30 and 32)\textsuperscript{[32]}. GP5+/6+ can detect the presence of DNA of 11 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 54 and 56) and 7 LR-HPV types (6, 11, 34, 40, 42, 43 and 44)\textsuperscript{[27]}. MY09/11 can detect the presence of DNA of 7 HR-HPV types (16, 18, 31, 32, 58, 66 and 81) and 2 LR-HPV types (6 and 61)\textsuperscript{[30]}

In the second PCR assay, the samples that were positive in the first PCR assay were analyzed using type-specific PCR with primers designed for the detection of HPV-6, -11, -16, -18 and -33 (Table 1).

These oligonucleotide primers were synthesized by Sigma-Aldrich, Japan (Tokyo, Japan). For the PCR reactions, 2×PCR Solution Premix Taq Polymerase (TaKaRa ExTaq Version2.0, Takara Bio Inc., Otsu, Japan) was used for the PCR using consensus primers, and 2×PCR Solution Premix Ex Taq Polymerase Hot Start Version (Takara Bio Inc., Otsu, Japan) was used for type-specific PCR. The thermal profiles used are described in Table 1. After the reaction, one-tenth of the reaction mixture was electrophoresed through a 3% NuSieve\textsuperscript{TM} GTG\textsuperscript{TM} agarose (Takara Bio Inc.) gel containing 1 μg/ml ethidium bromide and was visualized under an ultraviolet transilluminator. The gel was subsequently photographed.

In situ hybridization

The localization of HPV DNA in epithelium of all HPV-positive paraffin-embedded tissue samples was evaluated by in situ hybridization using the In situ Hybridization System for Biotinylated Probes (BCIP/NBT) (K6061, Dako North America, Carpinteria, CA, USA) with an HPV Type 16/18 Biotinylated DNA Probe (Y1412, Dako North America, Carpinteria, CA, USA), according to the product protocol.

Immunohistochemical examination

To immunohistochemically confirm the presence of the HPV protein, monoclonal mouse anti-HPV (Clone KH18, M3528, Dako Japan, Tokyo, Japan) antibodies were used in dilution 1:50. All HPV-positive paraffin-embedded tissue specimens were stained using the EnVision\textsuperscript{TM}/HRP method (K1392, DAKO North America, Carpinteria, CA, USA). To improve the staining pattern, antigen retrieval by heating in 10 mM citrate buffer was performed.
Table 1. The primers and conditions for PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence, 5'-3'</th>
<th>Size (bp)</th>
<th>PCR Cycle</th>
</tr>
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<tbody>
<tr>
<td>beta-globin</td>
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<td></td>
<td></td>
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<tr>
<td>PC03</td>
<td>ACACAACCTGTGTTTCACTAGC</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>
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<tr>
<td>PC04</td>
<td>CAACCTCATCCAGTTCACC</td>
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<td>GP</td>
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</tr>
<tr>
<td>GP5</td>
<td>TTTGTTACTGTGTTAGATACT</td>
<td>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>GP6</td>
<td>GAAAAATAAAACTGTAAATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP+</td>
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<td></td>
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</tr>
<tr>
<td>GP5+</td>
<td>TTTGTTACTGTGTTAGATACT</td>
<td>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>
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<tr>
<td>GP6+</td>
<td>GAAAAATAAAACTGTAAATCTATTCA</td>
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</tr>
<tr>
<td>MY09</td>
<td>CTTCGCCMARRGGAACAGTTGATC</td>
<td>450</td>
<td>94°C 5 min: 94°C 5 min, 54°C 2 min, 72°C 2 min (40 cycles): 72°C 10 min</td>
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<tr>
<td>MY11</td>
<td>GCMCAGGGWCATAAYAATGG</td>
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<tr>
<td>HPV-16L1F</td>
<td>ACGACCTGTACATCAAGGGCC</td>
<td>126</td>
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<td>HPV-18L1F</td>
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**Statistical analysis**

Statistical analysis was performed using the Chi-square test. When expectation was less than 5, Fisher’s exact test was used. Differences with a p value <0.05 (*) were considered statistically significant, and differences with a p value <0.01 (**) were considered highly significant.

This study was approved by the Ethics Committee of the School of Dentistry, Aichi Gakuin University (Approval Number No. 178).

**Results**

Eighty-three samples (41.5%) out of a total of 200 specimens were positive for HPV in PCR analysis using consensus primers. The HPV positive rates of males and females in all specimens were 39.0% (30/77) and 43.1% (53/123), respectively (Fig. 1). A statistically significant difference was not found between the percentage of HPV positive males and females. The HPV positive rates according to the primer set used were 13.0% (26/200), 35.5% (71/200) and 2.5% (5/200) for GP5/6, GP5+/6+ and MY09/11 primers, respectively (Fig.2).

In the HPV type-specific PCR assay, the frequency of positivity for each HPV type in all OLP specimens was as follows: HPV-16 (25.5%, 51/200), -18 (23.5%, 47/200), -11 (6.5%, 13/200), -6 (5.5%, 11/200) and -33 (3.5%, 7/200). The most frequent type of HPV was HPV-16, which is an HR-HPV that is associated with malignant disorders and is often detected in SCC (20). Statistically significant differences were found between the frequencies of HPV-6 and -16, -6 and -18, -11 and -16, -11and -18, -16 and -33, and -18 and -33 (p < 0.01) (Fig. 3).

The HPV-16 positive rates of males and females were 26.0% (20/77) and 25.2% (31/123), respectively (Fig. 4). A statistically significant difference was not found between the HPV-16 positive rates of males and females. HPV-16 positive rates according to the oral region were 20.5% (27/132), 50.0% (15/30), 15.8% (3/19), 40.0% (6/15) and 0.0% (0/4) for buccal mucosa, gingiva,
**Figure 1.** Analysis of the male/female ratio of HPV positivity using PCR with HPV consensus primers

**Figure 2.** Analysis of HPV positivity in the total population according to each HPV consensus PCR primer set

**Figure 3.** PCR analysis of all HPV positive OLP samples using type-specific HPV primers

**Figure 4.** PCR analysis of HPV-16 infection rates of males and females

**Figure 5.** PCR analysis of HPV-16 infection rates of different oral regions

**Figure 6.** PCR analysis of HPV-16 infection rates according to OLP clinical type

tongue, lip and palate, respectively. Statistically significant differences were found between the HPV-16 positive rates of buccal mucosa and gingiva (p < 0.01) and between those of gingiva and tongue (p < 0.05) (Fig. 5). HPV-16 positive rates according to OLP clinical type were 28.3 % (13/46), 18.6 % (8/43), 25.9 % (7/27) and 0.0 % (0/6) for erosive, reticular, plaque-type and atrophic, respectively (Fig. 6). Statistically significant differences were not found among HPV-16 positive rates according to OLP clinical type.

Positive staining for HPV DNA by in situ hybridization was observed in the nuclei of cells in all layers of the epithelium in all samples that were PCR positive for HPV (Fig. 7). The nuclei of cells in the upper layer of the epithelium in all samples that were
To date, many studies have reported that HPV is associated with the onset of benign lesions of the oral area including leukoplakia, oral papilloma and common warts, as well as with the onset of SCC, which is the most common malignant lesion of the same area. Furthermore, it has been reported that the HPV infection rate in OLP, which is an OPMD, is higher than that in the normal oral mucosa. The HPV infection rates in OLP that have been reported thus far range between 15.4% and 42.6%. These different rates might depend on the methods used for each analysis. These data were obtained outside of Japan, and, up to the present study, there has been no report regarding the HPV infection rate for OLP in Japan.

According to the PCR analysis using consensus primers in this study, the HPV positive rate was 41.5% (83/200) in the total samples for all three consensus primers. Although this result was within the range reported above, the HPV infection rate in OLP was higher in the Japanese population than in the populations of other countries.

The HPV positive rates for males and females were 39.0% (30/77) and 43.1% (53/123), respectively, and these rates were not statistically significantly different. It has been previously reported that the HPV infection rate in the normal human oral area is 4.5%, and that there is no difference between the infection rate of males and females. Although the nature of the infection differed between healthy people and OLP patients, the HPV infection rate in the oral area does not appear to be associated with sex.

It has been reported in many studies that the most common type of HPV infection in OLP is HPV-16 infection, which is considered as an HR-HPV, which is associated with cancer, and is often detected in SCC of the oral area. This finding suggests that HPV-16 infection is associated with malignant transformation of OLP.

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PCR positive for HPV showed positive staining for the anti-HPV antibody in immunohistochemical staining (Fig. 8).

Discussion

To date, many studies have reported that HPV is associated with the onset of benign lesions of the oral area including leukoplakia, oral papilloma and common warts, as well as with the onset of SCC, which is the most common malignant lesion of the same area. Furthermore, it has been reported that the HPV infection rate in OLP, which is an OPMD, is higher than that in the normal oral mucosa. The HPV infection rates in OLP that have been reported thus far range between 15.4% and 42.6%. These different rates might depend on the methods used for each analysis. These data were obtained outside of Japan, and, up to the present study, there has been no report regarding the HPV infection rate for OLP in Japan.

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In the present study, the HPV-16 positive rates in males and females were 26.0% (20/77) and 25.2% (31/123), respectively; no statistically significant difference was observed between the positive rates of these two groups, as was found in the PCR analyses using consensus primers. As for the site of infection, the oral region with the highest HPV-16 positive rate of 50.0% (15/30) was gingiva, followed by the buccal mucosa at 20.5% (27/132). It is notable that such a high HPV-16 infection rate was found in gingiva although OLP is most commonly observed in buccal mucosa. In recent years, junctional epithelium in the gingival pocket has been gathering attention as an oral reservoir of HPV infection. In addition, Anrudari Erkhembaatar et al. reported that HPV-16 infection was the most commonly observed HPV infection in epithelial hyperplasia of denture epulis in gingiva, suggesting that gingiva functions as an HPV reservoir in the oral cavity. Therefore, further studies are necessary to clarify the role of gingiva in HPV infection.

It has also been reported that HPV's infect epithelial basal cells, which allow epithelium to proliferate. In order for HPV's to infect epithelial basal cells, viral particles must enter through the cornified layer of the epithelium that was broken due to skin or mucosal injury, and subsequently reach the basal cell layer. Moreover, once the basal cells became infected, HPV's go through a particular lifestyle in which the number of viral copies is maintained at 20-50 while the infected cells leave the basal cell layer and proliferate or differentiate into the prickle cell layer.
It is said that this continuous infection requires proliferative stimulation and genomic amplification at the appropriate level in the infected cells\(^{42,43}\). Once the infected cells reach the epithelial granular layer that is approaching terminal differentiation, HPV is rapidly amplified to several hundreds or thousands of copies per cell, and the matured viral particles are freed into the cornified layer\(^{42,43}\). Considering these characteristic of HPV infection, it can be assumed that HPV can easily invade and infect cells of OLP lesions where the oral mucosa is often damaged due to erosion or ulcers. Nishimura et al.\(^{44}\) also reported that HPV infection was frequently observed in the oral mucosa of patients wearing dentures, suggesting that chronic stimulation of the mucosa by the denture and subsequent erosion of the mucosa are the reason for the frequent infection. In the present study, the highest HPV positive rate was observed in the erosive type of OLP. It has also been reported in other studies that HPV infection is most commonly observed in the erosive type of OLP\(^{23,24,45}\). These reports support the hypothesis that HPV might easily infect OLP where the oral mucosa is often damaged due to erosion or ulcers. In addition, Manuelle et al.\(^{46}\) strongly suggested that HPV infection and associated CD8+ T cell proliferation is related to the erosive type of OLP. It can therefore be considered that inflammation in OLP plays a role in inducing HPV genomic amplification and in proliferative stimulation at the appropriate level of the infected cells for continuous infection.

It has also been reported that the application of topical steroids, which is a common treatment for OLP, decreases immunity and reactivates potential HPV infection, resulting in an increase in infection\(^{49}\). This finding suggests that both the frequent occurrence of mucosal erosion or of ulcers in OLP, and the common use of steroids for OLP treatment, may facilitate HPV infection.

It is also known that OLP can undergo malignant transformation and become SCC, although this event is not very common\(^{2,24,25,47,48}\). However, the frequency of this event has been long debated. Fitzpatrick et al.\(^{47}\) performed meta-analyses of reports regarding the malignant transformation of OLP and systematically analyzed the 16 articles that were finally extracted. They then reported that the rate of malignant transformation of OLP was 0 % to 3.5 % in 7,806 patients. The rate of malignant transformation was 0 % when the subject population was small (less than 100), and was 0.8 % to 1.9 % in studies that were based on populations over 500. The mean rate of malignant transformation in the 16 articles was 1.1%, and the mean period from diagnosis of OLP to malignant transformation was 51.4 months. Smoking and alcohol intake are not usually associated with this malignant transformation\(^{49}\), and the cause of this event has not yet been revealed.

Regarding the association between malignant transformation of OLP and HPV infection, Szarka et al.\(^{26}\) and Gorsky et al.\(^{25}\) reported that HR-HPV infection might be a risk factor for OLP transformation. However, they did not investigate HPV infection in patients with OLP that had actually malignantly transformed into SCC. Their speculation was based on the result that the rate of HPV infection in OLP patients was higher than that of healthy people and similar to that of oral leukoplakia or SCC patients, and on the fact that HPV-16 and -18, which are associated with cancer, were frequently observed in OLP patients.

Based on the above data, it is considered necessary to further investigate the association between malignant transformation of OLP and HPV infection in detail in order to provide oral treatment that can contribute to the diagnosis, prevention and treatment of HPV-associated oral diseases. Therefore, further detailed studies, including an investigation into whether HPV DNA has been integrated into the host DNA or exists as an episome, with a larger number of cases will be necessary in the future.

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