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Characterization of High-Risk Human Papillomavirus according to Periodontitis Severity

Kanitsak Boonanantanasarn[1], Jeong-Hwa Baek[1], Seok-Mo Heo[2,3*]

[1]Department of Molecular Genetics, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, South Korea
[2]Department of Periodontology, School of Dentistry, Jeonbuk National University, Jeonju, South Korea
[3]Research Institute of Clinical Medicine of Jeonbuk National University, Jeonju, South Korea

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ORCID ID
Kanitsak Boonanantanasarn, http://orcid.org/0000-0001-9288-0786
Jeong-Hwa Baek, http://orcid.org/0000-0002-2032-1683
Seok-Mo Heo, http://orcid.org/0000-0002-2511-8123

ABSTRACT

Characterization of High-Risk Human Papillomavirus according to Periodontitis Severity

Kanitsak Boonanantanasarn[1], Jeong-Hwa Baek[1], Seok-Mo Heo[2,3*]

[1]Department of Molecular Genetics, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, South Korea
[2]Department of Periodontology, School of Dentistry, Jeonbuk National University, Jeonju, South Korea
[3]Research Institute of Clinical Medicine of Jeonbuk National University, Jeonju, South Korea

The oral microenvironment can be modulated by chronic exposure to microorganisms, their byproduct and host-derived inflammatory response. Recently, high-risk HPV has been reported in periodontitis patients, suggesting that periodontal disease may be a reservoir for high-risk human papillomavirus (HPV). We aimed to examine the relationship between the existence of high-risk HPV 16/18 and the severity of periodontal disease. We collected a total of 342 oral specimens from 20 healthy subjects and 37 periodontitis patients. The specimens included dental plaque, saliva, and tongue scrape samples. HPV 16 or 18 (high-risk HPV) were detected by real time PCR. The data showed that high-risk HPV in healthy, stage I (mild) periodontitis and stage II (moderate) to stage III/IV (severe) periodontitis were 1.75% (6/342), 2.92% (10/342), and 8.47% (29/342), respectively. Dental plaque and tongue scrape specimens from moderate and severe periodontitis patients showed a significant detection of high-risk HPV in comparison with specimens collected from mild periodontitis and healthy subjects ($p<0.01$). Collectively, the severity of periodontal disease significantly increased the odds of high-risk HPV-positive samples ($p<0.01$). Our data suggest that the presence of HPV 16 and 18 in plaque and tongue of periodontitis patient may provide a suitable ecosystem for high-risk HPV.

Key words : human papillomavirus (HPV), human papillomavirus 16, human papillomavirus 18, periodontitis

Corresponding Author
Seok-Mo Heo, DDS, PhD
Department of Periodontology, School of Dentistry, Jeonbuk National University, Jeonju, South Korea
Tel : +82-63-250-2018 / Fax : +82-63-250-2259 / E-mail : neoheo@jbnu.ac.kr

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Introduction

Periodontal disease is a chronic inflammatory disease caused by multiple factors including genetic, local, and systemic factors[1,2]. One of the most crucial factors has been known as microorganisms, which play a main role in host response induction resulting in inflammation and destruction within the supporting tissues of the teeth[1,3].

Several research studies have suggested that the oral cavity of patients with periodontal disease may serve as the reservoir of many viruses including herpes simplex virus, Epstein-Barr virus, human cytomegalovirus and corona virus[4~6]. The presence of these viruses in the oral cavity may play a synergistic role with other microorganisms for the pathogenesis of periodontal diseases[5,7].

Human papillomaviruses (HPVs) can also be found in the oral cavity from healthy newborn babies to adults[8,9], suggesting that oral HPV can be transiently transmitted and clearly eliminated by the host immune response. Chronic exposure to microorganism, its byproduct and host-derived inflammatory response to periodontal disease can likely modulate the oral microbial dysbiosis[1,3], thereby providing suitable ecology system for viral pathogens including HPV[10].

Human papillomaviruses (HPVs) are a group of at least 150 types known DNA viruses that can be classified based on oncogenic potential into high- and low-risk types[11~16]. Low-risk type of HPVs (e.g., HPV 6 and 11) are associated with benign epithelial lesions, while the high-risk type of HPVs (e.g., HPV 16 and 18) are significantly associated with malignant epithelial transformations in cervical, breast, lung, penile, anal, and also oral tissue[11~16].

The increased expression of HPV receptor candidates in the inflamed epithelium of periodontal tissue may contribute to not only colonization but also a chronic persistent infection of high-risk HPVs such as HPV 16 and 18[17]. However, additional evidence is needed to support that periodontal apparatus can serve as a reservoir for high-risk HPV in chronic periodontitis patients.

In this study, the possible link of etiology between periodontitis and high-risk HPVs (HPV16 and 18) would be characterized in the complex environment of human oral cavity with periodontitis.

2. Materials and Methods

2.1. Study design and clinical parameters

A total of 57 participants were enrolled in this study. Informed consent was obtained from all participants prior to the study. The study was performed after obtaining approval from the Research Subjects Institutional Review Board (No. 201303003) of Jeonbuk National University Hospital. All participants were ≥18 years old and had at least 12 teeth distributed in both maxillae were included, except the third molars. Study participants with uncontrolled diabetes, arthritis, ulcerative colitis, Crohn's disease, renal disease, HIV infection, cancer, and heart disease were excluded from this study. The exclusion

criteria also included use of following medication over the last 3 months prior to sample collection: systemic antibiotics, anti-inflammatory drugs, and immuno-suppressive drugs.

Under comprehensive oral examinations, 57 subjects were divided into two groups: 20 periodontally healthy subjects and 37 periodontitis patients (Table1). Clinical periodontal status was examined by determining bleeding on probing (BOP), clinical attachment level (CAL) and probing depth (PD) to the nearest millimeter at the mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual sites around each tooth. According to the classification systems for periodontal diseases[18,19], full-mouth CAL and PD values were used to place patients in the following categories: healthy (interdental CAL<1 mm, maximum PD≤3 mm), stage I or mild periodontitis (interdental CAL=1-2 mm, maximum PD≤4 mm), stage II or moderate periodontitis (interdental CAL=3-4 mm, maximum PD≤5 mm), stage III/IV or severe periodontitis (interdental CAL≥5 mm, maximum PD≥6 mm).

2.2. Oral specimen collection and DNA preparation

Samples for HPV analysis were collected seven days after the initial clinical examination. Before collection, the oral cavity was washed with sterile distilled water. Non-stimulated saliva was then collected[20]. After removal of the supragingival biofilm, the selected sites were isolated with cotton rolls and gently air-dried. A periodontist (SM Heo) collected dental plaque samples from four subgingival pockets from each subject by using sterile curettes. Sampling included both clinically healthy and diseased sites based on the criteria mentioned above. The dorsum and lateral surfaces of the tongue were also brushed using a soft-bristled toothbrush and the tongue plaque samples were kept in sterile PBS. Total genomic DNA was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol[21] and kept at -20oC. Genomic DNA from HeLa and SiHa, human cervical carcinoma cell lines, were also prepared and used as positive HPV 18 and HPV 16 controls, respectively[22].

Table 1. Characteristics of the clinical study groups.

Characteristics	Periodontally healthy group	Periodontitis group	*p*-Value
Total subjects, N (%)	20 (35%)	37 (65%)	
Male/Female	14/6	24/13	
Age	30.95±7.40	49.6±11.77	
Smoking/Non-smoking	5/15	18/19	0.080
PD(mm)	1.6±.50	4.32±1.58	<0.01
CAL(mm)	1.65±.59	3.88±2.99	<0.01
BOP(%)	27.50±30.24	72.14±33.08	<0.01

N, number of subjects CAL, clinical attachment level; BOP, bleeding on probing

2.3. Real-time polymerase chain reaction (PCR)

To detect HPV 16 and HPV 18 from periodontally healthy controls and patients with periodontal disease, real-time-PCR was performed using SYBR Premix EX Taq (Takara Biotechnology, Otsu, Japan) in an AB 7500 and AB 7300 Fast Real-Time system (Applied Biosystems, Foster City, CA, USA). As described previously, the reaction mixtures for real-time PCR were prepared and the standard curves were validated[23]. DNA extracted from SiHa and HeLa cells and ultrapure sterile water served as positive and negative controls for each set of reactions, respectively. The forward and reverse primers for each gene were as follows (5′→3′). The primer sequences used for real-time PCR were as follows: HPV 16 E6/7 (f) 5'-GAATGTGTGTACTGCAAGCA-3'and HPV 16 E6/7-(r) 5'-GTTGTATTGCTGTTCTAAGTTGT-3'; HPV 18 E6/7 (f) 5'-GTATGGACCTAAGGCAACA-3' and HPV 18 E6/7 (r) 5'-GTCGGGCTGGCAAA-3'[23]. The human β-globin gene served as an internal control for test validation for human samples from saliva and tongue. Universal bacterial 16S primers set were used to determine the suitability of the isolated DNA from dental plaque. The human β-globin and 16S primers were as follows: β-globin (f) 5'-AACAGCATCAGGAGTGGAC-3' and β-globin (r) 5'-CTGCCTATTGGTCTATTTTCC-3'; U16S-1020 (f) 5′-TTAAACTCAAAGGAATTGACGG-3′ and U16S-1190 (r) 5′-CTCACGRCACGAGCTGACGAC-3′[23,24].

2.4. Conventional PCR

Positive results from randomly selected real-time PCR data were confirmed by conventional PCR with the iNtRON's i-StarTaq™ DNA polymerase system (iNtRON Biotechnology, Sungnam, Korea). The PCR reaction used to determine the occurrence of HPV 16 and 18 was performed in a 20 μL reaction mixture containing the DNA template, 2 μL of 10X PCR buffer, 2 mM MgCl2, 0.5 μL i-StarTaqTM DNA polymerase, 0.2 mM dNTP, and 1μM specific primers. Specific primers were used as follow: HPV 16 E6 (f) 5'-CAGGACCCACAGGAGCGACC-3', and HPV 16 E6 (r) 5'-ATCGACCGGTCCACCGACCC-3'; HPV 18 E6 (f) 5'-GCTTTGAGGATCCAACACGG-3' and HPV 18 E6 (r) 5'-TGCAGCACGAATGGCACTGG-3'. Similar to real-time PCR, β-globin (PC04 andGH20)[16] and 16S universal bacterial primers (8F and 1510R)[25] were also included in the experiment as follow; PC04 (f) 5'-CAACTTCATCCACGTTCACC-3' and GH20 (r) 5'-GAAGAGCCAAGGACAGGTAC-3'; universal 8F (f) 5'-AGAGTTTGATCCTGGCTCAG-3' and universal 1510R (r) 5'-CGGTTACCTTGTTACGACTT-3'[16]. As an additional confirmatory step, all DNA amplicons seen (positive band) on an agarose gel were submitted for DNA sequence analysis. All sequenced products were then compared with published E6 sequences (Gene ID: 1489088) of known HPV 16 and 18 types.

2.5. Statistical analysis and sample size determination

The statistical significance was analyzed by Chi-

square and Fisher exact tests using Prism 6 (GraphPad Software). A p-value <0.05 was considered statistically significant.

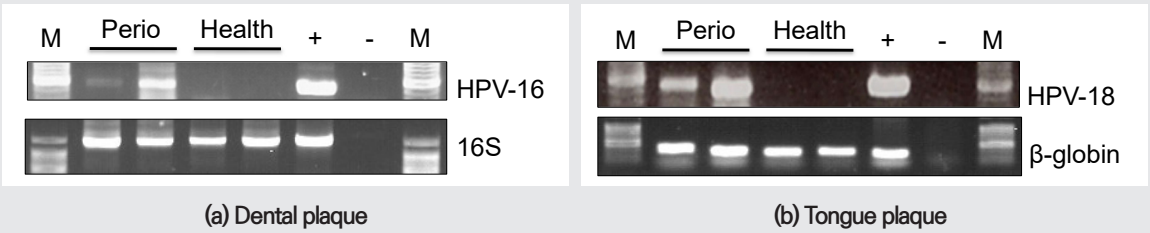
3. Results

3.1. Characteristics of periodontally healthy subjects and periodontal patients

Fifty-seven eligible patients were categorized as having periodontally healthy (20 cases), mild (15 cases), and moderate to severe (22 cases) periodontal conditions. Accordingly, the former status was allocated into periodontally healthy group and the latter two was referred to as periodontal group (Table1).

Four subgingival plaque samples, one tongue-scraping sample, and one salivary sample were collected for each patient. A total of 342 samples from the 57 subjects were subsequently tested by real-time PCR for the presence of high-risk HPV (HPV 16 and 18). HPV-positive samples by real-time PCR were randomly picked for further confirmation by conventional PCR and sequencing tests. An example of regular PCR from healthy and chronic periodontitis specimens was shown in Figure.1.

To indicate the severity of periodontal disease, clinical parameters have been used, including bleeding on probing (BOP), probing depth (PD), and clinical attachment loss (CAL). In the present study, the mean BOP (72.14±33.08), PD (4.32±1.58) and CAL (3.88±2.99) of patients in the chronic periodontitis group were considerably higher than the BOP (27.50±30.24), PD (1.6±0.50) and CAL (1.65±0.59) of the healthy control group (Table 1). These periodontal parameters suggest that the periodontal disease group has active and severe periodontal inflammation and destruction compared with the healthy group. Based on the case analysis of high-risk HPV detection (HPV 16 and 18) and periodontal



(a) Dental plaque (b) Tongue plaque

Figure 1. Identification of high-risk human papillomavirus (HPV) by polymerase chain reaction. HPV-16 E-6 and 16S rRNA from dental plaque samples (a) and HPV-18 E-6 and β-globin from tongue plaque samples (b). Electrophoresis on 2% agarose gel. M: Marker, Perio: moderate-severe periodontitis samples, Health: periodontally healthy samples, +: positive control, −: negative control, 16S: 16S rRNA

parameters, we found that enhancing the intensity of BOP, PD, and CAL significantly increased the detection of the high-risk HPV positive patient ($p<0.05$) (Table 2).

Since periodontal parameters may be associated with high-risk HPV positivity, next, we evaluated whether the periodontal status from patients with healthy or severity of periodontal conditions were associated with HPV 16 and HPV 18 status. HPV 16 was detected in 15%, 20%, and 50% of study participants with healthy, mild, and moderate to severe periodontal conditions, respectively. A similar trend of positivity was also found for HPV 18 detection (15% in healthy, 40% in mild, and 63.6% in moderate to severe periodontitis). Moreover, patients with moderate to severe periodontitis were associated with an approximately 6-fold (odds ratio, 5.67; 95% CI, 1.28 to 25.02) ($p=0.023$) and approximately 10-fold (odds ratio, 9.92; 95% CI, 2.20 to 44.62) ($p=0.002$), increased odds of HPV 16-positive and HPV 18-positive status, respectively. In contrast, no significant difference in the prevalence of HPV 16 in mild periodontitis cases was observed when compared with healthy group (odds ratio, 1.42; 95% CI, 0.24 to 8.26) ($p=1$) as well as HPV 18 (odds ratio, 3.78; 95% CI, 0.76 to 18.80), ($p=1.292$) (Table 3).

Other confounding factors such as age, gender, and smoking may also influence HPV status. Therefore, we determined whether these factors were relevant to the presence of HPV 16 and 18 in the oral cavities of the study participants. In our study, we considered volunteers with a history of smoking greater than one pack of cigarette/year as smokers. As shown in Table 3, around thirty-nine percent

Table 2. Periodontal parameters and high-risk HPV cases

Parameters	High-risk HPV cases		*p*-Value	
	Positive (%)	Negative (%)		
Subjective total, N (%)	34 (100%)	23 (100%)		
BOP			0.008	0.005
〈 25%	7 (20.6%)	14 (60.9%)		
≥25%	27 (79.4%)	9 (39.1%)		
PD			0.014	0.005
3 mm	12 (35.3%)	17 (73.9%)		
≥ 4 mm	22 (64.7%)	6 (26.1%)		
CAL			0.001	0.004
2 mm ≤	14 (41.2%)	19 (82.6%)		
3 mm -4 mm	6 (17.6%)	3 (13.0%)		
≥ 5 mm	14 (41.2%)	1 (4.4%)		

N, number of subjects; BOP, bleeding on probing; PD, pocket depth; CAL, clinical attachment level

Table 3. Periodontal status and smoking status related to the presence of HPV 16 and 18

Periodontal status	HPV 16 positive			HPV 18 positive		
	N (%)	*p*-Value	Odds ratio(95% CI)	N (%)	*p*-Value	Odds ratio(95% CI)
Mild periodontitis		n.s.			n.s.	
Healthy (n=20)	3 (15.0%)		1.42	3 (15.0%)		3.78
Mild (n=15)	3 (20.0%)		(0.24-8.26)	6 (40.0%)		(0.7-18.8)
Mod-Sev periodontitis		0.023*			0.002**	
Healthy (n=20)	3 (15.0%)		5.67	3 (15.0%)		9.92
Mod-Sev (n=22)	11 (50.0%)		(1.28-25.02)	14 (63.6%)		(2.2-44.6)
Smoking status						
Smoking (n=26)	10 (38.5%)	n.s.	2.65	14 (53.9%)	n.s.	3.35
Non-smoking (n=31)	7 (22.6%)		(0.82-8.5)	8 (25.8%)		(1.1-10.22)

N, number of participants; n.s., non-significance (*p*-value 〉 0.05); Mod-Sev, moderate to severe;

*, **: significance (*p*-Value 〈 0.05, 0.01, respectively)

and fifty-four percent of smokers were positive with HPV 16 and HPV 18, respectively, whereas lesser positive detection of HPV 16 (22.6%) and HPV 18 (25.8%) was observed in non-smokers. Even though HPV 16 positive in smoker was not statistically significant (p=0.143), the odds ratio was 2.65 (95% CI, 0.83 to 8.50) for smokers compared to non-smokers. For HPV 18, the result revealed that among smoking subjects, HPV 18 DNA was almost significantly detected with 3.35 times (95% CI, 1.1 to 10.22) higher than non-smokers (p =0.055) (Table 3).

To identify the presence of high-risk HPV in different periodontal status, we analyzed samples by PCR (Table 4). The data showed that approximately 13% (45/342) of all specimens were positive for high-risk HPV. Among all specimens (n=342), 20 specimens contained HPV 16 (5.8%), and 25 specimens contained HPV 18 (7.3%). Interestingly, one specimen contained both HPV-16 and 18 (0.3 %). Compared to participants with healthy periodontium and mild periodontitis, those with moderate to severe periodontitis had significantly higher levels of HPV 16 (2.5%, 4.4% vs 9.8%, p=0.007) and a greater detection of HPV 18 (2.5%, 6.7% vs 12.1%, p=0.002) (Table 4).

To determine whether the different microenvironments modified by periodontal disease play an important role in the presence of high-risk HPV (i.e. HPV 16 and 18) in the oral cavity, we examined 3 representative microenvironments from the oral cavity, including four different areas of dental plaque, the tongue surface and saliva. We found that tongue specimens from patients with moderate and severe periodontitis showed a significant increase of HPV 16 alone, HPV 18 alone or both high-risk HPVs (HPV 16 or 18). The corresponding result was also found in dental plaque specimens from patients

Table 4. The presence of HPV16 and 18 in the periodontally healthy and periodontal specimens

Periodontal status (N, %)	HPV 16 Positive	X2 (df) /*p*-Value	HPV 18 Positive	X2 (df) /*p*-Value
Healthy (120, 35.1%)	3 (2.5%)	7.319 df =1 / 0.007**	3 (2.5%)	10.06 df =1 / 0.002**
Mild peridontitis (90, 26.3%)	4 (4.4%)		6 (6.7%)	
Mod-Sev peridontitis (132, 38.6%)	13 (9.8%)		16 (12.1%)	
Total Specimens (342, 100%)	20 (5.8%)		25 (7.3%)	

N, number of specimens; Mod-Sev, moderate to severe;

** : significance (*p*-Value 〈 0.01)

Table 5. The presence of HPV16 and 18 in specimens from dental plaque, saliva and tongue

Periodontal status (N, %)	HPV 16 Positive	X2 (df) /*p*-Value	HPV 18 Positive	X2 (df) /*p*-Value
Dental plaque (228, 66%)				
Healthy (80)	3 (3.8%)	2.29 (df =1) /0.13	1 (1.3%)	5.83 (df =1) /0.015*
Mild peridontitis (60)	2 (3.3%)		4 (6.7%)	
Mod-Sev peridontitis (88)	8 (9.1%)		9 (10.2%)	
Saliva (57, 17%)				
Healthy (20)	0 (0%)	0.86 (df =1) /0.35	2 (10.0%)	0.009 (df =1) /0.92
Mild peridontitis (15)	1 (6.7%)		0 (0%)	
Mod-Sev peridontitis (22)	1 (4.5%)		2 (9.1%)	
Tongue plaque (57, 17%)				
Healthy (20)	0 (0%)	4.35 (df =1) /0.036*	0 (0%)	5 (df =1) /0.025*
Mild peridontitis (15)	1 (6.7%)		2 (13.3%)	
Mod-Sev peridontitis (22)	4 (18.2%)		5 (22.7%)	
Total Specimens (342, 100%)	20 (5.8%)		25 (7.3%)	

N, number of specimens; Mod-Sev, moderate to severe;

* : significance (*p*-Value 〈 0.05)

with moderate and severe periodontitis except for HPV 16 alone. In contrast, saliva and buccal specimens revealed no significant differences in the presence of HPV 16 or 18 and the severity of periodontal disease. These data indicate that microenvironment in the oral cavity from moderate to severe periodon-

titis patients may serve as reservoirs for HPV 16 and HPV 18 (Table 5).

4. Discussion

Periodontal disease is one of the most common diseases with a high incidence in Korea and many other parts of the world[26]. High-risk HPV infection is also speculated to exert a synergistic role with the chronic periodontal disease to enhance the risk of developing oral cancer[27~29]. However, the link between chronic periodontal disease and oral HPV acquisition is still unknown. Therefore, our hypothesis is that chronic periodontitis may promote the oral microenvironment to increase in the acquisition of high-risk HPV. To verify this hypothesis, 342 specimens were taken under the same condition from various oral specimens originating from different source and location (3 sites of dental plaques, saliva, and tongue surface).

The human oral cavity is a home to various kinds of bacteria, viruses, protozoa, fungi, and archaea assembled into a community harboring in different microenvironments and niches including saliva, supra- and sub-gingival plaques, and keratinized and non-keratinized oral mucosa[5,7]. Therefore, we determined whether the difference in oral microenvironments (e.g., saliva, dental plaques, and tongue mucosa) played a role in high-risk HPV positivity in chronic periodontitis. The result showed that the significant increase in high-risk HPV detection rate had been found only in specimens collected from the tongue (18.2%; p=0.004) and dental plaques (9.1%; p=0.001) of moderate to severe periodontitis. Our findings are in line with Fuster-Rossello et al., that high-risk HPV was present in the sample collected from the tongue (30%), mucosal epithelium (13%), and periodontal pocket (16.6%)[30]. Also, our findings confirmed the results of Parra and Slot which demonstrated that 17% of gingival fluid specimens collected from advanced periodontitis patients were positive for HPV[31].

It has been suggested that the periodontal pocket, which is composed of subgingival plaque and gingival fluid, could possibly serve as a reservoir for oral HPV[22,27]. This idea is based on the fact that one of the candidate receptors for oral HPV and cellular interacting receptors such as syndecan-1 were recently found to be highly expressed in the inflamed pocket epithelium of chronic periodontitis patient[32], thereby increase the susceptibility of high-risk HPV acquisition in periodontal pocket and subgingival plaques. In fact, the micro-ulceration of the pocket's epithelium may provide the great opportunity for high-risk HPV infection, transmission, and its persistence [17,27].

The tongue is the muscular organ in the oral cavity that is useful for tasting, licking, swallowing, chewing, and speaking. As a result, trauma and injury of the tongue can frequently occur during functioning. Also, recent evidence has revealed that tongue cancer may be caused by chronic dental trauma[33]. Thus, it is likely that micro-trauma on the tongue may result in increasing susceptibility to intraoral contamination from the HPV-infected periodontal

pocket[30] or direct transmission from oral sex practice[34].

The evidence that high-risk HPV infection may play a crucial role in oral cancer and oropharyngeal cancer development is increasingly appearing in the literature[23,27~29,35]. The prevention of these HPV-affected cancers using a vaccine is theoretically possible, even though many studies (e.g., vaccine efficacy and long-term protection) are still needed in this area[36,37]. The data from the present study provided the important evidence that an enhanced oral HPV positivity coincides with the severity of chronic periodontitis. Periodontal pocket, dental plaque, and tongue may act as reservoirs of high-risk HPV infection, suggesting that chronic periodontal disease would one of the predisposing risk factors for oral HPV infection. Additionally, prevention and treatment of periodontal disease are necessary and would be provided together with HPV vaccine implementation plan as following suggestions: 1. Multicenter randomized double-blind studies or large-scale national surveys would be performed since additional evidence is needed to support that increasing susceptibility to contamination from the HPV-infected mouth or direct transmission via oral sex. 2. According to the evidence-based data, national health insurance should support HPV vaccinations by broadening the age range (e.g. from teenagers to 30s) as well as more frequent periodontal maintenance (e.g. two times a year of dental recall, oral prophylaxis and scaling). 3. Dental healthcare providers would put on emphasis on the importance of HPV prevention in accordance with the periodontal treatment for microbial removal as one of potential risk factors.

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