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Human papillomavirus type 16 (HPV16) DNA copy number in oral rinse samples from oral cavity cancer patients

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Abstract

Objectives: The objective of this study is to examine the human papillomavirus type 16 (HPV16) DNA copy number in the oral cavity of cancer patients using oral rinse samples.

Methods: We analyzed the HPV16 DNA copy number of oral rinse samples in 13 primary oral cavity cancer patients (mean age 67.8 years, range 48–84 years) who visited the Department of Oral and Maxillofacial Reconstructive Surgery of Hiroshima University Hospital (Hiroshima, Japan). The 13 oral cavity cancers included 6 carcinomas in situ and 7 squamous cell carcinomas. Real-time PCR analysis was performed to examine the number of HPV16 E6 viral copies in oral rinse samples. Additionally, the HPV16 integration status was investigated using the real-time PCR findings for HPV16 E2 genes in HPV16 E6-positive oral rinse samples.

Results: HPV16 E6 copy numbers above the detection limit in a standard curve for HPV16 E6 DNA were assessed as HPV16 positive in oral rinse samples from 6 of 13 patients. The average number of HPV16 E6 viral copies was 1.71 $\pm$ 1.72 per cell (range, 0.39–4.96 copies/cell) in six oral rinse samples. The HPV16 E2 viral load was detected in four of the six HPV16-positive oral rinse samples. Two of the six HPV16-positive oral rinse samples showed HPV16 E2 copy numbers below the detection limit, indicating the full integration of HPV16 DNA.

Conclusions: The number of patients in this study was small; therefore, further investigation using a larger number of participants is required to clarify the level of HPV16 viral copy number in the oral cavity of cancer patients.

Keywords

HPV16, viral copy number, oral cavity cancer

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Introduction

Human papillomavirus (HPV) is a non-enveloped double-stranded DNA virus that belongs to the family of papillomaviridae.[1,2] Among HPV genotypes, HPV16 was identified as the cause of cervical cancer by zur Hausen and colleagues.[3] To date, more than 150 HPV genotypes have been reported, of which HPV16 is one of the most common genotypes in head and neck squamous cell carcinoma (HNSCC).[4] HPV16 E2 protein is a transcription factor that can regulate the expression of E6 protein.[5] HPV DNA integration into the host genome causes disruption of the E2 open reading frame, resulting in

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increased expression of E6. Therefore, HPV16 E6 DNA is commonly used for analysis of HPV16 DNA positivity and copy numbers.[5]

HPV infection remains in the basal cells of mucosa, though is discharged outside the body upon differentiation of the infected cells into keratinocytes.[6] Therefore, HPV DNA is thought to be detected in the exfoliated epithelial cells and saliva in the oral cavity. With regard to HPV infection in the oral and oropharyngeal regions, we have reported that HPV16 DNA positivity was higher in the gargle samples as compared to the oral rinse samples.[7] In addition, the average number of HPV16 E6 DNA copies was significantly higher in subjects with a high oral bacteria count than in those with a low count.[8] These results indicate that HPV16 infection is more common in oropharyngeal regions and oral HPV16 infection is associated with the oral hygiene status in cancer-free individuals. Conversely, few epidemiologic studies have been reported on the oral HPV copy number and its status in oral cavity cancer patients using oral rinse samples in Japan. Hence, we performed real-time PCR analysis to examine the HPV16 E6 DNA copy number using oral rinse samples obtained from primary oral cavity cancer patients.

Methods

Subjects

We analyzed 13 oral cavity cancer patients (mean age 67.8 years, range 48–84 years) who visited the Department of Oral and Maxillofacial Reconstructive Surgery of Hiroshima University Hospital (Hiroshima, Japan). The 13 primary oral cavity cancers included 6 carcinoma *in situ* and 7 squamous cell carcinomas (SCCs). Patients with recurrent disease were excluded from this study. The study design was approved by the Ethical Committee of Hiroshima University and all participants signed an informed consent agreement. Tumors were classified using the TNM staging system according to the 5th edition of the General Rules for Clinical Studies on Head and Neck Cancer.[9]

Oral rinse sample processing and DNA extraction

We collected oral samples by rinsing the oral cavity with 10 mL of saline for 30 s. The samples were centrifuged at 3000 $\times$ *g* for 10 min, the supernatants were removed, and the pellets were stored at -80°C until further processing. DNA was extracted and purified using a PureLink™ Microbiome DNA Purification Kit (Life Technologies, Gaithersburg, Maryland, USA).

Quantitation of human cell numbers

We quantitated the number of human cells in each sample using a real-time PCR assay of the human endogenous retrovirus group 3 member 1 (ERV3-1) gene. As we previously reported, a standard curve indicating CT value versus ERV3-1 copy number was used to estimate the number of human cells.[7] We prepared serial 10-fold dilutions of the pUC57 vector containing the ERV3-1 genome with copy numbers ranging from 10^0 to 10^9. Quantitation of DNA levels was performed using a CFX connect real-time PCR detection system (Bio-Rad, Hercules, California, USA). The reaction mixture was composed of 1.0 µl of DNA, 9.0 µl of THUNDERBIRD® SYBR qPCR Mix (TOYOBO, Osaka, Japan), and 10 µmol of each pair of oligonucleotide primers. Amplifications were performed with initial melting at 95°C for 5 min, then 40 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min.

Quantitation of HPV16 viral copy number

Real-time PCR was performed to determine the HPV16 viral load in oral rinse samples. A 600-bp fragment within HPV16 E6 and a 1920-bp fragment within HPV16 E2 were inserted into the pUC57 vector. Serial 10-fold dilutions of the vector were made with copy numbers ranging from 10^3 to 10^9. Those 10-fold dilutions were used to generate a standard curve (i.e. CT value vs. HPV16 E6 copy number). We then prepared DNA samples extracted from approximately 5000 to 10,000 human cells from each PCR mixture. The primer sequences for *HPV16 E6* were 5′-AAGGGCGTAACCGAAATCGGT-3′ (sense) and 5′-GTTTGCAGCTCTGTGCATA-3′ (antisense). DNA extracted from HPV16-positive CaSki cells was used as a positive control. HPV16 E6 copy numbers above the detection limit in a standard curve were assessed as HPV16 DNA positive.

Analysis of HPV16 physical status

The physical status of HPV16 was examined by real-time PCR, targeting the E2 open reading frame. Serial 10-fold dilutions of the vector were made with copy numbers ranging from 10^3 to 10^9. These 10-fold dilutions were then subjected to real-time PCR to generate a standard curve, which indicated the CT value versus copy number of HPV16 E2. The primer sequences for *HPV16 E2* were 5′-CAGACGACTATC-CAGCGACC-3′ (sense) and 5′-GCAGTGAGGATTGGAG-CACT-3′ (antisense). The loss of the E2 gene is considered to be a common consequence of HPV integration into the host genome.[10,11] Thus, we determined the HPV16 physical status as fully integrated DNA in the absence of an E2 signal in the E6 positive samples.

Statistical analysis

Statistical analysis was performed using SPSS software, version 24.0 (SPSS Inc., Chicago, Illinois, USA). Mann–Whitney *U* test was used to evaluate significant differences regarding HPV16 copy numbers. Fisher's exact test was used to evaluate the correlation between HPV16 DNA positivity and clinical factors. *P* values less than 0.05 were regarded as statistically significant.

Table 1. Correlation between HPV16 positivity in oral rinse samples and clinical parameters in oral cavity cancer patients.[a]

Clinical characteristics	HPV16 E6 positive	HPV16 E6 negative	*P* value
Gender			
Male (8)	3 (37.5%)	5 (62.5%)	
Female (5)	3 (60%)	2 (40%)	0.59
Age in years			
<65 (5)	3 (60%)	2 (40%)	
≥65 (8)	3 (37.5%)	5 (62.5%)	1.0
Tumor size			
Tis (6)	3 (50%)	3 (50%)	
T1/T2 (5)	3 (60%)	2 (40%)	
T3/T4 (2)	0 (0%)	2 (100%)	0.59
Anatomical site of tumor			
Tongue (10)	6 (60%)	4 (40%)	
Gingiva (2)	0 (0%)	2 (100%)	
Oral floor (1)	0 (0%)	1 (100%)	0.31
Diabetes			
(−) (13)	6 (46.2%)	7 (53.8%)	
(+) (0)	0 (0%)	0 (0%)	1.0
Hypertension			
(−) (10)	5 (50%)	5 (50%)	
(+) (3)	1 (33.3%)	2 (66.7%)	1.0
Smoking			
Current/former (3)	1 (33.3%)	2 (66.7%)	
Non (10)	5 (50%)	5 (50%)	1.0

HPV16: human papillomavirus type 16.
[a]Fisher's exact test was used for statistical analysis. *P* values less than 0.05 were regarded to be statistically significant.

Results

Quantitation of HPV16 E6 viral copy using real-time PCR

The HPV16 viral copy number was determined in a total of 13 oral rinse samples collected from oral cavity cancer patients, using real-time PCR with the HPV16 E6 primer. Furthermore, the number of HPV16 E6 DNA copies was evaluated as copy number per human cell. HPV16 E6 copy numbers above the detection limit in a standard curve were assessed as HPV16 DNA positive in 6 (3 SCC cases and 3 carcinoma in situ cases) of the 13 oral rinse samples tested. The average number of HPV16 E6 viral copies of oral rinse samples was 1.71 $\pm$ 1.72 per cell (range 0.39–4.96 copies/cell). The average number of HPV16 E6 viral copies of oral rinse samples was 0.66 $\pm$ 0.43 per cell and 2.77 $\pm$ 1.97 per cell in three SCCs and three carcinoma in situ cases, respectively. However, there was no significant difference in HPV16 E6 copy numbers of oral rinse samples between SCCs and carcinoma in situ cases ($p = 0.20$). In addition, there was no statistically significant association between HPV16 positivity of oral rinse samples and clinical parameters (Table 1).

Analysis of HPV16 physical status using real-time PCR

The HPV16 physical status was investigated using real-time PCR for HPV16 E2 in six HPV16 E6 positive oral rinse samples. The detection of E2 copy number was used to estimate the HPV16 physical state of each individual subject. The HPV16 E2 viral load was detected in four (average number, 0.027 $\pm$ 0.037 copies/cell) of the six HPV16 E6-positive oral rinse samples. Oral rinse samples of one SCC and one carcinoma in situ case (HPV16 E6 copy number, 0.43 and 1.14 per cell, respectively) showed HPV16 E2 copy numbers below the detection limit, indicating full integration of HPV16 DNA into the host genome.

Discussion

Oral HPV16 infection may be a significant risk factor for HNSCC, especially in oropharyngeal cancers.[12-14] As for oral cavity cancers, our previous study found that approximately 80% of patients with HPV16-positive moderate epithelial dysplasia progressed to oral SCC.[15] In addition, in vitro analysis suggested that HPV-positive oral cancer cell lines were no longer HPV DNA positive after several passages of cell culture.[15] It is hypothesized that oral HPV16 infection may be associated with the early development of oral cavity cancer. However, it remains controversial whether oral HPV infection is a significantly independent risk factor for oral cavity cancer. In addition to HPV, other risk factors such as smoking, alcohol consumption, and mechanical stress may play a vital role in the development of oral cavity cancer. E6 and E7 proteins are suppressed by the E2 protein and can be reactivated after the integration of HPV DNA into the host genome, inducing p53 degradation and cell cycle progression.[16,10] Therefore, HPV is thought to be involved in carcinogenesis in E6/E7-positive cancer. Although E6/E7 expression is common in cervical cancer, it is not frequently observed in oral cavity cancers.[12,17]

Oral rinsing is considered to be a noninvasive method compared with surgical resection or swabbing on the tumor surface. A previous study has demonstrated the efficacy of HPV16 DNA detection using oral rinse samples for the screening of HPV-positive HNSCC.[18] On the other hand, oral rinse samples showed low sensitivity of oral HPV16 to identify HPV16-positive oropharyngeal cancer.[19] There is a possibility that oral rinse samples may exhibit false negative results compared with surgically resected tissue samples in HNSCC patients. We could not examine the copy numbers of HPV16 E6 DNA in the surgically resected tissue samples from the 13 oral cavity cancer patients. Therefore, their HPV16 positivity of oral cavity cancer tissues remains unclear. Further examination using tissue samples is required to clarify the difference in HPV16 positivity between oral rinse samples and oral cavity cancer tissues.

We previously examined the HPV16 DNA copy number using oral rinse samples in individuals without oral cavity cancers and the number of HPV DNA copies was approximately 1.65 copies/cell (range 0.07–25.3 copies/cell).[8] The average copy number of HPV16 DNA in oral cavity cancer patients was comparable to individuals without cancer. This may suggest that the HPV16 DNA copy number is not especially high in oral cavity cancer patients. Interestingly, carcinoma in situ cases showed higher HPV16 E6 DNA copy number than SCC cases.

Smoking, in addition to sexual behavior, is considered to be a significant risk factor for oral HPV infection.[20] One possible reason is that smoking may suppress normal immunity in the oral cavity. Another reason is that smoking-induced periodontitis may be associated with oral HPV infection by providing the chance for HPV infection in the inflammatory periodontal pocket.[21,22] Therefore, a focus on smoking cessation and oral health care plays a vital role in its prevention. Interestingly, female smokers were more susceptible to oral HPV infection than male smokers, suggesting that the effect of smoking on female hormones may be involved in the risk of HPV infection.[23] With regard to current smokers with HPV-positive oral cavity cancer, the complex relationship between smoking and HPV is a critical issue. To clarify the effect of HPV16 on oral cavity cancers, it is necessary to take into consideration the effect of current smoking on oral HPV infection.

HPV DNA positivity was much higher in oropharyngeal cancers compared with oral cavity cancers, supporting the notion that the oropharynx is a more favorable site for HPV infection compared with the oral cavity.[12] We have previously reported a higher prevalence of HPV16 in gargle samples compared with oral rinse samples.[7] In addition, current smokers showed a higher percentage of HPV16 positivity than nonsmokers in gargle samples.[7] Our results indicate that smoking may be more strongly associated with HPV16 infection in the oropharyngeal region compared to the oral cavity.

Lorenzi et al.[24] reported that persistent oral HPV16 infection is associated with integrated HPV. Thus, HPV DNA integration into the host genome may be important for long-term HPV infection and progression of malignant changes in normal epithelial cells. In this study, complete integration was found in two cases of the HPV16-positive oral cavity cancer patients, indicating that complete integration of HPV16 DNA may not be common in oral cavity cancers. As this study had only a small number of oral rinse samples from patients with oral cavity cancer, further investigation using a greater number of oral rinse samples is required to clarify the level of HPV DNA copy number.

Conclusions

Complete integration of HPV16 DNA may not be common in oral cavity cancers. Since there was a small number of oral cavity cancer patients in this pilot study, further investigation using a greater number of oral rinse samples from oral cavity cancer patients is required to clarify the level of HPV DNA copy number in oral cavity cancer patients.

Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. *Vaccine* 2012; 30: F55–F70.
2. de Villiers EM, Fauquert C, Bernard HU, et al. Classification of papillomaviruses. *Virology* 2004; 324: 17–27.
3. Dürst M, Gissmann L, Ikenberg H, et al. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci U S A* 1983; 80: 3812–3815.
4. Shigeishi H and Sugiyama M. Association of human papillomavirus (HPV) infection with oral cancer: review of recent research. *J Dental Hlth* 2017; 67: 149–159.
5. Howley PM, Schiller JT and Lowy DR. Papillomaviruses. In: Knipe DM and Howley PM (eds) *Fields virology*. Philadelphia, Lippincott: Williams and Wilkins, 2013, pp. 1662–1703.
6. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci* 2006; 110: 525–541.
7. Shigeishi H, Sugiyama M, Ohta K, et al. Higher prevalence and gene amplification of HPV16 in oropharynx as compared to oral cavity. *J Appl Oral Sci* 2016; 24: 397–403.
8. Shigeishi H, Sugiyama M, Ohta K, et al. High HPV16 E6 viral load in the oral cavity is associated with an increased number of bacteria: a preliminary study. *Biomed Rep* 2018; 8: 59–64.
9. Japan Society for Head and Neck Cancer. *General rules for clinical studies on head and neck cancer*, 5th ed. Tokyo: Kanehara & Co Ltd, 2012.
10. Arias-Pulido H, Peyton CL, Joste NE, et al. Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. *J Clin Microbiol* 2006; 44: 1755–1762.
11. Saunier M, Monnier-Benoit S, Mauny F, et al. Analysis of human papillomavirus type 16 (HPV16) DNA load and physical state for identification of HPV16-infected women with high-grade lesions or cervical carcinoma. *J Clin Microbiol* 2008; 46: 3678–3685.
12. Ndiaye C, Mena M, Alemany L, et al. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. *Lancet Oncol* 2014; 15: 1319–1331.
13. Castellsagué X, Alemany L, Quer M, et al. HPV involvement in head and neck cancers: comprehensive assessment of biomarkers in 3680 patients. *J Natl Cancer Inst* 2016; 108: djv403.
14. Deng Z, Hasegawa M, Kiyuna A, et al. Viral load, physical status, and E6/E7 mRNA expression of human papillomavirus in head and neck squamous cell carcinoma. *Head Neck* 2013; 35: 800–808.
15. Sugiyama M, Bhawal UK, Dohmen T, et al. Detection of human papillomavirus-16 and HPV-18 DNA in normal, dysplastic, and malignant oral epithelium. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2003; 95: 594–600.
16. Moscicki AB, Schiffman M, Kjaer S, et al. Chapter 5: updating the natural history of HPV and anogenital cancer. *Vaccine* 2006; 24(Suppl 3): S3/42–51.
17. Lingen MW, Xiao W, Schmitt A, et al. Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral Oncol* 2013; 49: 1–8.

18. Chai RC, Lim Y, Frazer IH, et al. A pilot study to compare the detection of HPV-16 biomarkers in salivary oral rinses with tumour p16(INK4a) expression in head and neck squamous cell carcinoma patients. *BMC Cancer* 2016; 16: 178.
19. Ahn SM, Chan JY, Zhang Z, et al. Saliva and plasma quantitative polymerase chain reaction–based detection and surveillance of human papillomavirus–related head and neck cancer. *JAMA Otolaryngol Head Neck Surg* 2014; 140: 846–854.
20. Shigeishi H and Sugiyama M. Risk factors for oral human papillomavirus infection in healthy individuals: a systematic review and meta-analysis. *J Clin Med Res* 2016; 8: 721–729.
21. Hormia M, Willberg J, Ruokonen H, et al. Marginal periodontium as a potential reservoir of human papillomavirus in oral mucosa. *J Periodontol* 2005; 76: 358–363.
22. Tezal M, Sullivan Nasca M, et al. Chronic periodontitis-human papillomavirus synergy in base of tongue cancers. *Arch Otolaryngol Head Neck Surg* 2009; 135: 391–396.
23. Gillison ML, Broutian T, Pickard RK, et al. Prevalence of oral HPV infection in the United States, 2009–2010. *JAMA* 2012; 307: 693–703.
24. Lorenzi A, Rautava J, Kero K, et al. Physical state and copy numbers of HPV16 in oral asymptomatic infections that persisted or cleared during the 6-year follow-up. *J Gen Virol* 2017; 98: 681–689.

Translational Value

Human papillomavirus infection is a major cause of cervical cancer and is also associated with the development of head and neck squamous cell carcinoma. We examined the human papillomavirus type 16 (HPV16) DNA copy number in oral cavity cancer patients using oral rinse samples. The HPV16 copy number can be determined by real-time PCR using oral rinse samples. Complete integration of HPV16 DNA may not be common in oral rinse samples from oral cavity cancers. Further investigation using a greater number of participants is required to clarify the level of HPV16 copy number and physical status in oral cavity cancer patients.