Human Papillomavirus Type 16 Infection and Local Lymph Node Metastasis in Patients with Squamous Cell Carcinoma

Ryoko Kawai1, Madoka Isomura1, Nobuaki Sato1,2, Waka Yoshida1,2, Kei Kamiya1, Yoshitaka Nagaya1, Tomofumi Hattori1, Koji Suzuki1, Akiyoshi Funato1, Masanori Yoshiyama1, Yoshihiko Sugita1,2, Katsutoshi Kubo1,2 and Hatsuhiko Maeda1,2

1) Department of Oral Pathology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan
2) Research Institute of Advanced Oral Science, Graduate School of Dentistry, Aichi Gakuin University, Nagoya, Japan

(Accepted for publication, December 11, 2015)

Abstract: Human papillomavirus (HPV) infection is known to be an independent etiologic factor for oral squamous cell carcinoma (OSCC). Especially, HPV-16 is associated with a significant risk of developing OSCC. The most important prognostic factor in OSCC is local lymph node metastasis (LNM); therefore, knowledge of LNM status is crucial for selecting proper treatment plans. However, it is not clarify relationship between HPV-16 infection and LNM in OSCC. The purpose of this study was to determine the role of HPV-16 infection in LNM in OSCC. We analyzed 130 cases of OSCC (100 cases of OSCC without LNM; 30 cases of OSCC with LNM). HPV-16 infection was detected by PCR, immunohistochemical examination and in situ hybridization. HPV-16 positivity rates among primary tumor (PT) specimens without LNM were 43.0 % (43/100), and HPV-16 positivity rates among PT specimens with LNM were 26.7 % (8/30). In addition, HPV-16 positivity rates in both PT and LNM specimens in 30 OSCC patients with LNM were 10 % (3/30). OSCC with HPV-16 DNA detected by PCR showed positive staining on immunohistochemical examination and in situ hybridization. The HPV-16 infection rate in OSCC with LNM was significantly lower than that for OSCC without LNM. In the case of OSCC with LNM, HPV-16 infection rates for both in PT and LNM were low. This suggests that HPV-16 positive cases had a significantly lower risk of LNM when compared with patients having HPV-16 negative OSCC. The results of the present study suggest that HPV status in OSCC is able to act as a marker for risk of LNM.

Key words: Human papillomavirus, Oral squamous cell carcinoma, Lymph node metastasis

Introduction

Worldwide epidemiological data indicate that oral cancer caused 135,000 deaths in 2013, a significant increase over the 84,000 deaths in 1990, thus representing a major worldwide health problem1. Squamous cell carcinoma is most common malignancy of the oral cavity, and oral squamous cell carcinoma (OSCC) may be superficial as well as deep, destroying oral cavity tissues and metastasizing into local lymph nodes or distant organs, most frequently the lung2.

The primary risk factors for development of OSCC have not been fully elucidated. Tobacco use and alcohol consumption, however, are considered to be the main etiological factors3. With the decrease in the prevalence of these habits, there has been an increase in incidence of oropharynx, tongue and oral cancer in young adults. The increasing numbers of patients that develop oral and oropharyngeal cancers suggest etiologic factors other than tobacco and alcohol as its cause4. Human papillomavirus (HPV) infection is known to be an independent etiologic factor for OSCC4. According to recent studies in the United States and northern Europe, the incidence of HPV-related OSCC has increased, and it is expected that HPV will replace tobacco as the main causative agent5.

Recently more than 120 types of HPV have been identified in various lesions6. They are subdivided on the basis of cutaneous and mucosal site of involvement, and as low-risk (LR) groups and high-risk (HR) groups, depending on their association with malignancy. The LR-HPVs cause wart-like lesions of the skin, anogenital region and oral mucosa, while HR-HPVs are etiologically associated with cervical, anogenital and oral cancers7,8. HR-HPVs encode E6 and E7 oncoproteins, which bind and degrade p53 and pRb tumor suppressors9,10, respectively.
The most important prognostic factor in OSCC is local lymph node metastasis (LNM); therefore, knowledge of LNM status is crucial for selecting proper treatment plans. The purpose of this study was to determine the role of HR-HPV in LNM of OSCC and to investigate whether HPV status in OSCC is able to provide a marker for LNM risk.

Materials and Methods

Study population

Formalin-fixed and paraffin-embedded OSCC biopsy or excised specimens were obtained from the Aichi Gakuin University Dental Hospital in Nagoya, Japan. Of the 130 patients from which specimens were obtained, 100 had OSCC without LNM and the remaining 30 had OSCC with LNM.

Of the 100 specimens from OSCC without LNM, 59 specimens were derived from male patients, and 41 specimens from female patients ranging in age from 25 to 89 years, with a mean age of 63.5 years. In the 30 patients of OSCC with LNM, 17 were male and 13 were female, ranging in age from 45 to 94 years, with a mean of 67.4 years.

All specimens were grouped histologically into grade I according to the WHO classification.

Extraction of DNA

Analysis of specimens was performed on six adjacent 4-μm sections of each paraffin-embedded tissue, with the first section being stained with hematoxylin and eosin in order to visualize OSCC. The remaining slices were used for DNA extraction. DNA was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Tokyo, Japan) in accordance with the manufacturer’s protocol. To avoid false-negative results due to low DNA yield, extracted DNA quantity and purity were measured with the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE). Extracted DNA integrity was confirmed by PCR amplifying the 110 bp β-globin gene in all specimens. The CaSki cell line served as a positive control for HPV-16.

PCR analysis

In the first step of PCR assay, we used the L1 consensus primers GP5/6(12) and GP5+/6+(13). PCR products were further amplified using same primers by further PCR. GP5/6 is able to detect the presence of DNA of 4 HR-HPV types (16, 18, 31 and 33) and 7 LR-HPV types (1, 6, 11, 30 and 32)(12). GP5+/6+ is able to 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)(12). Secondly, HPV-positive PCR samples in the first step of the assay using consensus primers were analyzed by type-specific PCR with primers designed for detection of HPV-16, the most common HR-HPV.

Oligonucleotide primers were synthesized by Sigma Aldrich Japan (Tokyo, Japan). In this study, we used 2×PCR Solution Premix Ex Taq Polymerase Hot Start Version (Takara Bio Inc., Otsu, Japan). After the reaction, PCR products were electrophoresed on 3 % NuSieve 3:1 Agarose (Lonza Rockland, ME, USA) gel, followed by staining with ethidium bromide and visualization under an ultraviolet transilluminator. The gel was subsequently photographed.

Immunohistochemical examination (IHC)

In order to confirm the presence of HPV protein immunohistochemically, we used monoclonal mouse anti-HPV (Clone K1H8, M3528; Dako, Carpinteria, CA USA). Tissue specimens positive for HPV-16 by PCR methods were stained using the EnVision™/HRP method (K1392; Dako, Carpinteria, CA, USA).

In situ hybridization (ISH)

Distribution of HPV DNA was confirmed using the DNA ISH technique with formalin-fixed, paraffin-embedded tissue samples that were positive for HPV-16 by PCR methods, using the GenPoint HPV in situ hybridization System for Biotinylated Probes (K0620; Dako, Carpinteria, CA, USA) with Human Papillomavirus (HPV) DNA Probe Cocktails, Biotinylated (Y1412; Dako, Carpinteria, CA, USA), in accordance with the product protocols.

Statistical analysis

Statistical analysis was performed by Chi-squared test. Differences with a p value <0.05 (*) were considered to be statistically significant, and differences with a p value <0.01 (**) were highly significant.

This study was approved by the Ethics Committee of the
Results

On PCR analysis, HPV-16 positivity rates for all PT specimens were 39.2 % (51/130) and for LNM specimens were 10 % (3/30). A statistically significant association was confirmed between PT and LNM (p<0.05).

HPV-16 positivity rates for PT specimens without LNM were 43.0 % (43/100), and HPV-16 positivity rates for PT specimens with LNM were 26.7 % (8/30). In addition, HPV-16 positive rates for only PT specimens, and for both PT and LNM specimens in 30 OSCC patients with LNM were 16.7 % (5/30) and 10% (3/30), respectively (Fig. 1). Statistically significant differences were observed between the frequencies of OSCC without LNM and OSCC with LNM (HPV-16 was positive in only PT specimens), OSCC without LNM and OSCC with LNM (HPV-16 was positive in both PT and LNM specimens) (p<0.05).
There were 3 cases in which HPV-16 was positive in both PT and LNM specimens. The PT in these cases was derived from the base of the tongue (2 cases) and the floor of the mouth (1 case).

Histopathologically, all cases were well-differentiated OSCC that showed keratin pearls (Fig. 2a and 2b). Immunohistochemically, positive HPV staining of the nucleus in the epithelium layer was observed in specimens in which HPV DNA was detected by PCR (Fig. 2c).

On ISH, OSCC specimens that were positive for HPV-16 by PCR, showed positive staining in the nuclei of neoplastic epithelial cells. Episomal HPV DNA was evident from the diffuse nuclear staining pattern (Fig. 2d). On the other hand, integrated HPV DNA was denoted by the single punctate staining pattern by the ISH (Fig. 2e). In this study, episomal HPV DNA was observed most commonly.

**Discussion**

HPV is the most common sexually transmitted viral infection worldwide. Recent studies have shown that oral infection with HPV is associated with a significant risk of developing oropharyngeal cancer (OPSCC). There is also a potentially important causal association between HPV, OSCC and oral potentially malignant disorders (OPMD), as reported in a systematic review. The frequency of detection of HPV in tongue cancers ranges from 0 to 81%. HPV detection rates vary depending on the assay employed and geographic location of the study population. Most studies have been performed using PCR or ISH as detection methods. In this study, PCR, ISH and IHC were performed in order to detect HPV infections, and we confirmed that the reliability of these three methods is high.

In OSCC and OPSCC, the HR-HPV HPV-16 is considered to play an important role in malignant transformation. Recent studies have found that the frequency of HPV-16 infection in OSCC ranges from 10.6 to 81.3% using PCR. In this study, HPV-16 positivity rates among all PT specimens were 39.2% (51/130), which is within the range reported in the above-mentioned study. However, when compared with OPSCC, the prevalence of HPV-16 is slightly high. Histologically, OSCCs are classified into well, moderately and poorly differentiated types; among these, well-differentiated OSCCs are the most common. All well-differentiated samples in our study differed from OPSCC, which were poorly differentiated. Hauck et al. reported that HPV rates in well-differentiated head and neck squamous cell carcinomas were particularly high. This suggests that HPV positivity is correlated with low malignant potential in OSCC. Our results support the hypothesis that infection with HPV-16 is one of the etiologic factors in OSCC.

The incidence of neck node metastases in OSCC and OPSCC varies between 25% and 65% among different series. It has been accepted that local lymph node metastasis is an important prognostic factor in OSCC. Joo et al. reported that there was a significant correlation between HR-HPV positivity and cervical lymph node metastasis in OPSCC, but no significant relationship has been confirmed in OSCC patients with HR-HPV. In addition, HR-HPV-positive cases in OPSCC had a significantly higher risk of cervical lymph node metastasis when compared to OPSCC patients having HR-HPV-negative tumors. Likewise, Hoffmann reported that among 89.3% of patients with positive HPV cervical lymph nodes, metastasis was identified, while HPV-negative cases showed only a 64.4% rate of lymph node metastasis. Moreover, for cervical cancer, a higher frequency of pelvic lymph node metastasis was noted among the HPV-positive group, and deeper stromal invasion was more common in patients with HPV-positive tumors. These findings suggest that HR-HPV is associated with LNM in patients with OPSCC and cervical cancer. In our study, the HPV-16 positivity rates in PT specimens without LNM were 43.0%, and the HPV-16 positivity rates in PT specimens with LNM were 26.7%. In addition, HPV-16 positive rates in both PT and LNM specimens in 30 OSCC patients were 10%. From these results, it appears that HR-HPV-positive cases had a significantly lower risk of LNM when compared with patients having HR-HPV-negative OSCC. Because episomal HPV DNA was observed most commonly in this study, it also might be one of the reasons for the lower risk of LNM in OSCC.

HPV-related head and neck squamous cell carcinoma (HNSCC) has better prognosis than non-HPV HNSCC. The reason for a better survival in patients with HPV-positive tumors is unclear, but the better clinical outcome can be attributed either to higher radiosensitivity of HPV-positive tumors or active antiviral cellular immune responses in patients with these tumors. Furthermore, Weinberger et al. reported that HPV-positive/p16-expressing tumors had a low recurrence rate, as well as markedly improved disease-free survival and overall survival. These results are in agreement with our finding that HPV-16 infection was lower in patients with LNM. However, there remains much to clarify with regard to HPV activity during the invasion process in OSCC. Further studies into HR-HPV associated with the invasion of OSCC might aid in the treatment decisions of patients with oral cancer.

Based on the results of the present study, HPV status in OSCC appears to provide a marker for risk of LNM. However, it remains necessary to further investigate the association between HR-HPV infections and prognosis in OSCC patients. Moreover, further detailed studies with a larger number of OSCC cases will be necessary in the future.

**Acknowledgements**

This research was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, under a Grant-in-Aid for Scientific Research C (Grant No. 15K11031).
Conflict of Interest

The authors have declared that no COI exists.

References


34. Im SS, Wilczynski SP, Burger RA and Monk BJ. Early stage cervical cancers containing human papillomavirus type 18 DNA have more nodal metastasis and deeper stromal invasion. Clin Cancer Res 9: 4145-4150, 2003

