Human papillomavirus-16 infection and p16 expression in oral squamous cell carcinoma

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Abstract. Human papillomavirus (HPV) is a possible carcinogenetic factor in oral squamous cell carcinoma (OSCC). Previous studies have reported the prevalence of HPV in patients with OSCC. However, the association between HPV and OSCC remains controversial. The present study aimed to clarify the association between HPV infection, p16 protein expression and the clinicopathological characteristics of OSCC. The expression level of HPV-16E6 mRNA and p16 protein, a known surrogate marker of HPV infection, was investigated in 100 OSCC cases using TaqMan reverse transcription-quantitative PCR and immunohistochemistry staining, respectively. HPV-16E6 mRNA expression level was only detected in one case (1%), and positive expression of p16 was found in 10 cases (10%), including an HPV-positive case. Subsequently, the association between p16 expression level and clinicopathological characteristic factors were analyzed; however, no significant association was found. These results suggested that HPV-16 infection was less likely to cause OSCC in Japan and p16 expression was not a suitable marker for HPV infection in OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent type of head and neck squamous cell carcinoma (HNSCC), with >500,000 new cases annually worldwide (1). OSCC is more likely to invade local tissues and spread to the lymph nodes, and has a mortality rate of ~50% within five years (2). Despite the increasing knowledge into the etiology of OSCC and the advances in chemotherapy, radiation and surgery, there has been little improvement in the relative survival time in patients with OSCC in recent decades (2). Smoking and drinking are major risk factors for OSCC (3). In addition, infection with human papillomavirus (HPV) has been identified as another risk factor for developing carcinoma in the oral cavity (4).

HPV is a circular double-stranded DNA molecule, ~8 kb and over 100 genotypes have been reported (5). The high-risk types, HPV-16 and 18 have been associated with 90% of uterine cervical cancers (6-8). The HPV genome is composed of early and late genes, which encode the early proteins, E1 to E7, and the late proteins L1 and L2. Among these genes, E6 and E7 have critical functions in malignant transformation of squamous cells (9). E6 binds to TP53 and inactivates its function by ubiquitin-dependent degradation (10). E7 manipulates and degrades the retinoblastoma tumor suppressor protein (Rb), resulting in the activation of the transcription factor E2F, which enhances the expression of the cyclin-dependent kinase inhibitor 2A (CDKN2A; p16) (11). The expression of the p16 protein has been used as a surrogate marker for HPV infection in HNSCC (12).

The relevance of HPV infection in cervical cancer and HNSCC is well-known. Patients with HNSCC and are HPV-positive have an improved prognosis compared with those who are HPV-negative (13). According to a systematic review on HPV detection among 4,852 HNSCC cases worldwide, the overall prevalence rate of HPV was 34.5% (14). HPV-16 and 18 are the most common genotypes in HPV-positive OSCC, with a frequency rate of 32.4% (204/630) and 11.3% (71/630), respectively (15). Another systematic review on HPV infection, in patients with OSCC, reported that the prevalence rate of HPV was 55.5% (76/137) and the most common genotype was HPV-16 (16).

However, the association between HPV infection and OSCC remains unclear. For example, the prevalence of HPV in patients with OSCC varies in different regions worldwide (17,18). In addition, a wide range of HPV prevalence was observed among patients from Japan (0-78%) (19-23). The present study aimed to determine the prevalence of HPV and clarify the association between HPV-16 infection, p16 protein expression and clinicopathological characteristics of OSCC.

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Abbreviations: HPV, human papillomavirus; OSCC, oral squamous cell carcinoma

Key words: head and neck squamous cell carcinoma, human papillomavirus, infection, oral squamous cell carcinoma, p16

Materials and methods

Patients and samples. Tissue samples were obtained from 100 patients with OSCC, including basaloid squamous cell carcinoma (BSCC), who underwent surgical resection at the Department of Oral and Maxillofacial Surgery at Ehime University Hospital (Ehime, Japan) between April 2004 and March 2013. Tumor staging was assessed according to the Union for International Cancer Control TNM Classification of Malignant Tumors 7th Edition and histological grading was performed according to the World Health Organization criteria for OSCC (24). The Institutional Review Board of Ehime University Hospital (Ehime, Japan) approved the present study.

Immunohistochemistry. Surgically resected OSCC specimens were fixed in 10% phosphate-buffered formalin for 24 h at room temperature and embedded in paraffin. A series of 4-μm thick sections were prepared from each sample. Immunohistochemical (IHC) staining was performed using the avidin-biotin-peroxidase complex method. Briefly, the sections were deparaffinized and heated at 121˚C in an autoclave for 20 min in 10 mM citrate buffer (pH 6.0) to regenerate epitopes. The sections were incubated with 0.3% hydrogen peroxide in distilled water for 5 min at room temperature to block endogenous peroxidase activity. The sections were then incubated overnight at 4˚C with a specific mouse monoclonal antibody to anti-human p16 (cat. no. 550834; diluted 1:50; BD Pharmingen; BD Biosciences). After washing with TBS+Tween-20 (TBS-T; Sigma-Aldrich; Merck KGaA), the sections were overlaid with biotinylated anti-mouse antibody (Maravai LifeSciences) at room temperature for 60 min, washed with TBS-T, then labeled with streptavidin-peroxidase complex (Maravai LifeSciences). The sections were subsequently counterstained with hematoxylin for 10 sec at room temperature, dehydrated with a series of graded ethanols (75, 95, 100 and 100%) for 5 min each at room temperature, treated with xylene and enclosed in synthetic resin. IHC staining was observed at x40 and x100 magnifications under a light microscope (Nikon Corporation). Positive expression of p16 protein was determined as when >70% of tumor cells showed strong and diffuse nuclear and cytoplasmic staining.

RNA extraction and RT-qPCR. Total RNA was extracted using ISOGEN (Nippon Gene) from lysing the tissues, after homogenization, with a TissueLyser (Qiagen), according to the manufacturer's protocol.

The presence of HPV-16E6 mRNA in the tissues was determined using RT-qPCR and the TaqMan® RNA-to-C_T™ 1-Step kit (Thermo Fisher Scientific, Inc.). PCR amplification was performed in a 10-μl final reaction mixture containing 0.25 μl TaqMan® RT Enzyme Mix (40X), 5 μl TaqMan® RT-PCR Mix (2X), 0.4 μl each forward and reverse primers (10 μM each), 0.2 μl TaqMan® probe (10 μM) and 1 μl total RNA (100 ng/μl). The following thermocycling conditions were used: RT at 48˚C for 15 min, then initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 min and 60˚C for 1 min. Oropharyngeal cancer tissues from biopsy materials obtained from patients with tonsil cancer treated in the Department of Oral and Maxillofacial Surgery at Ehime University Hospital were used as the positive control. Written informed patient consent was obtained.

The expression of HPV-18E6 mRNA was detected using RT-qPCR and the SYBR® system. PCR amplification was performed in a 10-μl final reaction mixture containing 5 μl 2X One Step SYBR® RT-PCR Buffer 4, 0.4 μl PrimeScript® One Step Enzyme Mix 2, 0.2 μl ROX reference Dye II (50X), 2.6 μl RNase-free distilled water (all from Takara Bio, Inc.), 0.4 μl forward and reverse primers (10 μM each) and 1 μl total RNA (100 ng/μl). The following thermocycling conditions were used: RT at 45˚C for 5 min and 95˚C for 10 sec, followed by 40 cycles at 95˚C for 5 sec and 55˚C for 30 sec.

Amplification and detection were performed using the ViiA™ 7 real-time PCR system (Thermo Fisher Scientific, Inc.). Hydroxymethylbilane synthase (HMBS) was used as an internal control. To confirm the amplicon size, the PCR products were electrophoresed on 3% agarose gels (Bio-Rad Laboratories), stained with ethidium bromide for 5 min at room temperature, and visualized under an ultraviolet transilluminator (FAS-III; Toyobo Life Science).

The following primers and TaqMan® probe were used: HPV-16E6 forward, 5'-GAATGTGTGTACAAGCAACAG-3', reverse, 5'-TGGATTCCCATCTCTATATACTATGCAT-3' and TaqMan® probe, 5'-CGACGTGAGGTATATGACTTTGCTTTTCGG-3' (25); HPV-18E6 forward, 5'-CAGAAACCGTTGAATCCAGCA-3' and reverse, 5'-TTTCTCTGCGTCGTTGGAGTC-3' (25) and HMBS forward, 5'-CATGCAGGCTACCATCCATGTC-3' and reverse, 5'-GTTACGAGCAGTGATGCCTACCAA-3'.

Genomic DNA extraction and exonuclease V-qPCR. Genomic DNA was extracted from formalin fixed paraffin embedded (FFPE) OSCC tissues using a GeneRead DNA FFPE kit (Qiagen GmbH). Exonuclease V (ExoV; New England BioLabs, Inc.) digestion was performed, as previously described (26). For detecting HPV-16E6 DNA, qPCR amplification was conducted in a 10-μl final reaction mixture containing 5 μl 2X PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc.), 0.5 μl each forward and reverse primers (8 μM each), 3 μl nuclease-free distilled water and 1 μl genomic DNA (10 ng/μl) with or without ExoV digestion. The following thermocycling conditions were used: Enzyme activation at 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Amplification and detection was performed using a ViiA™ 7 real-time PCR system (Thermo Fisher Scientific, Inc.). Human ribosomal 18S DNA (rDNA) was used as an internal control. To confirm the amplicon size, the PCR products were electrophoresed and visualized using an Agilent 2100 Bioanalyzer with the DNA 1000 kit (Agilent Technologies). The following primers were used: HPV-16E6 forward, 5'-GAGAACTGCAATGTTTCAGGACC-3' and reverse, 5'-TGTATAGTTTGCAGCTCTGTGC-3'; rDNA forward, 5'-GCAATTCCCCATGAACG-3' and reverse, 5'-GGGACTTAATCAACGCAAGC-3' (26).

Statistical analysis. χ^2 or Fisher's exact tests was used to determine significant differences between 2 groups. The Kaplan-Meier method was applied for survival analysis after follow-up for 36 months. Differences in patient survival were determined using the log-rank test. $P<0.05$ was considered

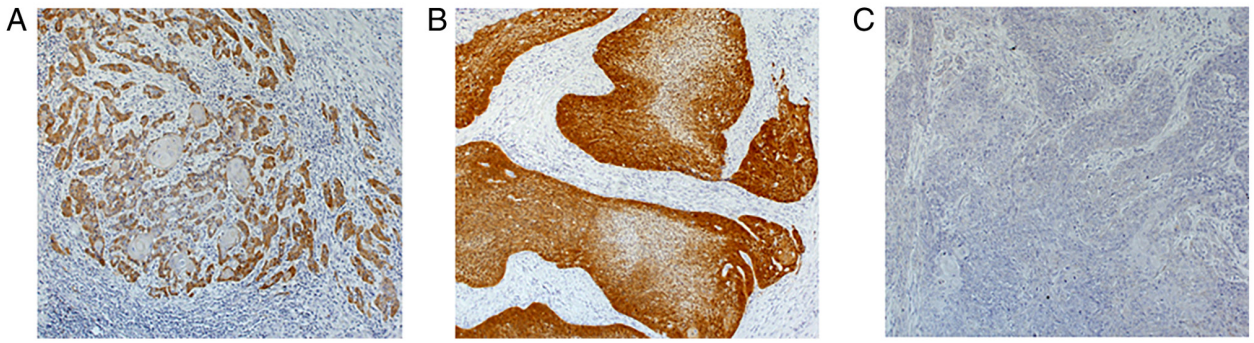

Figure 1. Immunohistochemical staining for p16 protein in oral squamous cell carcinoma tissues. (A) Positive expression in an HPV-16E6 mRNA negative case. (B) HPV-16E6 mRNA positive case showing strong and diffuse staining in the nucleus and cytoplasm of the tumor cells. (C) A representative image showing negative expression. Magnification, x40

to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism software (v5.04; GraphPad Software, Inc.).

Results

Characteristics of the patients. The 100 OSCC cases included 54 men and 46 women, ranging in age from 39 to 93 years (median, 70.3 years). The primary tumor was located in the tongue (n=36), mandibular gingiva (n=31), maxillary gingiva (n=13), floor of the mouth (n=9), buccal mucosa (n=9), or lower lip (n=2). All the patients were histopathologically diagnosed with squamous cell carcinoma, including BSCC (n=1).

Immunohistochemistry staining of p16 protein. Positive expression of p16 protein was observed in 10 out of 100 OSCC cases (10%). Most p16-positive cases showed strong and diffuse staining in the nucleus and the cytoplasm of the tumor cells (Fig. 1A and B). The association between p16 immunohistochemistry expression in the tumors from 100 patients with OSCC and their clinicopathological parameters was investigated; however, the differences were not significant (Table I). Furthermore, following the analysis between p16 expression and survival using the Kaplan-Meier method, no significant association between overall and disease-free survival times was found (Fig. S1).

Prevalence of HPV-16 in all patients with OSCC. To clarify HPV-16 infection, the expression level of HPV-16E6 mRNA in 100 OSCC cases was determined using RT-qPCR. HPV-16E6 mRNA expression was only detected in one case (1%), which was also positive for p16 expression. PCR products of HPV-16E6 and HMBS were visualized using agarose gel electrophoresis (Fig. 2). HPV-18E6 mRNA expression was investigated in p16 positive OSCC tissues using RT-qPCR; however, it was not detected (data not shown). Furthermore, the possibility of persistent or silent infection with HPV-16 was investigated using genomic DNA derived from p16 positive OSCC cases using qPCR. HPV-16E6 DNA was detected in 3 out of 10 cases (Cases 1, 3 and 10; Fig. S2A). One of these cases expressed HPV-16E6 mRNA (Case 1), but the other cases did not (Case 3 and 10). Subsequently, genomic DNA was digested by ExoV, which preserved nicked and supercoiled DNAs but degraded linear DNAs, followed by qPCR for the detection of HPV-16E6 DNA. HPV-16E6 DNA was ExoV-resistant, suggesting that the circular episome was observed only in the HPV-16E6 DNA and mRNA positive case (Case 1; Fig. S2B). Since neither HPV-16E6 DNA nor RNA was detected in 7 of the 10 cases of p16 overexpression (Case 2, 4, 5, 6, 7, 8 and 9), the false-positive rate of HPV-16 infection was 70%. Finally, only the HPV-16E6 DNA- and mRNA-positive case (Case 1) was histopathologically determined as BSCC, which is a rare variant of OSCC. The other cases (Case 3 and 10) were determined to be keratinized squamous cell carcinomas.

Discussion

HPV has been recognized as a possible pathogen of oral cancer (27-29). HPV has been associated with cancer of the uterus and cervix and was found in most cases (6-8). However, its role in oral carcinogenesis is still unknown (30-33). In oropharyngeal HNSCC, HPV-positive tumors may have different clinical and biological functions, with improved overall survival time and favorable prognosis (34). It has also been associated with therapeutic response in patients with HNSCC of the oropharynx (35). Another study showed that cases positive for HPV-16 had lower recurrence rates compared with that for their negative counterparts, indicating an association between HPV-16 infection and a good prognosis in OSCC (36). In contrast, HPV-16 infection reportedly enhanced the risk of distant metastasis and poor survival in patients with advanced OSCC (37). In the present study, the prevalence of HPV-16 in patients with OSCC was only 1%. Therefore, the association between HPV infection and prognosis could not be evaluated.

Several techniques have been used to detect HPV. Examples include PCR, an HPV genotyping test, morphology, *in situ* hybridization and p16 immunohistochemistry. HPV detection methods, such as morphology, *in situ* hybridization and p16 immunohistochemistry lack sensitivity and specificity, as well as the ability to detect high-risk HPV types. Therefore, PCR was considered the most sensitive method (38,39). The prevalence of HPV infection varied from 0% (19,40) to 100% (41), even in oral cavity cases. In the present study, p16 protein expression, which was used as a surrogate marker of HPV infection, was detected in 10% of cases, and the rate of HPV-16 infection with E6 expression was only 1% in 100 OSCC cases. The one case of HPV-16E6 mRNA positive expression also

Table I. Association between p16 status and the characteristics of patients with oral squamous cell carcinoma.

Characteristic	p16-positive (n=10)	p16-negative (n=90)	P-value
Median age, years	71	70	0.383
Sex			
Male	7	47	0.335
Female	3	43	
Primary site			0.185
Tongue	1	35	
Maxillary gingiva	2	11	
Mandibular gingiva	3	28	
Floor of mouth	2	7	
Buccal mucosa	1	8	
Lip	1	1	
Histological grading			0.465
G1	7	56	
G2	3	22	
G3	0	12	
T-status			0.077
½	4	63	
¾	6	27	
N-status			0.515
0	5	55	
1-3	5	35	
Clinical stage			0.504
I/II	3	42	
III/IV	7	48	
Recurrence/metastasis			>0.999
No	6	56	
Yes	4	34	

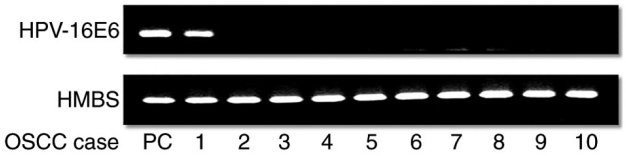

Figure 2. Expression of HPV-16E6 mRNA in p16 positive OSCC cases. The expression level of HPV-16E6 mRNA was analyzed using reverse transcription-quantitative PCR. Only 1 case was found to be positive. HMBS was used as a loading control. PC, positive control; HMBS, hydroxymethylbilane synthase; OSCC, oral squamous cell carcinoma; HPV, human papillomavirus.

had expression of the p16 protein. To investigate the association between HPV-16 infection and p16 expression, the presence of HPV-16E6 DNA in genomic DNA samples, which were also p16 positive was determined and HPV-16E6 DNA was found not only in the E6 mRNA positive case, but also in additional 2 cases without E6 expression. The expression of p16 was also 70% HPV-16 false-positive, indicating the low reliability of the surrogate marker of HPV infection in OSCC. A previous study showed 100% HPV false-positive results in mobile tongue cancer (19). It suggested that although p16 protein expression was a biomarker for cervical or tonsillar cancer arising from carcinogenic HPV infection, it was not applicable for tongue cancer (19). Furthermore, another study indicated that p16 expression was not a suitable surrogate marker of HPV infection in oral lesions and HPV-16 infection was associated with BSCC (42). In fact, only one HPV-16E6 mRNA-positive case was histopathologically determined to be BSCC in the present study. However, both HPV-16E6 DNA positive cases, without E6 expression, were keratinized squamous cell carcinomas.

Most cervical and oropharyngeal cancers show high expression of E6 and E7; however, OSCC has a lower positive rate of E6 and E7 mRNA expression compared with that for HPV DNA positive rate (43). We have hypothesized that there are two types of HPV DNA positive OSCCs. One is HPV-related OSCC, which is a silent infection with no expression of E6 and E7, but is caused by the integration of HPV DNA into the genome. The other is non-keratinized OSCC with E6 and E7 expression, in which HPV DNA is actively infected as a nuclear episome and/or genome integration.

In summary, the results from the present study indicated that there were few OSCC cases due to HPV-16 infection. The expression of p16 protein was not an appropriate surrogate marker for HPV-16 infection in OSCC. In addition, HPV-16 DNA may also be detected in p16 negative OSCC cases. As the number of HPV-16 DNA positive cases was extremely low in the present study, further investigation is required to examine the presence of episomal and integrated HPV DNA and the expression of E6 and E7 mRNA, regardless of p16 expression, using the large number of fresh frozen OSCC tissues.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NT and KN confirm the authenticity of all the raw data. KN, NK and DU advised and supervised the study. KN designed the experiments. NT and ST performed the experiments. NT, HG, NK and DU analyzed the data. NT and KN wrote the manuscript. All authors read and approved the final version of manuscript.

Ethics approval and consent to participate

The present study was approved by the Institute Research Ethics Committee of the Ehime University Hospital (approval

number, 1607005) and written informed consent was provided by all the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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