

RESEARCH ARTICLE

Lack of Association between High-risk Human Papillomaviruses and Oral Squamous Cell Carcinoma in Young Japanese Patients

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Abstract

Background: Human papillomaviruses (HPV) may play an important role as one of the possible etiologies of oral squamous cell carcinoma (OSCC). The present study aimed to investigate the association between HPV and OSCC in young Japanese patients by examining the presence of HPV DNA and surrogate markers in OSCC tissues. **Materials and Methods:** Forty young patients with OSCC whose surgical specimens were available were analyzed and compared with 40 patients randomly recruited from a pool of patients aged >40 years. HPV DNA was detected using the polymerase chain reaction-based AMPLICOR® HPV test, and surrogate markers of HPV infection were analyzed using immunohistochemical techniques to detect p16^{INK4a} and p53. **Results:** Only two (5%) young patients and one (2.5%) older patient were positive for HPV DNA. p16^{INK4a} overexpression was identified in six (15%) young patients. p53 staining levels were not high in tissues of most young patients (27 patients, 67.5%). HPV DNA status did not significantly correlate with p16^{INK4a} expression levels. Profiles of increased levels of p16^{INK4a} expression with diminished levels of p53 staining were not associated with the presence of HPV DNA. The combined p53 with p16^{INK4a} profiles were significantly correlated with alcohol consumption in younger patients ($p=0.006$). **Conclusions:** Results of the present study indicate that HPV is less likely to cause OSCC in young Japanese patients, and the p16^{INK4a} expression level is not an appropriate surrogate marker for HPV infection in OSCC.

Keywords: Oral squamous cell carcinoma - human papillomavirus - p16^{INK4a} - p53 - young

Asian Pac J Cancer Prev, 15 (10), 4135-4141

Introduction

Cancer of the oral cavity is a common cancer in males (10th most common) and females (14th most common) worldwide (Soerjomataram, 2012), leading to approximately 263,000 new patients and 127,000 deaths annually (Ferlay, 2010). Oral cancer is ranked one of the sixth most frequent malignancies in Asia. High incidence rates are reported from developing nations situated in South-Central and South-East regions like India, Pakistan, Bangladesh, Taiwan and Sri Lanka (Krishna et al., 2013). Approximately 11-15 males and five females in every 100,000 people in the Japanese population present with this disease (Matsuda, 2012). The majority of oral malignancies arise from epithelial tissue, and squamous cell carcinoma is the predominant tumor type (Barnes, 2005). Oral squamous cell carcinoma (OSCC) typically occurs in individuals aged between 60 and 70 years with prolonged exposure to known risk factors such as tobacco, alcohol, or betel quid (Barnes, 2005). However, the prevalence of OSCC in patients aged <45

years has increased during the past decade from <3% to 3.6-6.7% (Llewellyn, 2001; Annertz, 2002; Schantz & Yu, 2002; Patel, 2011). This trend may be attributed to multiple causes. While some reports suggested exposure to traditional risk factors (Llewellyn, 2004), the others indicate a trend in nonsmokers the young group. Among the possible etiologies of OSCC, human papillomavirus (HPV) has been speculated to have an important role in the pathogenesis of OSCC in the young patient group.

Human papillomavirus (HPV), a DNA virus, is a member of the *Papillomaviridae* family, with more than 50 subtypes. E6 and E7, its oncoproteins, are jointly involved in the immortalization of infected cells (Finzer, 2002). E7 manipulates and degrades the retinoblastoma tumor suppressor protein (pRb), resulting in the release of the transcription factor E2F that induces elevated production of the cyclin-dependent kinase inhibitor INK4a (p16^{INK4a}) (McLaughlin-Drubin and Münger, 2009). E6 promotes p53 degradation through its interaction with the E3 ubiquitin ligase E6AP (Narisawa-Saito and Kiyono, 2007). Although HPV and mutated p53 may coexist in a

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subset of squamous cell carcinomas of the head and neck, HPV and disruptive p53 mutations appear to represent nonoverlapping factors (Westra, 2008).

HPV causes cervical cancer (Harald, 2002) and is a factor that predicts better prognosis of patients with oropharyngeal cancer (Gillison, 2000; D'Souza, 2007; Fakhry, 2008; Ang, 2010; Chaturvedi, 2011; Huang et al., 2012). HPV positive oropharyngeal cancers are increasing in Turkish patients as in the Western world (Tural et al., 2013). However, a correlation between HPV and OSCC has not been established. For example, there are discrepancies in the prevalence of HPV in patients with OSCC residing in different regions worldwide (El-Mofty and Lu, 2003; Lopes, 2011; Duray, 2012; Kaminagakura, 2012; Lingen, 2013; Reuschenbach, 2013; van Monsjou, 2013). Moreover, patients residing in Japan exhibit a wide range for HPV prevalence (0-78%) (Shima, 2000; Kojima, 2002; Sugiyama, 2007; Kabeya, 2012). However, a few of these studies analyzed mixed sub-sites of head and neck squamous cell carcinomas (Shima, 2000; Kojima, 2002; Sugiyama, 2007; Kabeya, 2012). Furthermore, few studies focus specifically on HPV in young Japanese patients with OSCC (Kabeya, 2012).

Therefore, the present study aimed to investigate the association between HPV and OSCC in young Japanese patients by examining the presence of HPV DNA and surrogate markers in formaldehyde-fixed paraffin-embedded (FFPE) OSCC specimens.

Materials and Methods

OSCC prevalence in young patients

We conducted a preliminary survey of clinical details of patients enrolled for treatment of OSCC between January 2001 and December 2009 at the Department of Oral and Maxillofacial Surgery of the Tokyo Medical and Dental University, Tokyo, Japan. Data for 1 113 patients were retrospectively collected from medical records. Patients who were not aged >40 years at time of diagnosis were identified as young, and the proportion of young patients in the total population was calculated. We included 40 young patients with OSCC with available surgical specimens and compared them with 40 randomly recruited older patients who were over 40 years old. The older patients were recruited from patients with advanced cancer to investigate the mutational status of p53.

After obtaining approval from the ethics committee of the Faculty of Dentistry, Tokyo Medical and Dental University, FFPE surgical specimens were retrieved, which included anatomical sections of the tongue, upper and lower gingiva, floor of the mouth, retromolar trigone, palate, and buccal mucosa. The study was done according to the Helsinki Declaration. Squamous cell carcinoma was histopathologically confirmed by pathologists, and was classified according to the World Health Organization's (WHO) Classification (The World Health Organization's Classification of Tumour, Pathology and Genetics of Head and Neck Tumours, 2005) as follows: well differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III). With reference to several sections of hematoxylin and eosin (HE)-stained slides,

tumor areas in FFPE specimens were marked and hand-dissected using macrodissection methods to ensure tumor tissue inclusion. Five 4- μ m curls and three slices of 10- μ m sections were prepared for HPV DNA detection and immunohistochemical (IHC) staining, respectively.

DNA extraction and HPV DNA detection

Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen NV, Hilden, Germany) according to the manufacturer's protocol. Samples including HPV-positive cervical carcinomas, a tissue microarray of human cervical carcinomas containing 52 individual tissue samples (e.g., squamous cell carcinoma, carcinoma *in situ*, and normal mucosa) on a single slide (cat #401 2203, lot #017P240105.40; Provitro, Berlin, Germany), were lysed into a single tube, and the genomic DNA extracted from the lysate was termed the "cervical carcinomas" (Figure 1). DNA isolated from the cervical carcinomas and FFPE HeLa cells were used as positive external controls. Sterile water was used as the negative control. HPV DNA was detected using the polymerase chain reaction (PCR)-based AMPLICOR® HPV test (Roche Molecular Systems, Branchburg, NJ, USA), which is designed to detect 13 high-risk HPV genotypes. HPV detection procedures were performed according to the manufacturer's protocol. For the AMPLICOR® HPV test, pools of primers targeting a nucleotide sequence within the L1 region of the HPV genome were utilized to amplify HPV DNA from genotypes 16, 18, 31, 33, 39, 45, 51, 52, 56, 59, and 68. Additional primer pairs designed to detect the human β -globin gene were used as indicators of the integrity of DNA preparations. Taq DNA polymerase was used to elongate the annealed primers to produce 159-167 base pair (bp) HPV or 268-bp β -globin amplicons. The HPV and β -globin amplicons were subsequently denatured. Biotin-labeled HPV and β -globin amplicons were then hybridized with oligonucleotide probes bound to the surface of microwell plates. After hybridization, the plates were repeatedly washed to remove unhybridized material. Subsequently, an avidin-horseradish peroxidase conjugate was added to each well to bind biotin-labeled HPV and β -globin DNAs hybridized with the oligonucleotide probes. Hybrids were detected using the AMPLICOR substrate containing tetramethylbenzidine and hydrogen peroxide for color development. The optical density of the reaction mixtures was determined at 450 nm using a ChroMate® Microplate Reader (Awareness Technology, Inc., FL, USA). Samples with absorbance readings exceeding 0.2 were considered positive for the presence of HPV DNA.

IHC detection of p16^{INK4a}

p16^{INK4a} expression was determined using the CINtec® Histology kit (mtm laboratories AG, Heidelberg, Germany) according to the manufacturer's protocol with minor modifications. Three pieces of 4- μ m FFPE sections from each surgical specimen were incubated at 40°C for 12h, deparaffinized in a graded series of ethanol solutions, rehydrated, and washed in phosphate-buffered saline (PBS). All subsequent steps were performed using the same manufacturer's protocol.

Antigen retrieval was performed by immersing slides in Tris–ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris base, 1 mM EDTA, 1.5 mM NaN₃, pH 9.0), incubating them in a temperature-controlled bath at 98 °C for 10 min, and cooling them at room temperature for 20 min. After blocking endogenous peroxidase activity for 10 min, two slides from each specimen were covered with an anti-human p16^{INK4a} monoclonal antibody (clone E6H4). Another slide that served as a negative control was incubated with a mouse monoclonal anti-rat oxytocin-related neurophysin antibody. All sections were incubated in a humidified chamber at 25°C for 30 min. Antigen–antibody complexes were visualized by the conversion of 3,3 diaminobenzidine (DAB) following incubation with a polymer reagent conjugated to horseradish peroxidase and affinity-purified goat anti-mouse Fab’ antibody fragments. All slides were counterstained with hematoxylin and dehydrated.

IHC detection of p53

Deparaffinization and rehydration were performed using the same protocol as that used for p16^{INK4a} detection. All washing steps were performed using PBS. Epitope retrieval was performed by immersing slides in 0.01 M sodium citrate buffer (pH 0.6), incubating them in a controlled water bath for 20 min at 98°C, and cooling them for 20 min at room temperature. The sections were the immersed in 0.3% hydrogen peroxide in methanol for 20 min followed by blocking of nonspecific binding using 10% normal serum (Histofine SAB PO kit; Nichirei, Tokyo, Japan) for 10 min. Subsequently, the slides were covered with a mouse monoclonal anti-human p53 antibody (clone DO-7, DakoCytomation, Denmark) and incubated in a humidified chamber at 25°C for 60 min. After washing with PBS, tissue sections were covered with biotinylated primary antibodies (Histofine SAB PO kit code 424022) at 25°C for 30 min. Rinsing with PBS was repeated, and sections were reincubated with streptavidin–biotin–peroxidase (Histofine SAB PO kit) and DAB. The sections were counterstained using Mayer’s hematoxylin

Evaluation of IHC staining

IHC staining was evaluated in reference to negative control slides stained with HE. All cell counting was performed using micrographs acquired at 400× magnification. More than 1,000 cells in five areas of (borders and middle) the carcinomas were assessed on each slide, and the proportion of positive cells was calculated. Scoring of p16^{INK4a} and p53 was based on the percentage of positive cells. Frequencies of p16^{INK4a} expression were assigned scores as follows: <10%, 10%–40%, and >40% for scores of 1, 2, and 3, respectively, and a score of 3 was considered “p16^{INK4a} positive” (Figure 2). No p53-positive staining was assigned a score of 0. A frequency <25% of p53-positive cells was assigned a score of 1, and p53-positive staining frequencies of 25%–50% and >50% were assigned scores of 2 and 3, respectively. A “p53-positive” result was defined as a score of 2 or 3 (Figure 2).

Statistical analysis

The correlation between HPV DNA status and IHC profiles with clinicopathological variables was determined using the Chi-square or Fisher’s exact test. Kaplan–Meier and log-rank tests were used to define the association between HPV status, IHC profiles, and disease-specific survival. Probabilities of <0.05 were considered significant. Statistical analyzes were performed using IBM SPSS version 20.0 software (IBM SPSS, Inc., Chicago, IL, USA).

Results

Prevalence of young patients and characteristics of the study population

We enrolled 1 113 OSCC patients enrolled between January 2001 and December 2009. Among these patients, we identified 86 patients aged <40 years at the time of diagnosis. The overall prevalence of young patients was

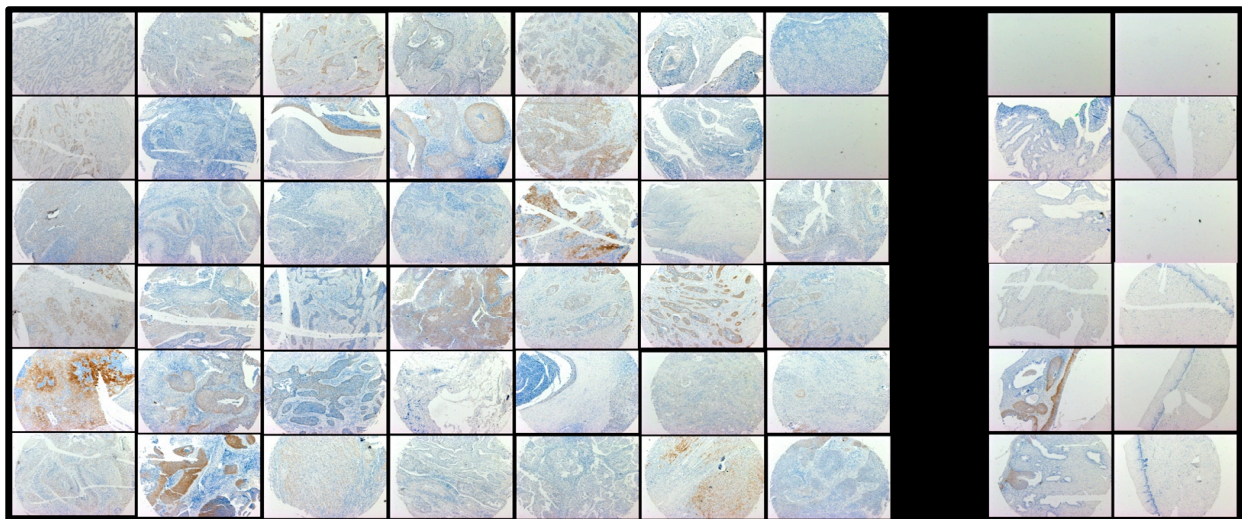


Figure 1. p16^{INK4a} Immunohistochemistry (IHC) of a Human Cervical Carcinoma Tissue Microarray Containing 52 Individual Tissue Samples. p16^{INK4a} IHC was performed using the same protocol as that used for the OSCC samples. Samples on a slide from the same lot (lot #017P240105.40) were lysed into a single tube, and the genomic DNA extracted from this lysate was termed the “cervical carcinomas” for detecting human papilloma virus (HPV) DNA

7.7%, and the annual prevalence ranged from 3.4% to 9.8% during the last decade. The cohort of 40 young patients with complete FFPE specimens was compared with 40 older patients; mean age \pm standard deviation and range were 31.5 \pm 4.5 (21–38) years and 61.0 \pm 9.8 (41–80) years, respectively. The gender ratio was 1.86:1 (26 men, 14 women) in the young group and 2.33:1 (28 men, 12 women) in the older group. Oral risk factors were for

young and older groups were as follows: 18 (45%) and 22 (55%) patients were tobacco smokers and 24 (60%) and 21 (52.5%) patients consumed alcohol, respectively.

The tongue was identified as the most common tumor site (36 patients, 90%) in young patients. Other sites included the gingiva (three patients) and buccal mucosa (one patient). In contrast, 17 (42.5%) patients in the older group had cancer of the tongue and 23 (57.5%) had cancer at other sites. Most tumors in young patients were well differentiated (21 patients, 52.5%).

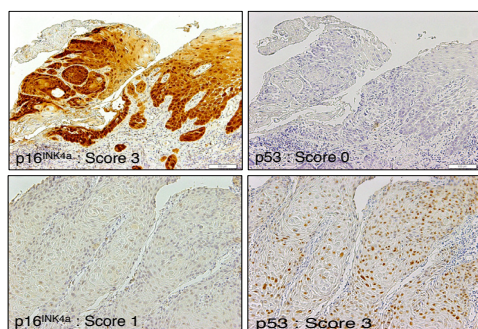


Figure 2. p16^{INK4a} and p53 Immunohistochemistry. (A) Case no.19. p16^{INK4a} overexpression with diminished levels of p53 staining at 100 \times magnification (Bar=100 μ m). Left panel: p16^{INK4a} staining=score 3. Right panel: p53 staining=score 0. (B) Case no.20. Diminished p16^{INK4a} expression levels with high p53 staining levels at 100 \times magnification (Bar=100 μ m). Left panel: p16^{INK4a} staining=score 1. Right panel: p53 staining=score 3

Detection of HPV DNA

Only two (5%) young patients and one (2.5%) older patient were positive for HPV DNA. The presence of HPV DNA was not significantly different between the two groups. Further, the presence of HPV DNA did not significantly correlate with any clinical profile.

IHC analysis of p16^{INK4a} and p53

p16^{INK4a} overexpression was identified in six young patients (15%) and four older patients (10%). p16^{INK4a} expression levels did not differ significantly between the two groups. p16^{INK4a} expression levels were significantly correlated with the tumor differentiation ($p=0.030$) in younger patients (Table 1), whereas they were significantly correlated with tumor differentiation ($p=0.013$) and alcohol consumption ($p=0.004$) in the entire cohort

Table 1. Correlation between Tumor Characteristics and Results of Human Papilloma Virus (HPV) DNA Detection and Immunohistochemistry (p16^{INK4a} and p53) Status

Tumor characteristics	HPV DNA			No. of Cases in all patients (in young patients)									
	Negative	Positive	Significant p value	p16 ^{INK4a} Staining		p53 Staining		p16↑p53↓	p16 ^{INK4a} + p53		Significant p value		
				Score 1,2	3	Score 0,1	2,3		p16↓p53↑	p16↑p53↑			
Age (years)													
≤40	38	2		34	6		27	13	0.025	5	23	12	
>40	39	1		36	4		17	23		2	17	21	
Gender													
Male	52(24)	2 (2)		47 (23)	7 (3)		29 (16)	25 (10)		4 (2)	28 (15)	22(9)	
Female	25(14)	1 (0)		23 (11)	3 (3)		15 (11)	11 (3)		3 (3)	12 (8)	11(3)	
Tumour sites													
Tongue	50(34)	3 (2)		46 (30)	7 (6)		29 (24)	24 (12)		6 (5)	24 (20)	23(11)	
Others	27 (4)	0 (0)		24 (4)	3 (0)		15 (3)	12 (1)		1 (0)	16 (3)	10(1)	
T classification													
T1	17(15)	0 (0)		14 (12)	3 (3)		12 (11)	5 (4)		2 (2)	11 (10)	4(3)	
T2 - T4	60(23)	3 (2)		56(22)	7 (3)		32 (16)	31 (9)		5 (3)	29 (13)	29(9)	
Tumor differentiation													
Well	31(21)	2 (2)		25 (17)	8 (6)	0.013	19 (15)	14 (8)		6 (5)	15 (11)	12(7)	0.044
Moderately, Poorly	46(17)	1 (0)		45 (17)	2 (0)	0.030	25 (12)	22 (5)		1 (0)	25 (12)	21(5)	
Neck node metastasis													
Yes	31 (8)	1 (1)		29 (8)	3 (1)		16 (8)	16 (1)		1 (1)	17 (7)	14(1)	
No	46(30)	2 (1)		41 (26)	7 (5)		28 (19)	20 (12)		6 (4)	23 (16)	19(11)	
Distant metastasis													
Yes	5 (0)	0 (0)		5 (0)	0 (0)		1 (0)	4 (0)		0 (0)	1 (0)	4(0)	
No	72(38)	3 (2)		65 (34)	10 (6)		43 (27)	32 (13)		7 (5)	39 (23)	29(12)	
Tobacco													
Yes	39(17)	1 (1)		33 (15)	7 (3)		24 (11)	16 (7)		4 (2)	19 (10)	17(6)	
No	38(21)	2 (1)		37 (19)	3 (3)		20 (16)	20 (6)		3 (3)	21 (13)	16(6)	
Alcohol													
Yes	44(23)	1 (1)		35 (18)	10 (6)	0.004	30 (20)	15 (4)	0.017	7 (5)	26 (16)	12(3)	0.002
No	33(15)	2 (1)		35 (16)	0 (0)		14 (7)	21 (9)	0.015	0 (0)	14 (7)	21(9)	0.006
Outcome													
DOD	12 (3)	0 (0)		11 (2)	1 (1)		5 (2)	7 (1)		1 (1)	4 (1)	7(1)	
DID	6 (0)	0 (0)		6 (0)	0 (0)		3 (0)	3 (0)		0 (0)	3 (0)	3(0)	
NED	59(35)	3 (2)		53 (32)	9 (5)		36 (25)	26 (12)		6 (4)	33 (22)	23(11)	

*DOD, died of disease; DID, died of intercurrent disease; NED, no evidence of disease; p16^{INK4a} p53⁺ p16^{INK4a} overexpression with diminished levels of p53 staining; p16^{INK4a} p53⁺, Low staining levels of p16^{INK4a} and p53; p16^{INK4a} p53⁺, High staining levels of p16^{INK4a} and p53; p16^{INK4a} p53⁺, diminished levels of p16^{INK4a} expression with high staining levels of p53

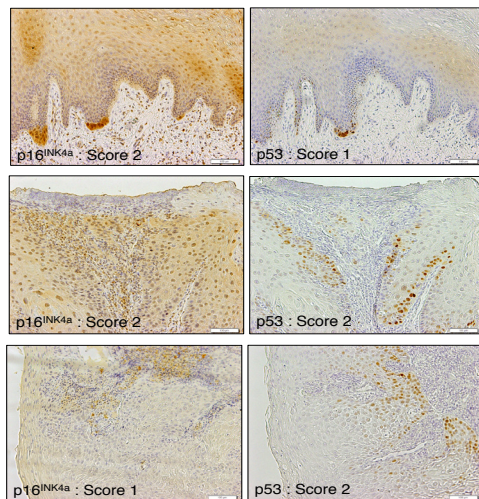


Figure 3. p16^{INK4a} and p53 Immunohistochemistry in the Human Papilloma Virus (HPV) DNA Positive Cases. (A) Case no.1 (Bar=100 μ m). Left panel: p16^{INK4a} staining=score 2. Right panel: p53 staining=score 1. (A) Case no.2 (Bar=100 μ m). Left panel: p16^{INK4a} staining=score 2. Right panel: p53 staining=score 2. (A) Case no. 73 (Bar=100 μ m). Left panel: p16^{INK4a} staining=score 1. Right panel: p53 staining=score 2

(Table 1) and were not correlated with various clinical characteristics in older patients.

p53 staining levels were not high for most young patients (27 cases, 67.5%). In contrast, high p53 staining levels were detected in 23 of the older patients (57.5%). A significant inverse correlation was observed between p53 expression levels and alcohol consumption in younger patients and in the entire cohort ($p=0.015$ and 0.017 , respectively) (Tables 1), whereas no significant correlation was observed in older patients. p16^{INK4a} overexpression with diminished levels of p53 staining was observed in 5 (12.5%) young patients and in two (5%) older patients. In contrast, diminished levels of p16^{INK4a} expression with high staining levels of p53 were detected in 12 (30%) young and 21 (52.5%) older patients. The combined p53 with p16^{INK4a} profiles were significantly correlated with alcohol consumption in younger patients ($p=0.006$; Table 1), whereas they were significantly correlated with the tumor differentiation ($p=0.044$) and alcohol consumption ($p=0.002$) in the total cohort (Table 1) and were not correlated with various clinical characteristics in older patients.

HPV DNA status did not significantly correlate with p16^{INK4a} expression, p53 staining levels or combined p53 with p16^{INK4a} profiles in any cohort (Table 1).

Analyzes of HPV DNA status, IHC results, and survival time

Next, we analyzed the effect of HPV DNA status or IHC staining profiles on prognosis, and did not find a significant correlation with 5-year disease-specific survival rates.

Discussion

Although global anti-smoking campaigns have reduced the prevalence of typical OSCC patients, the

number of young patients with cancer of the oral cavity cancer has gradually increased during the past two decades (Llewellyn, 2001; 2004; Annertz, 2002; Schantz and Yu, 2002; Patel, 2011). In Japan, percentages of men and women diagnosed from 2004 to 2006 with OSCC at ≤ 40 years of age were 3.46% and 5%, respectively (Matsuda, 2010; 2011; 2012). Descriptive data from our department show a prevalence of 7.7% between 2001 and 2009 and may be higher than other published data because of an urban location in Tokyo. Unlike typical patients with OSCC who were exposed to risk factors such as alcohol consumption and smoking, these young patients usually have no obvious etiological factors (Llewellyn, 2001).

To identify the possible cause of OSCC in this patient population, we considered many probable risk factors, particularly HPV. HPV is associated with SCC in other organs such as the cervix, oropharynx, and tonsils (Harald, 2000; 2002; Annertz, 2002; Begum, 2003; Ang, 2010; Mehanna, 2013). For example, HPV is detected in cervical and oropharyngeal SCC at frequencies of 90% (Harald, 2002; Clifford, 2003; de Sanjose, 2010) and 47.7% (Mehanna, 2013), respectively, with evidence of functional E6 and E7 oncoproteins. The study in India demonstrated a positive correlation of HPV infection with oral tongue cancer (Elango et al., 2011), while HPV may not play an important role in Thai and Malaysian OSCC (Khovidhunkit et al., 2008; Goot-Heah et al., 2012). Therefore, the present study aimed to investigate whether the presence of HPV correlates with OSCC in Japanese patients aged <40 years.

We chose relatively simple and reproducible methods that can be performed in smaller laboratories. Therefore, we used the AMPLICOR[®] human papillomavirus detection kit, and analyzed the expressions of p16^{INK4a} and p53 using immunohistochemistry as surrogates for functional HPV E6 and E7 oncoproteins. The AMPLICOR[®] HPV detection kit is widely used to identify the L1 genomic region of 13 common types of high-risk HPV serotypes. Furthermore, its reliability, sensitivity, specificity, and positive and negative predictive values compare favorably with other PCR-based commercial kits such as the Hybrid Capture II HPV detection assay (Monsonogo, 2005; van Ham, 2005; Wentzensen, 2009; Ovestad, 2011).

In contrast to the relatively high prevalence of HPV in the young populations of North America and Scandinavia, the prevalence is low in young population of Japan (Kabeya, 2012). In the present study, we detected HPV DNA in two young patients and in one older patient. Moreover, our experiments were well controlled, as indicated by the detection of β -globin DNA as well as HPV DNA in the cervical carcinoma samples used as positive control.

The expression profiles of p16^{INK4a} and p53 did not correlate with the presence of HPV DNA in OSCC analyzed in this study (Figure 3). Whether these findings were caused by either contamination of tumor with normal tissue or because the HPV genome was present in its episomal form or partial disruptive form (Deng, 2013; Hamid, 2009; Munger, 2004), it is unlikely that HPV caused these tumors. Although p16^{INK4a} was not overexpressed, the trend of increasing p16^{INK4a} expression

correlates with the oncogenicity of HPV E6. The relatively low levels of HPV DNA indicate low copy numbers of HPV in the specimens, and explain the finding of relatively low levels of p16^{INK4a} in two young patients. In contrast to the association of HPV DNA and p16^{INK4a} in other diseases, the correlation between the detection of HPV did not significantly correlate with p16^{INK4a} expression or the expression patterns of p16^{INK4a} and p53. Although there are some reports of discrepancies between the IHC detection of p16^{INK4a} and p53 with HPV status (Balz, 2003; Yamakawa-Kakuta, 2009), the findings of this study can be explained by the very low prevalence of HPV DNA in the tissue specimens.

Our present findings are consistent with those of others regarding the prevalence of HPV in oral cavity cancers in young patients (Boy, 2006; Kabeya, 2012; Reuschenbach, 2013). Because of the high specificity and predictive value of AMPLICOR[®] kit, it is unlikely that many cases of OSCC in the young are influenced by HPV infection. However, further investigation is required to confirm the association between HPV status and OSCC in young patients. Moreover, future studies should re-evaluate the expression level of p16^{INK4a} and p53 in combination with that of p16^{INK4a}-p53 as a marker of HPV in OSCC. Our findings presented here indicate that the combined p53 with p16^{INK4a} profiles were significantly correlated with alcohol consumption in the younger patients (p=0.006; Table 1). However, only a few studies have documented the p53 expression pattern in primarily related to alcohol (Matthews, 1993; Thongsuksai, 2003), therefore further studies are required.

In conclusion, our findings indicate that HPV is less likely a cause of OSCC in young Japanese patients and that the p16^{INK4a} expression level is not an appropriate surrogate marker for HPV infection in OSCC.

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