RESEARCH ARTICLE

p16 - a Possible Surrogate Marker for High-Risk Human Papillomaviruses in Oral Cancer?

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Abstract

Background: High-risk human papillomaviruses (HR-HPV), particularly types 16 and 18, have been found to play an important role in head and neck cancer, including oropharyngeal squamous cell carcinoma (OPSCC) and oral squamous cell carcinoma (OSCC). p16, a cell cycle inhibitor, has been postulated as a surrogate marker for HR-HPV, since p16 is aberrantly overexpressed in such lesions, especially in HR-HPV-positive OPSCC. However, p16 as a surrogate marker for HR-HPV infection in cancers of the oral cavity remains controversial. **Objective:** The objectives of the study were to investigate the expression of p16 and the presence of HR-HPV in OSCC and oral verrucous carcinoma (VC) and to determine if p16 could be used as a surrogate marker for HR-HPV. **Materials and Methods:** Forty one formalin-fixed, paraffin-embedded tissues of OSCC (n=37) or VC (n=4) with clinical and histopathologic data of each case were collected. Expression of p16 was determined by immunohistochemistry, focusing on both staining intensity and numbers of positive cells. The presence of HPV types 16 and 18 was detected by polymerase chain reaction (PCR). Descriptive statistics were employed to describe the demographic, clinical, and histopathologic parameters. Associations between p16 overexpression, HR-HPV and all variables were determined by Fisher's exact test, odds ratios (ORs) and corresponding 95% confidence intervals (CIs). In addition, the use of p16 as a surrogate marker for HR-HPV was analyzed by sensitivity and specificity tests. **Results:** p16 was overexpressed in 8/37 cases (21.6%) of OSCC and 2/4 cases (50%) of VC. HPV-16 was detected in 4/34 OSCC cases (11.8%) and HPV-18 was detected in 1/34 OSCC cases (2.9%). Co-infection of HPV-16/18 was detected in 1/4 VC cases (25%). Both p16 overexpression and HR-HPV were significantly associated with young patients with both OSCC and VC (p<0.05, OR 20, 95% CI 1.9-211.8; p<0.05, OR 23.3, 95% CI 2.4-229.7, respectively). p16 was able to predict the presence of HPV-16/18 in OSCC with 40% sensitivity and 79.3% specificity and in VC with 100% sensitivity and 66.7% specificity, respectively. **Conclusions:** p16 overexpression was found in 24.4% of both OSCC and VC. HR-HPV, regardless of type, was detected in 15.8% in cases of OSCC and VC combined. The results of sensitivity and specificity tests suggest that p16 can be used as a surrogate marker for HR-HPV in OSCC and VC.

Keywords: High-risk human papillomaviruses - oral SCC - p16 - surrogate marker - verrucous carcinoma

Introduction

Globally, oral cancer is a major public health problem accounting for approximately 3% of all cancers (Bixofis et al., 2014; Hubbers and Akgul, 2015; Komolmalai et al., 2015). More than 90% of malignant tumors in the oral cavity consist of oral squamous cell carcinoma (OSCC) (Johnson et al., 2011). Recently, we have found a significant increase in the number patients with OSCC from 587 cases during 1991-2000 to 874 cases during 2001-2010 at Maharaj Nakorn Chiang Mai University Hospital, which is regarded as the largest cancer treatment center in northern Thailand (Iamaroon et al., 2004a;

Komolmalai et al., 2015). These findings indicate that OSCC remains a constant, unsolved problem in Thailand. It is well accepted that both genetic and environmental factors play a pivotal role in OSCC carcinogenesis (Pannone et al., 2011). Well-known major environmental risk factors for OSCC include tobacco use in various forms, heavy alcohol consumption, areca nut/betel quid chewing, and small intake of fresh vegetables and fruits (Komolmalai et al., 2015; Sritippho et al., 2015). However, approximately 15-20% of patients with OSCC in the western population are not linked with those conventional risks, and lately, high-risk human papillomaviruses (HR-HPV) have been associated with such patients (Vargas-

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Ferreira et al., 2012). HR-HPV comprises HPV types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (Rautava and Syrjänen, 2012). Of all types, HPV-16 is the most frequent genotype found in OSCC, accounting for more than 90% of all cases (Kreimer et al., 2005; Shaw and Robinson, 2011; Syrjänen et al., 2011; Isayeva et al., 2012).

Interestingly, patients with HR-HPV-related OSCC are genetically and clinically different from those with conventional risks, since HR-HPV-related OSCC occurs predominantly in younger white male patients who may have multiple sexual partners in their lifetimes and oral sexual habits (Bixofis et al., 2014; Antonsson et al., 2015). More importantly, the clinical outcomes of patients with HR-HPV-related OSCC have been shown to be better than those without HR-HPV infection, since the patients with HR-HPV-related OSCC are more responsive to radiotherapy and certain forms of chemotherapy. Collectively, risk assessment in each OSCC patient is of importance, since patients with different risks may need individualized treatment modalities and have different prognoses.

The HPV genome comprises approximately 8,000 base pairs and is classified as early gene encoding (E), late gene encoding (L), and viral capsid proteins. Particularly, E6 and E7 viral proteins are believed to play an essential role in malignant transformation of infected host cells. E6 can abrogate functions of p53, a well-known tumor suppressor protein, and also prohibit telomere erosion. These results lead to unlimited growth, genomic instability and progression toward malignancy of the HPV-infected host cells (Rautava and Syrjänen, 2012; Abogunrin et al., 2014). E7 protein, in particular, can compete with the transcription factor E2F to bind the retinoblastoma protein (Rb), another tumor suppressor protein, leading to favoring cell cycle progression. Loss of Rb-E2F complexes results in overexpression of p16 protein, a cell cycle inhibitor (Muirhead, 2006; Rautava and Syrjänen, 2012; Patil et al., 2014).

Under normal circumstances, p16 protein can inhibit cell cycle progression by restraining Rb phosphorylation during the G1 and S phases of the cell cycle. Loss of p16 through the mechanisms of hypermethylation of the promoter region, homozygous deletion or loss of heterozygosity is commonly found in patients with OSCC with conventional risk factors. (Nemes et al., 2006; Stephen et al., 2013). Moreover, loss of p16 function can be found in potentially malignant disorders, such as oral leukoplakia and erythoplakia, suggesting a role for p16 in the early stage of carcinogenesis (Muirhead, 2006; Grobe et al., 2013; Stephen et al., 2013). On the other hand, in patients with cervical cancer and head and neck squamous cell carcinoma (HNSCC) with HR-HPV infection, p16 is aberrantly overexpressed as aforementioned. Taking advantage of this phenomenon, p16 is widely used as a surrogate marker for HPV-related cervical cancer and HNSCC (Singhi and Westra, 2010; Stephen et al., 2013; Chung et al., 2014; Patil et al., 2014). For example, Laco et al. found the overexpression of p16 in 17/48 (35%) cases of OSCC and in 36/44 (82%) cases of oropharyngeal squamous cell carcinoma (OPSCC) and the presence of HPV DNA in 7/48 (15%) cases of OSCC and 35/44 (80%) cases of OPSCCs (Laco et al., 2012). The estimated analytical sensitivity of p16 as a surrogate marker for HPV infection ranges from 85% to 100% and the specificity ranges from 74% to 81% (Snietura et al., 2010; Laco et al., 2012). Patients with p16-positive HNSCC have also been shown to have advanced clinical stages of the condition, but favorable prognoses (Snietura et al., 2010; Perez-Sayans et al., 2011). However, a correlation between HPV and p16 in HNSCC appears to be significant in OPSCC, since only a small number of non-OPSCC cases, including OSCC cases, have been investigated. Thus, using p16 as a surrogate marker is suitable for OPSCC and remains debatable in OSCC (Stephen et al., 2013; Ndiaye et al., 2014).

The objectives of this study were (1) to determine the expression of p16 in OSCC, and VC, (2) to detect HPV-16/18 in OSCC and VC, a variant of OSCC, and (3) to analyze the correlation between p16 and HPV-16/18 in OSCC and VC.

Materials and Methods

Sample selection

Thirty-seven formalin-fixed, paraffin-embedded (FFPE) OSCC and four FFPE oral VC tissues were collected from the archive of the Oral Pathology Laboratory, Faculty of Dentistry, Chiang Mai University. This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University (Clearance No. 54/2014).

Immunohistochemical technique

The sections were deparaffinized in xylene, rehydrated in graded alcohols and washed in distilled water. Endogenous peroxidase was blocked by 3% hydrogen peroxide. Antigen unmasking was performed by incubating the sections in citrate buffer at 90-100°C and allowing the sections to cool down to room temperature. The sections were subsequently incubated in 2.5% normal horse serum (Vectastain™ ABC Staining Systems, Burlingame, CA, USA) and a mouse monoclonal antibody against p16 (dilution 1: 100, Chemicon™, (Temecula, CA, USA) at 4°C overnight. For negative control sections, the blocking serum was incubated as a replacement for the primary antibody. On the following day, the sections were washed in Tris-base saline (TBS), incubated with a secondary anti-mouse antibody (dilution 1: 100, Vectastain™ ABC Staining Systems) in TBS, then washed in TBS. The sections were incubated with Vectastain™ ABC reagent. To develop the color reaction, the sections were incubated with a solution of 3, 3'-diaminobenzidine. The sections were counterstained in hematoxylin solution, washed in running tap water for five minutes, air dried, and coverslipped.

Immunohistochemical scoring

The immunostained sections were scored under a light microscope at 400x magnification by two observes trained by an oral pathologist. The observer was blinded to the clinico-pathologic data of each case. Scoring was

performed using the Image J program® (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). Each cell was counted with the use of a 10 x 10 grid to avoid counting duplication.

The staining intensity scores were categorized as 0 (no staining), 1+ (weak staining or light brown staining, visible only with high magnification), 2+ (intermediate staining, the staining intensity falling between scores 1+ and 3+), and 3+ (strong staining or dark brown staining, visible with low magnification), modified from the criteria used in a study of non-small-cell lung cancers (Pirker et al., 2012). The immunostaining of each case was scored on a continuous scale of 0–300. By integration of the data relating to the intensity and frequency of staining, the score was calculated with the formula: 1 × (percentage of cells staining weakly [1+]) + 2 × (percentage of cells staining moderately [2+]) + 3 × (percentage of cells staining strongly [3+]). A score ≥ 200 was considered as positive staining for p16 expression.

Statistical analyses

All data were processed and analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL., USA). Descriptive statistics were used to analyze demographic, clinical, and histopathologic data. The intra-observer and inter-observer calibrations were determined, using the kappa value. Fisher's exact test was employed to determine the association between p16 expression and demographic, clinical, and histopathologic data. $p < 0.05$ was considered significant. The associations between p16, HPV-16/18, sex, age of the patients and risks for both OSCC and VC were assessed by calculating odds ratios (ORs) and their corresponding 95% confidence intervals (CIs). To determine if p16 could be used as a surrogate marker for HR-HPV, the sensitivity and specificity tests were performed.

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Polymerase chain reaction

DNA extraction: Forty-one FFPE tissue samples of OSCC and one FFPE tissue sample of cervical carcinoma in situ, as a positive control, were collected, and each sample was cut into eight five-micron sections. The QIAamp® DNA FFPE Tissue Kit (QIAGEN GmbH, Hilden, Germany) was used to extract DNA from FFPE tissue sections according to the manufacturer's instructions. DNA yields were quantitatively measured by absorbance at 260 nm (A260), and DNA integrity was determined by agarose gel electrophoresis. The

concentration of DNA in each sample was prepared as 20 ng/µl.

Genotyping HPV-16 and -18 strains using real-time PCR

All samples were tested for HPV-16 and -18 strains, using real-time PCR. The reaction mixture for real-time PCR containing 5 µl of 2xSensiFAST SYBR® No-ROX mix, 0.3 µM forward primer, 0.3 µM reverse primer and 20 ng/µl of genomic DNA was prepared in a total volume of 10 µl. The specific oligonucleotide primer pairs for the E6 region of each of HPV-16 and -18, of β-actin and of GAPDH, employed as the housekeeping genes, are listed in Table 1. All assays were performed using the Eco™ Real-Time PCR system (illumina®, San Diego, CA, USA). The PCR reactions, conducted in 48-well plates, consisted of the initial denaturing temperature at 95ºC for 5 minutes, followed by 40 PCR cycles that comprised the denaturing temperature at 95ºC for 30 seconds, the annealing temperature at 60ºC for 30 seconds, and the extension temperature at 72ºC for 30 seconds. The fluorescence data were acquired at the end of each extension step during the PCR cycles. After the amplification cycle, the melting curve was generated by increasing temperature from 55ºC to 95ºC to characterize the PCR product. A no-template control was included on each plate for each assay. To confirm the expected PCR size of each HPV genotypes, the amplified PCR products were resolved onto 2% agarose gel with a 100-base pair DNA ladder (Research Organic Inc., Cleveland, OH, USA) in Tris-borate EDTA (TBE) buffer. The plasmid of HPV-16 and HPV-16-positive carcinoma in situ of the cervix were used as the positive controls. The negative control, sterile water in place of DNA, shows no DNA templates The digitalized images from the stained agarose gel with ethidium bromide were captured by a charge-coupled device (CCD) camera, attached to the ChemiDoc XRS gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

Results

Demographic, clinical, and histopathologic data show that the patients included 19 males (46.3%) and 22 females (53.7%) (Table 2). Their ages ranged from 31 to 82 years (mean = 52.2 years). The histopathologic diagnoses were VC (n = 4, 9.7%), microinvasive OSCC (n = 2, 4.9%), well-differentiated OSCC (n = 16, 39.0%), moderately-differentiated OSCC (n = 10, 24.4%), poorly-

Table 1. Primer Sequences of the E6 Region of Human Papillomaviruses (HPV) Types 16 and 18, the Beta-Actin and GAPDH Genes

Gene	Primer sequence (forward and reverse)	PCR fragment length (bp*)
HPV-16 E6	5'-ATGCACCAAAAGAGAACTGCAA 5'-TCACATACAGCATATGGATTCCCATC	212
HPV-18 E6	5'ATGGACCTAAGGCAACATTGCAAGACA 5'-TCGGCTCGTCGGGCTGGAA	150
Beta-actin	5'-CATGTACGTTGCTATCCAGGC 5'-CTCCTTAATGTCACGCACGAT	250
GAPDH	5'-TGGTATCGTG GAAGGACTCAT 5'-GTGGGTGTCGCTGTTG AAGTC	370

*bp = based-pair

differentiated OSCC (n = 2, 4.9%), and unidentifiable (n = 7, 17.1%). The most common site was the gingiva and alveolar mucosa (n = 17, 41.5%), followed by the tongue (n = 12, 29.3%), buccal mucosa (n = 6, 14.7%), palate (n = 3, 7.3%), labial mucosa (n = 1, 2.4%), maxilla (n = 1, 2.4%), and mandible (n = 1, 2.4%).

Detection of p16 by immunohistochemical staining: The immunostaining pattern of p16 in VC and OSCC cases varied from very low to high expression. The p16 expression in the tumor islands of OSCC was mainly stained in the cytoplasm of the tumor cells (Figures 1 and 2). The peripheral cells of the tumor islands showed weaker staining than did the inner cells. The keratin pearls lacked staining. In addition, the chronic inflammatory cells in the stroma were occasionally p16-positive. The immunostaining of p16 in VC was predominantly in the cytoplasm of all epithelial cell layers except the keratinized layer (Figure 3). Upon scoring, 10 cases of OSCC (24.4%)
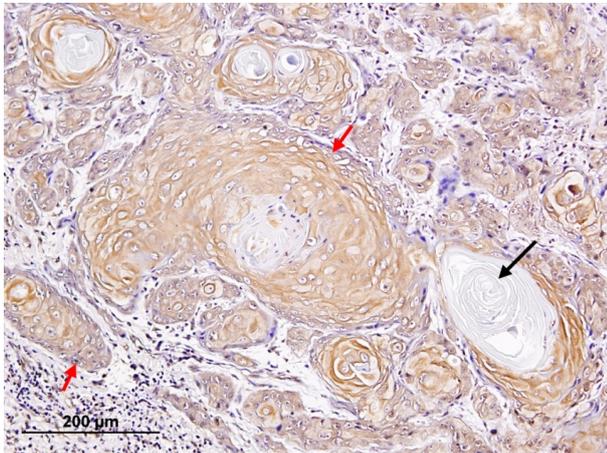

Figure 1. p16 Staining in Tumor Islands (red arrows) of an Oral Squamous Cell Carcinoma. Note lack of p16 staining in a keratin pearl (black arrow)
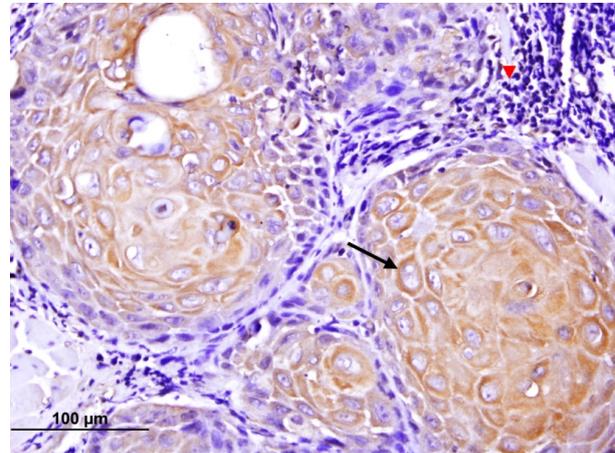

Figure 2. p16 Intense Staining in the Cytoplasm of Tumor Cells (black arrow) of an Oral SCC. Also note +-ve inflammatory cells in stroma (red arrowhead)

Table 2. Demographic Data and p16 staining in oral squamous cell carcinoma (OSCC) and and verrucous carcinoma (VC)

| Characteristics	n	%	p16 positive (n=10)	%	p
Age, years (n=41)					
30-39	5	12.2	4	40	0.009*
40-49	15	36.6	2	20	0.277
50-59	4	9.8	1	10	1
60-69	5	12.2	0	0	0.31
70-79	9	22	2	20	1
80-89	3	7.3	1	10	1
Sex (n=41)					
Female	22	53.7	4	40	0.469
Male	19	46.3	6	60	
Primary tumor site (n=41)					
Alveolar mucosa/gingiva	17	41.5	3	30	0.48
Tongue	12	29.3	3	30	1
Buccal mucosa	6	14.7	2	20	0.622
Palate	3	7.3	2	20	0.142
Labial mucosa	1	2.4	0	0	1
Maxilla	1	2.4	0	0	1
Mandible	1	2.4	0	0	1
Grade (n=41)					
Verrucous carcinoma	4	9.7	2	20	0.245
Micro-invasive	2	4.9	2	20	0.055
Well differentiated	16	39	6	60	0.15
Moderately differentiated	10	24.4	0	0	0.084
Poorly-differentiated	2	4.9	0	0	1
Unidentifiable	7	17.1	0	0	0.164
p16 staining (n=41)					
Positive	10	24.4	10	100	N/A
HPV (n=38)					
HPV-16	5	13.2	N/A	N/A	N/A
HPV-18	2	5.3	N/A	N/A	N/A
Co-infection	1	2.6	N/A	N/A	N/A
HPV regardless of types	6	15.8	N/A	N/A	N/A

N/A = non-available data
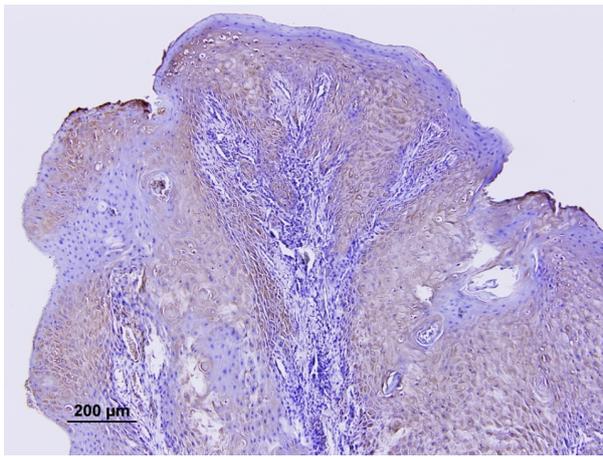
Figure 3. Expression of p16 in All Epithelial Cell Layers Except the Keratinized Layer in a Verrucous Carcinoma

were p16 overexpressed and the rest (n=31, 75.61%) were considered negative. The overexpression of p16 was significantly associated with patients aged below 40 years (p<0.05, OR 20, 95% CI 1.9-211.8). There was no significant association between p16 overexpression and the sex of the patients (p = 0.47, OR 2.1, 95% CI 0.5-8.9). Eight out of ten p16-positive cases were OSCC (80%) and two out of ten p-16-positive cases were VC (20%). Of all p16-positive cases, the tongue (30%) and the gingiva and alveolar mucosa (30%) were the most common locations, followed by the buccal mucosa (20%) and the palate (20%). Regarding the histologic grading of OSCC, p16 was overexpressed only in 20% of microinvasive and 60% of well-differentiated OSCC cases.

Upon testing intra-observer and inter-observer reliability, the Kappa values for the intra-observer and inter-observer calibrations were 0.89 and 0.80, respectively.

Table 3. p16 Staining and Specific Types of Human Papillomavoruses (HPV) Detection in oral Squamous Cell Carcinoma (OSCC) and Verrucous Carcinoma (VC)

Case	Sex	Age	Diagnosis	Site	p16 staining	HPV
1	Female	46	OSCC	Tongue	Positive	Negative
2	Female	34	OSCC	Tongue	Positive	Negative
3	Male	34	OSCC	Tongue	Positive	HPV-16
4	Male	36	OSCC	Buccal mucosa	Positive	Negative
5	Female	54	OSCC	Buccal mucosa	Positive	Negative
6	Male	31	OSCC	Palate	Positive	HPV-18
7	Female	42	OSCC	Palate	Positive	Negative
8	Male	82	OSCC	Alveolar mucosa	Positive	Negative
9	Female	78	VC	Alveolar mucosa	Positive	Negative
10	Female	74	VC	Gingiva	Positive	HPV-16,-18
11	Male	55	VC	Buccal mucosa	Negative	Negative
12	Female	61	VC	Buccal mucosa	Negative	Negative
13	Male	77	OSCC	Buccal mucosa	Negative	Negative
14	Female	72	OSCC	Buccal mucosa	Negative	Negative
15	Male	49	OSCC	Tongue	Negative	Negative
16	Female	67	OSCC	Tongue	Negative	Negative
17	Male	47	OSCC	Tongue	Negative	Negative
18	Female	49	OSCC	Tongue	Negative	Negative
19	Male	48	OSCC	Tongue	Negative	Negative
20	Male	42	OSCC	Tongue	Negative	Negative
21	Female	49	OSCC	Tongue	Negative	N/A
22	Male	33	OSCC	Tongue	Negative	HPV-16
23	Male	43	OSCC	Tongue	Negative	Negative
24	Female	54	OSCC	Alveolar mucosa	Negative	Negative
25	Female	50	OSCC	Alveolar mucosa	Negative	Negative
26	Male	63	OSCC	Alveolar mucosa	Negative	HPV-16
27	Female	80	OSCC	Alveolar mucosa	Negative	Negative
28	Male	70	OSCC	Alveolar mucosa	Negative	Negative
29	Female	69	OSCC	Alveolar mucosa	Negative	Negative
30	Male	76	OSCC	Alveolar mucosa	Negative	Negative
31	Female	76	OSCC	Alveolar mucosa	Negative	Negative
32	Male	45	OSCC	Gingiva	Negative	Negative
33	Female	46	OSCC	Gingiva	Negative	Negative
34	Female	42	OSCC	Gingiva	Negative	Negative
35	Female	70	OSCC	Gingiva	Negative	Negative
36	Female	66	OSCC	Gingiva	Negative	N/A
37	Female	48	OSCC	Gingiva	Negative	Negative
38	Male	49	OSCC	Palate	Negative	Negative
39	Female	73	OSCC	Buccal mucosa	Negative	Negative
40	Male	43	OSCC	Maxilla	Negative	N/A
41	Male	82	OSCC	Mandible	Negative	Negative

N/A = non-available data

Figure 4. HPV-16 Specific Primer-mediated PCR of DNA Extracted from Oral Squamous Cell Carcinomas. The arrows show the 212-bp amplicons representing HPV-16 (lanes 4, 9, 19, and 20). The DNA ladder marker (M) was used as the DNA standard. The plasmid of HPV-16 (P) and HPV-16-positive carcinoma in situ of the cervix (lane 1) were used as the positive controls. The negative control (N), sterile water in place of DNA, shows no DNA template

Detection of HPV-16 and -18 by real-time PCR: Based on real-time PCR, both HPV-16, and -18 were detected in the positive control tissue, cervical carcinoma in situ. Of all samples, the DNA could not be extracted from three out of 41. HPV-16 was detected in 4/5 cases (80%) and HPV-18 in 1/5 cases (20%) of OSCC (Figure 4). A co-infection of HPV-16/18 was found in 1/4 cases (25%) of VC (Table 3). Both HPV-16 and -18 detected in OSCC and VC were significantly associated with younger patients (30-39 years of age) ($p < 0.05$, OR 23.3, 95% CI 2.4-229.7). There was no significant association between HR-HPV and the sex of the patients ($p = 0.15$, OR 6.2, 95% CI 0.6-61.4). However, 4/5 cases (80%) of both OSCC and VC with HPV-16/18 infection were found in male patients. Of all HPV detected cases, the tongue (40%) and the gingiva (40%) were the most frequent sites. HR-HPVs-16 or -18 were detected in 3/10 cases of p16-positive OSCC and VC. To determine whether or not p16 overexpression can be used as a surrogate marker for HR-HPV infection, the sensitivity and specificity tests were analyzed. For cases with both OSCC and VC cases, the sensitivity and specificity were 50% and 78.1%, respectively. For cases of OSCC alone, the sensitivity and specificity were 40% and 79.3%, respectively and for cases of VC alone, the sensitivity and specificity were 100% and 66.7%, respectively.

Discussion

In our study, the age of patients with VC and OSCC ranged from 31 to 82 years (mean = 52.2 years), supporting previous studies that OSCC and VC are a disease of middle-aged or older adults (Iamaroon et al., 2004a; American Cancer Society, 2014; Komolmalai et al., 2015). In recent decades, many studies have attempted to look for diagnostic and prognostic markers for OSCC, for example COX-2, epidermal growth factor receptors (EGFR), H-ras, c-myc, p53, cyclin D1, p21, Rb, Ki-67, Bcl-2, p16, etc. (Iamaroon et al., 2004b; Perez-Sayans et al., 2011; Iamaroon, 2016). p16, in particular, has drawn interest from many investigators for its role in HPV-related OSCC carcinogenesis (Pande et al., 1998; Fregonesi et al., 2003; Perez-Sayans et al., 2011; Dragomir et al., 2012; Prakash et al., 2013; Chung et al., 2014). Under normal circumstances, p16 regulates the cell cycle during the G1-S phases. Loss of p16 is often seen in many cancers, including OSCC (Perez-Sayans et al., 2011). However, in HPV-related cervical cancer, OSCC and OPSCC, p16 is

markedly overexpressed. This paradoxical phenomenon is believed to occur due to the fact that the E7 oncoprotein of HR-HPV can interfere with Rb protein in the cell cycle, resulting in unlimited cell division and overexpression of p16 protein. Collectively, p16 is, therefore, broadly used as a surrogate marker for HPV-related carcinomas (Patil et al., 2014; Sritippho et al., 2015).

In our study, the expression of p16 protein in OSCC and VC was determined by the immunohistochemical method. We found that 21.6% of OSCC and 50% of VC cases were p16 overexpressed, suggesting that those cases were HPV-infected. Previous studies have revealed that the rate of p16 overexpression in OSCC is somewhat variable. The higher rates have been found in Japanese (28.7% and 29.1%) (Nakahara et al., 2001; Suzuki et al., 2006), Romanian (64.7%) (Dragomir et al., 2012), and Spanish (68%) (Gonzales-Moles et al., 2002) patients, whereas the lower rates have been found in Indian (15.4%) (Ramshankar et al., 2014), U.S.A (13%) (Muirhead, 2006), and Canadian (13%) (Chandarana et al., 2013) patients. The discrepancy in results may be due to differences in age, race, sex, risk factor, location of the disease, case selection, techniques used and positive criteria for immunochemical staining (Brouwer et al., 2016).

Notably, numerous investigators have developed various criteria to proclaim the overexpression of p16, resulting in varying rates of p16 positive cases, ranging from 12.8%-100% in patients with OSCC (Perez-Sayans et al., 2011; Chung et al., 2014). However, a systematic review of p16 in OPSCC revealed that the highest correlation between p16 and HPV detection occurred when p16 was expressed in more than 70% of all tumor cells (Grønhøj Larsen et al., 2014). In our study, we adopted the stringent criteria of Pirker et al. for immunohistochemical staining, where a staining score ≥ 200 was considered positive, to judge p16 overexpression. These criteria were originally used in a study of EGFR as a predictor of survival rate in patients with non-small-cell lung cancer (Pirker et al., 2012). By using these criteria, we found p16 was overexpressed in 21.6% of OSCC and 50% VC cases. Interestingly, OSCC cases with higher grades, including moderately and poorly differentiated, were not at all overexpressed. These findings were in line with those of a previous study (Cao et al., 2014) in which cases of p16-positive esophageal carcinoma were correlated with higher differentiated grading. These results may also reflect that p16-positive patients with OSCC may have a better prognosis, since patients with higher differentiation of OSCC have better survival outcomes (Sawazaki-Calone et al., 2015). In fact, patients with HNSCC with p16-positive tumors have favorable outcomes, good prognosis, low recurrence rates, and reduced risk of death (Laco et al., 2012; Prakash et al., 2013; Chung et al., 2014). Moreover, HNSCC patients who are HPV-negative but p16-positive have a better survival rate than those who are HPV-negative and p16-negative (Stephen et al., 2013).

The HPV family consists of more than 170 diverse types and preferentially infects the mucosa of genitals, upper-respiratory tract, and the epithelium of the skin. (Rautava and Syrjänen, 2012; Ghittoni et al., 2015). In the last decade, it has become obvious that HPV is not only

an etiological factor in cervical cancer, but also HNSCC (Antonsson et al., 2015; Hubbers and Akgul, 2015). A recent study in Australian patients has shown interesting findings that the prevalence of HPV-related OPSCC, in particular, was elevated from 19% during 1987-1990 to 66% during 2005-2006 (Hong et al., 2010). Consistently, a Swedish study in patients with OPSCC showed a significant increased trend of HPV-related OPSCC over almost four decades from 23% in 1970s to 93% during 2006-2007 (Nasman et al., 2009).

The prevalence of HPV infection in OSCC is variable from study to study. A systematic review by Kreimer et al. revealed that the pooled prevalence of HPV DNA detection in 2,642 patients with OSCC was 23.5% (Kreimer et al., 2005). A more recent systematic review, analyzing 4,195 patients with OSCC from 60 publications, also showed a similar prevalence of HPV DNA detection (20.2%) (Isayeva et al., 2012). Interestingly, we found a similar trend of the prevalence of HR-HPV infection in OSCC and VC (14.7% and 25%, respectively). However, our results were much lower than those of the Indian (48.3%) (Elango et al., 2011), the Japanese (74%) (Shima et al., 2000) and two Chinese (40.4% and 74%) (Zhang et al., 2004; Zhao et al., 2009) studies. These discrepant findings may be due to differences in ethnicity and geography, sample size, HPV detecting technique, and sexual behavior. Notably, a prior investigation in Thai patients with OSCC showed that the rate of HPV detection was only 3.1%. The low prevalence in this study may be due to most patients being elderly and having a long history of betel nut chewing, smoking or alcohol consumption (Khovidhunkit et al., 2008). In terms of genotype distribution, a recent systematic review of HPV in HNSCC demonstrated that HPV-16 was the most common HPV type (more than 80% of all HPV cases detected) (Ndiaye et al., 2014). Similarly, we found HPV-16 in 83.3% and HPV-18 in 16.7% of all OSCC and VC cases, respectively, confirming that HPV-16 is the most common HPV genotype in OSCC and VC.

To predict if p16 could be used as a surrogate marker for HNSCC, many investigators have explored the correlation between p16 overexpression and HR-HPV. A systematic review of HNSCC illustrated that p16 overexpression using an immunohistochemical technique showed a high sensitivity, but moderate to high specificity for HPV-related HNSCC (Ndiaye et al., 2014). For example, p16 was found to be a reliable surrogate marker for HPV-related OPSCC, showing high sensitivity and high specificity (92% and 92%, respectively) (Liu et al., 2015). These findings have been consistent with those of many other studies on OPSCC (Laco et al., 2012; Ndiaye et al., 2014). Previous reports of p16 as a surrogate marker for HR-HPV in OSCC, however, appeared controversial, probably due to the small number of studies on OSCC (Smeets et al., 2007). Some investigations have found a positive correlation (Laco et al., 2012; Patil et al., 2014), whereas others have not (Nemes et al., 2006; Greer et al., 2008). In our study, we demonstrated that p16 could predict the presence of HR-HPV for both OSCC and VC with a moderate sensitivity and moderate-to-high specificity. Interestingly, for VC alone, the sensitivity was as high as 100% and the specificity was 66.7%.

Collectively, these findings suggest p16 could be used as a surrogate marker for oral cancer, especially VC.

In conclusions, HR-HPV was detected in 14.7% of OSCC and 25% of VC. The overexpression of p16 and HR-HPV was significantly associated with younger patients with both OSCC and VC. p16 overexpression was concordant with HR-HPV testing with a moderate sensitivity and high specificity, suggesting that p16 can be used as a surrogate marker for HR-HPV in OSCC and VC.

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