# RESEARCH ARTICLE

# Methylation Status of P16[Ink4a] in Human Papillomavirus-Associated Cancer of Oral Cavity and Oropharynx in Northeastern Thailand

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## Abstract

**Background:** Over-expression of p16[INK4a] protein is a biomarker for human papillomavirus (HPV)-associated cervical cancer. However, absence of p16[INK4a] protein expression in HPV-associated cancer of the oral cavity and oropharynx has been reported. Among a number of possible reasons for this is methylation, which is frequently noted in the promoter region of p16[INK4a] and is associated with silencing of the gene and disease severity. **Methods:** We investigated the relationships between p16[INK4a] protein expression, HPV infection and methylation status of the p16[INK4a] promoter in cancers of the oral cavity and oropharynx. Fifty-three formalin-fixed paraffin-embedded (FFPE) cancer tissue samples from the oral cavity (49 cases) and oropharynx (4 cases) were studied. P16[INK4a] protein expression was determined using immunohistochemical staining (IHC). Additional oral tissues lacking squamous intraepithelial lesions (SILs), and cervical tissues with high-level SILs, were used as negative and positive controls, respectively. High-risk HPV infection was detected using HPV E6/E7 mRNA in situ hybridization. Methylation status of the p16[INK4a] promoter was investigated using sodium bisulfite treatment and methylation-specific PCR (MS-PCR). **Results:** HPV infection was found in 40.8% (20/49) and 50.0% (2/4) of oral cavity and oropharynx cancers, respectively. Promoter methylation of p16[INK4a] occurred in 73.6 % of all cases and differed significantly in frequency between HPV-positive (90.9%, 20/22) and HPV-negative (61.3%, 19/31) samples. Expression of p16[INK4a] was found in 35.8% (19/53) and commonly detected in samples with p16[INK4a] unmethylation (79.5%). Interestingly, the silencing of p16[INK4a] (64.2%, 34/53) was significantly associated with methylation status (91.2%, 31/34), especially in HPV-infected samples in which the p16[INK4a] promoter was methylated (52.9%, 18/34). **Conclusions:** This result demonstrated high frequency of p16[INK4a] promoter methylation status in HPV-associated HNSCC subsets that could influence the silent p16[INK4a] expression and might promote disease severity.

**Keywords:** Human papillomavirus- promoter methylation- oral cavity- oropharynx- p16[INK4a]

## Introduction

Cancers of the oral cavity, nasal cavity, sinonasal tract, pharynx and larynx are subsets of head and neck cancers. Approximately 90% of these cancers are squamous cell carcinomas. More than 550,000 cases of head and neck squamous cell carcinoma (HNSCC) occur globally each year, causing around 300,000 deaths (Jemal et al., 2011). It is possible that environmental risk factors or cofactors play an important role in onset of HNSCC. Such factors include genetic susceptibility, nutritional factors, tobacco and alcohol interaction as well as chewing of betel quid and areca nut which is common in Asian countries (Krishna Rao et al., 2013). Infections with human papillomavirus (HPV) are also found increasingly in HNSCC, especially tonsillar or oropharyngeal subsites (Dahlstrand and Dalianis, 2005; Mirghani et al., 2015) The prevalence of HPV in HNSCC differs regionally. It is higher in western countries than in Asian countries (Kreimer et al., 2005). Prevalence of HPV also differs among HNSCC subsites: 16%, 55% and 26% prevalence in cancers of the oral cavity, oropharynx and larynx, respectively (Gillison et al., 2000). Biological distinctions among HPV-positive and -negative HNSCC cases have been reported.

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Infection with high-risk HPV (HR-HPV) is well-known as a cause of cervical cancer. HPV-positive oral squamous cell carcinoma (OSCC) is characterized by inactivation of p53 and retinoblastoma protein (pRb) via HPV oncoproteins E6 and E7, respectively. E7 overexpression induces the expression of a protein that slows cell-cycle, cyclin-dependent kinase inhibitor protein 2A (CDKN2A or p16$^{INK4a}$) by disruption of interaction between pRb and E2F and release of E2F. In contrast, tobacco-related oropharyngeal cancer is characterized by p53 mutation and down-regulation of CDKN2A (encoding p16$^{INK4a}$). HPV-positive OSCC seems to be more responsive to chemotherapy and radiation than is HPV-negative disease (Jung et al., 2010).

Elevated expression of p16$^{INK4a}$, a surrogate marker of HPV-related cervical neoplasia, occurs in nearly 100% of such cancers to an extent proportional to cervical lesion severity (Kalof and Cooper, 2006). Overexpression of p16$^{INK4a}$, reported in 24-61% of HNSCC cases, is associated with good prognosis and response to treatment with chemotherapy and radiation (Rautava et al., 2012). Overexpression of p16$^{INK4a}$ varies across subsets of HNSCC. It has been reported in 30% of cases involving the oral cavity, but in up to 86.3% of cases of oropharynx cancer (Bixofis et al., 2014; Zafereo et al., 2016). Previous studies have reported that not all HPV-positive HNSCCs showed p16$^{INK4a}$ overexpression (Nemes et al., 2006; Bishop et al., 2012). Some studies showed a strong association between HPV infection and p16$^{INK4a}$ overexpression, but 37-52% of HPV-positive HNSCC were p16$^{INK6a}$-negative and associated with poor prognosis (Ukpo et al., 2011; Lingen et al., 2013; Melkane et al., 2014). In such cases, epigenetic events may influence p16$^{INK4a}$ expression. Promoter methylation of p16$^{INK4a}$ might explain the lack of p16$^{INK4a}$ overexpression in many cancers (Sanchez-Cespedes et al., 2000).

Expression of the p16$^{INK4a}$ gene, located at the INK4A/ARF locus on chromosome 9p21, can be controlled by many molecular pathways. Increase of transcriptional factors or host oncoproteins can up-regulate expression of this gene (Sharpless and DePinho, 1999). Some reports have shown that the INK4A/ARF locus is controlled by epigenetic mechanisms such as histone modification and promoter methylation pathways. Promoter methylation of p16$^{INK4a}$ is found commonly in prostate, colorectal and gastric cancers and the degree of methylation is proportional to disease severity (Goto et al., 2009).

HR-HPV oncoproteins are involved in up-regulation processes of the DNA methyltransferase (DNMTs) enzymes DNMT1 and DNMT3b. HPV16 E6 is identified as a factor for induction of DNMT1 expression in SiHa and CaSki cells (Au Yeung et al., 2010). HPV16 E7 was also found to up-regulate DNMT1 expression in a human keratinocyte cell line (Laurson et al., 2010). HPV18-transfected organotypic raft culture showed the expression of DNMT1 and DNMT3b in basal and suprabasal layers (Leonard et al., 2012). Moreover, promoter methylation of various genes accumulated in HR-HPV-infected keratinocytes with HPV integration form before cell immortalization (Henken et al., 2007).

Promoter methylation of p16$^{INK4a}$ gene is a frequent event in primary HNSCCs and plays a major role in the silencing of this gene during tumor development (Demokan et al., 2012). Although promoter methylation leads to down-regulation of p16$^{INK4a}$ expression, in oral cancer cell lines treated with a DNMT inhibitor (5-aza-deoxycytidine), re-activation of p16$^{INK4a}$ expression was shown (Yakushiji et al., 2003).

To explore the INK4A/ARF promoter methylation affecting p16$^{INK4a}$ protein expression in HPV-associated HNSCC subsets (cancers of oral cavity and oropharynx), we first investigated HPV infection in formalin-fixed paraffin-embedded (FFPE) tissues of HNSCC subsets from northeastern Thai patients. Presence of HPV infection was determined by HR-HPV E6/E7 mRNA in situ hybridization (HR-HPV E6/E7 mRNA ISH). The methylation status of the p16$^{INK4a}$ promoter was evaluated by sodium bisulfite treatment and methylation-specific polymerase chain reaction (MS-PCR). Finally, the expression of p16$^{INK4a}$ protein was observed with immunohistochemistry (IHC) staining.

## Materials and Methods

*Patients and Samples*

Studied samples were 53 FFPE tissue samples collected from various HNSCC subsets including cancer of the oral cavity (49 cases) and oropharynx (4 cases). Additional 6 FFPE tissue samples from the oral cavity with no squamous intraepithelial lesions (No-SIL) and FFPE cervical tissues with high-SILs were included as negative and positive controls for p16$^{INK4a}$ IHC, respectively. All samples were collected from participating patients at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University and Khon Kaen Central Hospital. This study was approved by Khon Kaen University Ethics Committee in Human Research (No.HE521344).

*High risk (HR)-HPV E6/E7 mRNA detection by in situ hybridization (ISH)*

RNAscope® Reagent Kit (Advanced Cell Diagnostics, Hayward, CA) was used to detect 18 types of HR-HPV E6/E7 RNA as described in the manufacturer's instructions. This detection by ISH was performed on a tissue microarray (TMA) platform. FFPE blocks were cut into 5 μm sections that were put on glass slides and stained with hematoxylin and eosin (H&E) dyes. The tumor areas were identified using a light microscope. To prepare the TMA blocks, FFPE tissues were punched with a biopsy-gauge needle in at least two areas of the tumor. Punched tumor tissues were re-embedded in paraffin. Five μm sections of re-embedded TMAs were cut and put on SuperFrost Plus glass slides. TMA slides were de-paraffinized in xylene and hybridized with a cocktail of 18 HR-HPV E6/E7 mRNA probes (HPV type 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59,66, 68, 73 and 82) and hybridization signal was developed with the RNAscopeTM 2.0 HD (26) system (Advanced Cell Diagnostic, Hayward, CA, USA). The human cyclophilin B probe (PPIB), which is the internal control targeting a constitutively expressing gene and bacterial dihydrodipicolinate reductase (DapB) negative control probe were also used. HPV E6/E7

mRNA hybridization signals were observed under a light microscope. Specific signals were observed as brown dots in the cytoplasm or in both cytoplasm and nucleus of tumor cells.

### p16$^{INK4a}$ Immunohistochemical staining (IHC)

Sections 2 µm thick were cut from each FFPE block and adhered to SuperFrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany). The sections were de-paraffinized in xylene (5 min x 3 washes) followed by re-hydration in absolute ethanol (5 min x 2), 95% ethanol (5 min), 70 % ethanol (5 min) and distilled water (at least 10 min). IHC was performed using a CINtec histology kit (mtm labs, Heidelberg, Germany) following the manufacturer's instructions. The stained tissues were observed under a light microscope. In cells positive for p16$^{INK4a}$, stain was present in nuclei or nuclei/cytoplasm. Over 500 tumor cells were evaluated in each sample and scored according to the intensity of p16$^{INK4a}$ staining: 0=no expression; 1=1-20% of cells were stained; 2=21-40% of cells were stained and 3=41-100% of cells were stained (Pande et al., 1998). In cervical tissues used as controls, cells were scored in three categories; no expression, focal staining and diffuse staining for p16$^{INK4a}$.

### Sodium bisulfite modification and p16$^{INK4a}$-methylation-specific polymerase chain reaction (MS-PCR)

Before performing the experiments, control DNA for the p16$^{INK4a}$ MS-PCR was prepared and tested. DNA was extracted from SiHa cells, which are known to be unmethylated. The methylated DNA control was prepared by treatment of 1 µg of SiHa DNA with 100 units of M. SssI DNA CpG methyltransferase (New England Biolabs, Ipswich, MA, USA) together with S-adenosylmethionine (Monzon et al., 1998) substrate and incubated at 37°C for 16 hours for complete methylation. Fifty-three selected DNA samples from HNSCC subsets (HPV-positive or HPV-negative) were treated using the sodium bisulfite modification method. A quantity (200-500 ng) of DNA was treated with Epitect Bisulfite kit (QIAGEN, Hilden, Germany) following the instruction manual. The treated DNA samples were checked by PCR using p16$^{INK4a}$ wild-type primers (p16$^{INK4a}$ WT) to monitor the gene modification after bisulfite treatment (no amplification should occur). The primers for p16$^{INK4a}$ MS-PCR were designed to distinguish methylated from unmethylated DNA and produced amplicons of 150 and 151 bp, respectively. The details of each primer pair are shown in Table SI (Herman et al., 1996) All MS-PCR reactions were performed with Platinum Taq polymerase (Life Technologies). PCR products were separated on a 2% agarose gel and visualized under UV light.

### Statistical analysis

Statistical analysis was done using SPSS version 16.0 (IBM, Armonk, NY, USA). Relationships of HPV infection, p16$^{INK4a}$ expression and p16$^{INK4a}$ promoter methylation were analyzed using the Chi-square test. Any P-value < 0.05 was considered as statistically significant. The impact of potential factors on p16$^{INK4a}$ methylation was evaluated by univariate analysis.
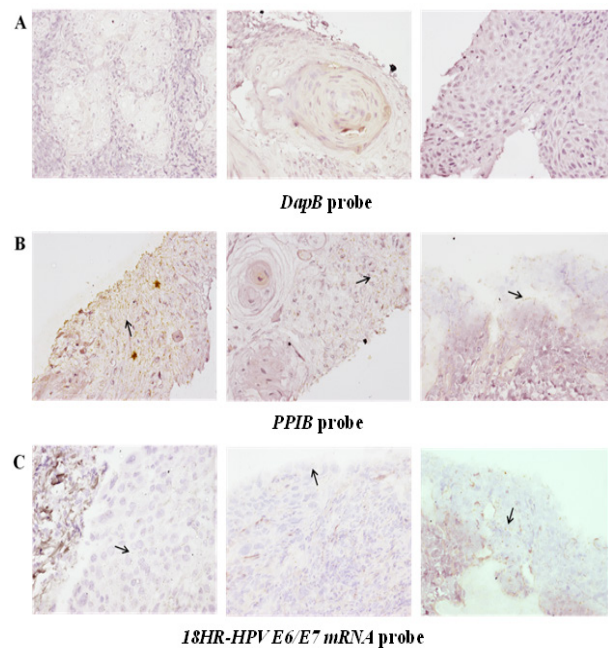


Figure 1. HPV Detection in Various HNSCC Subsets. RNA in situ hybridization methods (RISH) were used to identify the genotypes of HR-HPV E6/E7 present. Probes for 17 different genotypes were used. (A) DapB and (B) PPIB probes were used as internal negative and positive controls respectively for the in situ hybridization. (C) Hybridization signals of HR-HPV E6/E7 mRNA are shown. The signals appear as brown dots and are indicated with black arrows. DapB: bacterial dihydrodipicolinate reductase, PPIB: human peptidylpropyl isomerase B, HR-HPV: high-risk human papillomavirus.

## Results

### Characteristics of studied samples

Table 1 describes the characteristics of samples used in this study. Median age of patients was 70 years (ranging from 41 to 90 years). Female patients were in the majority (69.8%).

### HPV infection in HNSCC subsets

A total of 53 HNSCC subset samples and 6 oral samples with No-SIL were tested for HPV infection by detection of HR-HPV E6/E7 mRNA expression using the ISH method. The mRNA hybridization was performed with different three probes; DapB (Figure 1A.), PPIB (Figure 1B.) and HR-HPV E6/E7 mRNA (Figure 1C.). HPV infection was found in 41.5% (22/53) of HNSCC subsets (Table 1). All oral samples with No-SIL were negative for HPV infection.

### Methylation of p16$^{INK4a}$ promoter in HNSCC subsets

Methylation was observed on the p16$^{INK4a}$ promoter located at the INK4A/ARF locus by MS-PCR. The PCR products were separated using agarose gel electrophoresis and visualized under ultraviolet light (Figure 2A.). The association between p16$^{INK4a}$ promoter methylation and HPV infection was investigated. Fifty-three HNSCC subset samples were evaluated according to their HPV infection status: 22 positive cases and 31 negative cases. Methylation of the p16$^{INK4a}$ promoter methylation was found in 73.6% (39/53) of all samples. The promoter
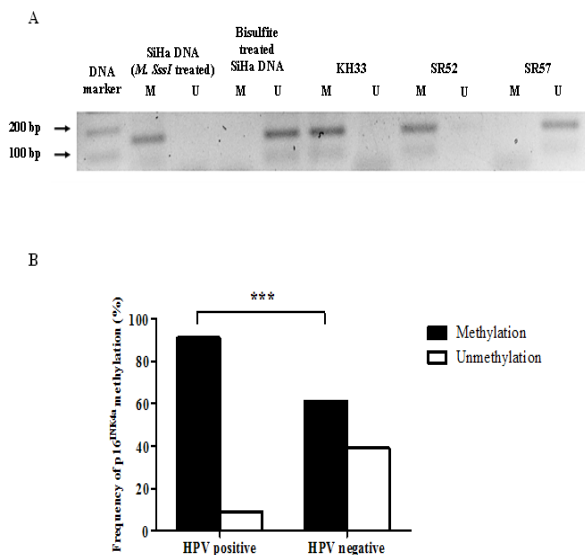
A



B



Figure 2. p16$^{INK4a}$ Promoter Methylation in HNSCC Samples. DNAs of OSCCs were treated with sodium bisulfite before evaluation of p16$^{INK4a}$ promoter methylation by MS-PCR. (A) Representative examples of MS-PCR for p16$^{INK4a}$. PCR products (approx 150 bp) were separated in an agarose gel. SiHa DNA treated with DNA methylatransferase (M. SssI) was used as a positve control of p16$^{INK4a}$ methylated promoter. (B) Frequency of p16$^{INK4a}$ promoter methylation catagorized by HPV infection status. MS-PCR: methylation-specific polymerase chain reaction, M. SssI, DNA methyltranferase; U, unmethylation; M, methylation

of p16$^{INK4a}$ gene was more frequently methylated in HPV-positive (90.9%) than in HPV-negative (61.3%) HNSCC cases (P<0.001) (Figure 2B. and Table 2.). This result demonstrated that a high frequency of p16$^{INK4a}$
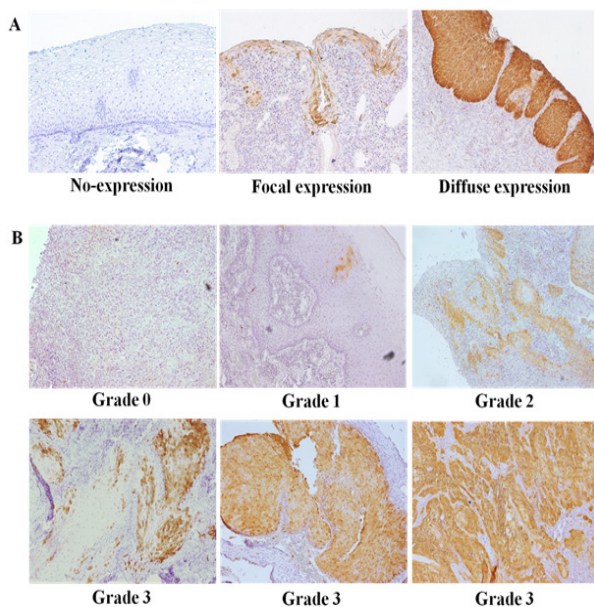


Figure 3. Immunohistochemical (IHC) Staining to Demonstrate Expression of p16$^{INK4a}$ in Representative Cervical and Oral Tissues. (A) IHC expression in cervical tissues exhibited 3 patterns: no-expression, focal expression and diffuse expression. (B) IHC staining patterns of p16$^{INK4a}$ fell into 4 categories: At least 500 tumor cells, in which nuclei and cytoplasm were stained, were counted in each section

Table 1. Characteristics of Samples (n=53)

| Characteristics | Number (%) |
|---|---|
| Sex | |
| Female | 37 (69.8) |
| Male | 16 (30.2) |
| Anatomical site | |
| Oral cavity | 49 (92.5) |
| Buccal mucosa | 33 (62.3) |
| Tongue | 3 (5.7) |
| Lip | 3 (5.7) |
| Floor of mouth | 5 (9.4) |
| Gum | 2 (3.8) |
| Hard palate | 3 (5.7) |
| Oropharynx | 4 (7.5) |
| Soft palate | 2 (3.8) |
| Uvular | 2 (3.8) |
| Histological classification | |
| Well differentiated | 36 (67.9) |
| Moderately differentiated | 9 (17.0) |
| Poorly differentiated | 8 (15.1) |
| HPV status | |
| HPV positive | 22 (41.5) |
| HPV negative | 31 (58.5) |
| Molecular features of p16$^{INK4a}$ in patients | |
| p16$^{INK4a}$ methylation | 39 (73.6) |
| p16$^{INK4a}$ expression | 19 (35.8) |

methylation in HNSCC subsets is associated with HPV infection.

*p16$^{INK4a}$ protein expression in HNSCC subsets with HPV infection and p16$^{INK4a}$ methylation*

To elucidate the relationship of HPV associated p16$^{INK4a}$ methylation to p16$^{INK4a}$ protein expression in HNSCC subsets, p16$^{INK4a}$ expression was investigated in oral cavity SCC (49 cases) and oropharynx SCC (4 cases). The correlation between p16$^{INK4a}$ expression and HPV-associated p16$^{INK4a}$ methylation is shown in Table 3. P16$^{INK4a}$ protein expression was demonstrated in controls (FFPE cervical tissues) and showed 3 patterns; no-expression, focal and diffuse expression (Figure 3A.). Expression of p16$^{INK4a}$ in FFPE HNSCC subsets showed different patterns and at least 500 cells per section with positive staining of nuclei/cytoplasm were scored (Figure 3B.). P16$^{INK4a}$ expression in HNSCC subsets was 35.8%

Table 2. HPV Infection and p16$^{INK4a}$ Promoter Methylation in HNSCC Subset Samples (n=53)

| HPV infection | p16$^{INK4a}$ | (%) | P | OR | 95% CI |
|---|---|---|---|---|---|
| | M | U | | | |
| Positive | 20 (90.9) | 2 (9.1) | <0.0001 | 6.46 | 2.9-14.3 |
| Negative | 19 (61.3) | 12 (38.7) | | | |
| Total | 39 (73.6) | 14 (26.4) | | | |

M, methylated; U, unmethylated

Table 3. HPV Status and Molecular Features of p16$^{INK4a}$ in HNSCC Subset Samples (n=53)

| | HPV infection | | Total | P | OR | 95% CI |
|---|---|---|---|---|---|---|
| | Positive | Negative | | | | |
| Positive p16$^{INK4a}$ expression (n=19) | | | | | | |
| p16$^{INK4a}$ (M) | 2 (5.1) | 6 (15.4) | 8 (20.5) | 0.53 | 1.52 | 0.47-4.89 |
| p16$^{INK4a}$ (U) | 2 (14.3) | 9 (64.3) | 11 (79.5) | | | |
| Negative p16$^{INK4a}$ expression (n=34) | | | | | | |
| p16$^{INK4a}$ (M) | 18 (52.9) | 13 (38.2) | 31 (91.2) | <0.001 | 26.4 | 1.49-467.8 |
| p16$^{INK4a}$ (U) | 0 (0) | 3 (8.8) | 3 (8.8) | | | |
| Total | 22 (41.5) | 31 (58.5) | 53 (100) | | | |

M, methylated; U, unmethylated

(19/53, Table 3.). Interestingly, p16$^{INK4a}$ expression was significantly more frequent in HPV-negative (78.9%, 15/19) than in HPV-positive HNSCC subsets (21.1%, 4/19) (P<0.05). Lack of p16$^{INK4a}$ expression was significantly associated with a higher frequency of methylation (91.2%, 31/34), especially in HPV-associated cases 52.9% (18/34). This result indicates that silencing p16$^{INK4a}$ protein expression in HPV-infected HNSCC subsets is associated with p16$^{INK4a}$ promoter methylation.

## Discussion

This study investigated relationships between HPV-infected HNSCC subsets in northeastern Thai patients and molecular features of p16$^{INK4a}$. We found that 90.9% of HPV-infected HNSCC cases exhibited p16$^{INK4a}$ promoter methylation. A possible effect of epigenetic involvement via promoter methylation was further investigated using p16INK4 IHC. This study demonstrated that 91.2% of promoter methylation in most HNSCC subsets lacked an express p16$^{INK4a}$. Interestingly, approximately 50% of HPV-associated HNSCC cases exhibiting p16$^{INK4a}$ methylation lacked p16$^{INK4a}$ protein expression.

Reported prevalences of HPV infection in HNSCC patients range from 0 to 75%, depending on sample types and methods used (Mirghani et al., 2015). We found an HPV prevalence in HNSCC subsets of 41.5% when a "gold standard" diagnostic method was used (Table 1). Although many studies have used a combination of methods to increase sensitivity and specificity of HPV detection in OSCC samples, detection of viral transcripts using ISH is suggested to be the most reliable approach. This method can indicate viral transcriptional activity and increased sensitivity of HPV detection since more copies of target mRNA are presented than of viral genomes (Weinberger et al., 2006). Evans et al., (2014) reported that the patterns of HPV E6/E7 staining in cervical intraepithelial neoplasia (CIN) related to grade of cervical lesion. In addition, a pattern of multiple dot-like staining in nucleus and cytoplasm suggests the transformative phase of HPV. Therefore, HPV E6/E7 RNA ISH might indicate an important role for the transformative phase of HPV infection in development of oral carcinogenesis.

Previous reports showed considerable variation of p16$^{INK4a}$ expression (approximately 18.75-86.3%) in OSCC. In part, this may be a function of different geographical regions and/or antibody clone used (Buajeeb

et al., 2009; Fischer et al., 2010; Thomas and Primeaux, 2012; Bixofis et al., 2014). In this study, p16$^{INK4a}$ expression was detected in 35.8% of HNSCC cases (Table 1. and Table 3.). A previous report from Thailand noted expression of p16$^{INK4a}$ in 18.75% of OSCC (a subset of HNSCC) cases and in 26.7% of oral leukoplakia tissues, but no expression of p16$^{INK4a}$ was detected in any cases of dysplasia or in normal mucosa (Buajeeb et al., 2009). HPV infection is a significant factor influencing p16$^{INK4a}$ expression via oncogenes (Bussu et al., 2013). A positive relationship between HPV DNA, E6/E7 mRNA and p16$^{INK4a}$ expression in OSCC samples are 96.4% and 78.7%, respectively (Ukpo et al., 2011). However, as many as 41.8% of HPV-positive OSCC cases lacked expression of p16$^{INK4a}$ (Junor et al., 2012). This matches our study, in which half of all HPV-infected HNSCC cases lacked p16$^{INK4a}$ expression (Table 3): we demonstrated that p16$^{INK4a}$ expression was more frequent in HPV-negative HNSCC cases (15/19) than in HPV-infected HNSCC cases (4/19).

In this study, about half of HPV-positive HNSCC cases did not express p16$^{INK4a}$. Promoter methylation has been reported as a key factor controlling p16$^{INK4a}$ expression in many cancers, such as colorectal and prostate cancers (Goessl et al., 2000; Goto et al., 2009). In addition to the p16$^{INK4a}$ promoter, methylation of many other promoters, such as those for retinoic acid receptor-beta (RARβ), p14$^{ARF}$, p15$^{INK4a}$ and the E-cadherin family, has been reported in HNSCC cases (Viswanathan et al., 2003; Shaw et al., 2006). We found a high frequency of methylation of the p16$^{INK4a}$ promoter (73.6%) in HNSCC cases, most of which (91.2%) were found in the p16$^{INK4a}$-negative group (Table 3.). Interestingly, we found expression of p16$^{INK4a}$ in eight cases exhibiting promoter methylation (20.5%). Similar findings have been reported for cervical carcinomas, in which promoter hypermethylation did not affect p16$^{INK4a}$ expression (Nehls et al., 2008). Moreover, hypermethylation of p14$^{ARF}$, located at the same locus as p16$^{INK4a}$, is usually high in oral cancer and not correlated with p16$^{INK4a}$ promoter methylation status (Esteller et al., 2000). We have also found a high frequency (98.21%) of methylation of the p14$^{ARF}$ promoter (data not shown). Promoter methylation was found in 52.9% of HNSCC cases that were HPV positive/p16$^{INK4a}$-negative (Table 3). Previous evidence has demonstrated that HPV oncoproteins can up-regulate the DNA methyltransferase enzymes (DNMTs) including DNMT1 and DNMT3b

(D'Costa et al., 2012; Leonard et al., 2012). Therefore, HPV infection involving expression of viral oncoproteins may influence methylation status in HPV-positive HNSCC subsets. In HPV-negative HNSCC subsets, other risk factors, such as chewing of betel quid and areca nut, may affect promoter hypermethylation (Lee-Chen et al., 1996; Lai and Lee, 2006). The study of arecoline in mouse models showed suppression of RARβ, which regulates DNMT expression in human HNSCC (Lai et al., 2014). Previous evidence has indicated good prognosis and better survival rate in HPV-positive and p16$^{INK4a}$-expressing HNSCC cases (Deng et al., 2014). Moreover, methylation of p16$^{INK4a}$ promoter was associated with poor prognosis in anal and colorectal and cancers (Kim et al., 2005; Koerber et al., 2014). HNSCC cases that are HPV-positive and lacking p16$^{INK4a}$ expression might experience a worse prognosis due to DNMT expression and promoter methylation. However, our sample size was small. Larger sample sizes are required for further investigation.

In conclusion, aberrant p16$^{INK4a}$ expression was usually associated with promoter methylation in HNSCC cases. Methylation of the p16$^{INK4a}$ promoter was also commonly found in HPV-positive HNSCC cases lacking expression of p16$^{INK4a}$, a situation that may be related to disease severity.

*Statement conflict of Interest*

The authors have no conflicts of interest to declare.

## Acknowledgments

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