

## ORIGINAL ARTICLE

# Absence of human papillomavirus in oral cavity squamous cell carcinomas among Saudi patients

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**Abstract**

This study aimed to examine the possible association of human papillomavirus (HPV) with oral cavity squamous cell carcinomas (OCSCCs) in Saudi Arabia. Forty-five paraffin-embedded tumor blocks that represent different subsets of OCSCCs between 2010 and 2014 were retrieved and histologically evaluated. The presence of high-risk HPV (16, 18, 31, and 33) was assessed by p16-immunohistochemistry followed by DNA detection using in situ hybridization technique. Twenty-four patients were male with the mean age of 59.3 years, and 21 patients were female with the mean age of 61.2 years. Forty-one cases were positive for p16 immunostaining, and the remaining four cases were negative. However, none of the 45 cases showed DNA-expression for any HPV subtypes (16, 18, 31, and 33). High-risk HPV appears not to be involved in the etiology of OCSCCs in older Saudi patients, but further studies with cross section of a younger age group are still required.

**KEYWORDS**

HPV, oral cavity squamous cell carcinoma, prevalence, Saudi Arabia

## 1 | INTRODUCTION

The estimated age-standardized incidence rate of oral cancer is 2.7 per 100,000, and this rate is expected to increase globally because of population aging and growth (Shield et al., 2017). The description of oral cavity squamous cell carcinomas (OCSCCs) usually consists of oral cavity that includes the anterior 2/3 of the tongue (C02–C06; World Health Organization, 2016).

It is well known that tobacco and alcohol are considered risk factors for oral cancer (Tuyns et al., 1988). However, accumulating evidences consider human papillomavirus (HPV) as a new etiologic factor for different subsets of head and neck squamous cell carcinoma (HNSCCs; D'Souza et al., 2007; Gillison et al., 2000; International Agency for Research on Cancer, 2007).

HPV-associated OCSCCs seem to be a separate clinical entity. HPV-positive cancers are likely to appear at young age, with reduced exposure to tobacco products and alcohol. Furthermore, the prognosis of HPV-positive tumors is better than HPV-negative tumors, which

is partly attributed to enhanced sensitivity to radiation and chemotherapy (Chaturvedi, Engels, Anderson, & Gillison, 2008).

Although more than 100 different types of HPV are known (International Agency for Research on Cancer, 2007), the viral genotypes, which are often associated with leukoplakia and squamous cell carcinoma, are classified as high-risk category such as HPV 16, 18, 31, and 33. In addition, HPV 16 genome is considered the most frequently encountered (84–90%) in HPV-positive patients (D'Souza et al., 2007; Gillison et al., 2000).

Previous studies from different regions and countries on HPV association in OCSCCs have reported widely different estimates ranging from 0% to 100% (Castro & Bussoloti Filho, 2006; Elango et al., 2011; Kreimer, Cifford, Boyle, & Franceschi, 2005; Lopes et al., 2011).

In Saudi Arabia, no study has been previously investigated the possible association of high-risk HPV with OCSCCs; therefore, the present study was designated to investigate this possibility using archived OCSCCs specimens from the Department of Pathology at King Fahad Medical City (KFMC) in Riyadh, Saudi Arabia.

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## 2 | MATERIAL AND METHODS

### 2.1 | Tumor blocks

Following approval of the Ethics Committee at King Abdullah International Medical Research Center in Riyadh, Saudi Arabia (Ref. RO/326/2013, Date: 24/06/2013), a total of 88 formalin-fixed paraffin-embedded (FFPE) tumor blocks were retrospectively retrieved from pathology archives at KFMC between 2010 and 2014. These tumor blocks were cut into 3-µm sections, and the availability of representative tumor tissue together with preservation of nucleic acids in the tissue was reconfirmed by an experienced pathologist certified by Saudi Council for Health Specialists. Accordingly, 45 tumor blocks were included in this study because of the following exclusion criteria:

1. The histological sections do not contain representative tumor tissue.
2. The histological sections fail to demonstrate positive staining when DNA positive control probe (PBO682) was used in place of the HPV probe (subtypes 16, 18, 31, and 33).

According to the International Classification of Disease (ICD-10; World Health Organization, 2016), the distribution of specimens by anatomic subsite is shown in Table 1.

### 2.2 | P16-immunohistochemistry

An automated immunostainer (Leica BOND-III) was used to perform P16 immunostaining with antibody anti-P16 (Thermo Scientific, clone 5A8A4, dilution 1 : 3,000, pretreatment: Bond Epitope Retrieval Solution 1/97°C) and Bond Polymer Refine Detection on Leica BOND-III. If staining attains at least 10% of the nucleus and cytoplasm, P16 immunostaining was considered positive; otherwise, P16 was considered negative (Rodrigo et al., 2015).

### 2.3 | HPV—In situ hybridization

HPV-DNA detection by in situ hybridization was completed using Leica HPV probe (subtypes 16, 18, 31, and 33), Catalogue No. PB0829, and Bond Polymer Refine Detection kit (DS9800) on Leica BOND-III. Leica HPV probe was ready to be used; therefore, no further necessities to reconstitute, mix, dilute, or titrate this reagent.

DNA positive control probe (PB0682) was used in place of the HPV probe (subtypes 16, 18, 31, and 33) with a section of each patient's specimen. This was performed to check the preservation of nucleic acids in the tissue as well as accessibility of the probe to these nucleic acids. If the DNA positive control probe did not reveal positive staining, results were considered invalid, and the patient's specimen was excluded from this study. Furthermore, HPV probe (subtypes 16, 18, 31, and 33) was validated using previously confirmed 10 HPV-positive and 10 HPV-negative cervical cancer specimens, which were retrieved from pathology archives at KFMC.

DNA negative control (PB0731) was used in place of the HPV probe (subtypes 16, 18, 31, and 33) with a section of each patient's specimen. This was done to improve interpretation of specific versus nonspecific staining.

### 2.4 | Statistical analysis

Sensitivity analysis was done by taking HPV as confirmatory test (gold standard) for the specimens suspected to be HPV positive in OCSCCs (Table 3).

## 3 | RESULTS

All patients received their treatment at otolaryngology department at KFMC. Twenty-four patients were male with the mean age of 59.3 years, and 21 patients were female with the mean age of 61.2 years. The stage of the tumors was determined according to the classification of TNM system of the International Union Against Cancer (Sobin, Gospodarwicz, & Wittekind, 2009; Table 2).

Forty-one cases were positive for P16 immunostaining, and the remaining four cases were negative. DNA detection for HPV subtypes (16, 18, 31, and 33) by in situ hybridization demonstrates negative staining for all 45 specimens used in the present study (Table 2).

### 3.1 | Statistical analysis

Sensitivity analysis, which was done by taking HPV as confirmatory test (gold standard) for the specimens suspected to be HPV positive in OCSCCs (Table 3), revealed 0% sensitivity, 8.89% specificity, 0% positive predictive values, and 100% negative predictive value (Table 3).

**TABLE 1** Distribution of oral cavity squamous cell carcinomas by anatomic subsite

Squamous cell carcinomas		
Site	Subsite	Total number of specimens
Oral cavity (C02-C06)	Tongue: (C02.0, 1 & C02.2) = 21	45
	Mucosa of upper and lower lips (C00.3, 4) = 1	
	Upper alveolus and gingiva (upper gum) (C03.0) = 4	
	Lower alveolus and gingiva (lower gum) (C03.1) = 4	
	Hard palate (C05.0) = 1	
	Soft palate (C05.1) = 1	
	Cheek mucosa (C06.0) = 11	
	Retromolar areas (C06.2) = 2	

**TABLE 2** Detection of P16 and HPV in oral cavity squamous cell carcinomas

Tumor stage	Oral cavity squamous cell carcinomas				
	P16		HPV		
	Positive	Negative	16, 18, 31, 33	Positive	Negative
I	5	0	0	0	5
II	3	1	0	0	4
III	6	0	0	0	6
IV	27	3	0	0	30
Total	41	4	0	0	45

Note. TP: true positive; FP: false positive; FN: false negative; TN: true negative. Sensitivity = TP/(TP + FN) = 0%. Specificity = TN/(FP + TN) = 8.89%. Positive predictive value (PPV) = TP/(TP + FP) = 0%. Negative predictive value (NPV) = TN/(FN + TN) = 100%.

## 4 | DISCUSSION

Reports have shown that association of HPV with OCSCCs varies significantly from one geographic location to another (Table 4). This heterogeneity has been clearly demonstrated in a recent systematic review by Anantharaman et al. (2017), who reported that HPV16-associated oral cancer is 10.6% in the United States, 6.1% in Europe, and 0% in Brazil. Similarly, another literature review in Asia Pacific (Shaikha, McMillan, & Johnson, 2015) has reported that HPV positivity in oral cancer attained 100% in Japan and Malaysia, 82.7% in Taiwan, 3.1% in Thailand, and 2.9% in Bangladesh.

In the light of the above-mentioned reports, the absence of high-risk HPV subtypes (16, 18, 31, and 33) in OCSCCs among Saudi patients is not a unique phenomenon. It is suggested that this absence might be attributed to social and cultural prohibitions of certain sexual behavior such as oro-genital contact. It is noteworthy to mention that similar suggestion has been previously proposed by Li et al. (2003). The possible implication of this sexual behavior in oral cancer has been supported by many reports showing strong association between sexual behavior and HNSCCs (D'Souza, Agrawal, Halpern, Bodison, & Gillison, 2009; Schwartz et al., 1998; Smith et al., 2004). Unfortunately, sexual behavior data are not available in our patients' file. However, in this regard, it is speculated that the results of our present study might be related to the sexual behavior status of older patients, but not necessarily represent the status of a younger and modern Saudi patients who possibly have different sexual behaviors. Accordingly, it is suggested that further studies with cross section of young and modern Saudi Arabian population are needed.

It is noteworthy to mention that prevalence of HPV-associated oropharyngeal, hypopharyngeal, tonsils, and laryngeal cancers varies largely in the literature, ranging from 0% up to 75% (Duray et al., 2011; Halec et al., 2013; Isayeva, Li, Maswahu, & Brandwein-Gensler,

2012; Kreimer et al., 2005; Li et al., 2003; Ribeiro et al., 2011; Rodrigo et al., 2015; Shaikha et al., 2015; Singhi & Westra, 2010). Therefore, it is suggested that large multicenter studies for HPV-associated different subsets of HNSCCs are needed for better understanding of the existing heterogeneity between different geographic locations.

In the present study, p16 has been used as a predictor for HPV status because previous reports have shown that p16 overexpression is associated with HPV infection in oropharyngeal squamous cell carcinoma and malignant oral lesions (Fregonesi et al., 2003). However, our results have shown that majority of OCSCCs specimens (91%) was positive for p16 immunostaining with complete absence (100%) of HPV subtypes (16, 18, 31, and 33). This outcome is in line with a recent report by Belobrov et al. (2017) who demonstrated that p16 is rarely associated with HPV-positive OCSCCs, and its overexpression might be related to nonviral mechanisms. In this regard, it has been suggested by Witkiewicz, Knudsen, Dicker, and Knudsen (2011) that p16 expression in the absence of HPV-DNA detection might be due to RB signaling pathway disturbance, which is not related to HPV infection, such as in the case of lymphoma and small cell lung cancer. However, further studies are required for confirmation.

The type of biopsy specimens (FFPE blocks) or the technique (in situ hybridization) used in the present study is not likely to affect the results, because previous reports have shown that using paraffin-embedded versus fresh frozen biopsies (Kreimer et al., 2005), or PCR versus in situ hybridization techniques (Mehanna et al., 2013), did not alter the positivity of HPV-associated HNSCCs. However, it is suggested that further comparative studies using different types of biopsy specimens in combination with various analytical approach such as microarray, real-time PCR, and in situ hybridization techniques are needed for confirmation.

P16 immunohistochemistry together with in situ hybridization technique is commonly used by pathologists for HPV-DNA detection (Klingenberg et al., 2010; Kumar et al., 2008; Licitra et al., 2006). However, DNA degradation when using formalin fixation and paraffin-embedded materials might be of concern because detection rates of larger HPV-DNA fragments in FFPE samples have been found to be less when compared with shorter DNA segments (Baay et al., 1996); therefore, in our study, preservation of nucleic acids in the tissue as well as accessibility of the probe to these nucleic acids has been tested using DNA positive control probe (PBO682). It is noteworthy to

**TABLE 3** Sensitivity analysis

P16	HPV		Total
	16, 18, 31, 33	Positive	
Positive	0 (TP)	41 (FP)	41
Negative	0 (FN)	4 (TN)	4
Total	0	45	45

**TABLE 4** Prevalence of HPV positivity in oral cancer by country/region

PubMed ID	First author	Country/region	Number of oral cancer cases	Overall HPV positivity (%)	HPV genotypes
28108990	Anantharaman D. et al.	Brazil	107	0	16
23617206	Akhter M. et al.	Bangladesh	34	2.9	16, 18, 6, 11
19256774	Khovidhunkit S.O. et al.	Thailand	32	3.1	16
28108990	Anantharaman D. et al.	Europe	165	6.1	16
28108990	Anantharaman D. et al.	USA	123	10.6	16
28612284	Qatouseh L.A. et al.	Jordan	65	21.5	16,18
12190813	Chen P.C. et al.	Taiwan	29	82.7	16, 18, 6, 11
17181737	Koyama K. et al.	Japan	13	100	16, 18, 22, 38, 70
17487385	Lim K.P. et al.	Malaysia	20	100	16, 18

mention that the newest HPV detection technique such as RNAscope™ HPV test might be promising (Mirghani et al., 2014), but further research still required regarding this issue.

It has been reported that the mean age of oral cancer in Saudi Arabia is 62 years with the highest burden in Jizan and Najran (Brown, Ravichandran, & Warnakulasuriya, 2006). Furthermore, a recent report from eight different regions in southern Saudi Arabia has shown that 33% of oral cancer patients were at the age group of >70 years, 44.7% between 51 and 70 years, 16% between 31 and 50 years, 4.3% between 10 and 30 years, and 2.1% of <10 years (Hesham et al., 2017). These later data showed that the burden of oral cancer among young Saudi patients is not large. However, the increased incidence of HPV infection in Saudi Arabia from 3.5% to 5.8% (Memish, Filemban, Al-Hakeem, Hassan, & Al-Tawfiq, 2016) suggests the need to establish a preventive educational programs regarding sexually transmitted infections.

It is noteworthy to mention that there are only few studies dealing with risk factors of head and neck cancer in Saudi Arabia, but cigarette smoking, shisha, betel nuts, khat, and smokeless tobacco such as shamma are considered common risk factors for head and neck cancer occurrence in this country (Alhazzazi & Alghamdi, 2016). The habits of chewing tobacco and khat are likely to be associated with higher rate of oral cancer (Sawair et al., 2007). Furthermore, shamma, which is frequently used among oral cancer patients in the southern province of Saudi Arabia (Allard, DeVol, & Te, 1999; Ibrahim et al., 1986; Quadri et al., 2015), was found to increase the incidence of oral cancer by 29-fold (Quadri et al., 2015).

In the light of the above-mentioned findings, it is suggested that prevention of oral cancer in this country should combine educational programs about sexually transmitted infections and tobacco cessation.

To our knowledge, this is the first study in Saudi Arabia, which report that high-risk HPV (16, 18, 31, and 33) appears not to be involved in the etiology of different subsets of OSCCs in older Saudi patients. However, due to the selected sample of the present study, further investigations using large sample size with cross section of a younger age group are still required.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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