

## Heterogeneity: A Stumbling Block in the Expression of Human Papillomaviruses 16 in Oral Squamous Cell Carcinoma

### Abstract

**Background:** Tobacco and smoking are the established risk factors for oral cancer, but studies have reported the occurrence of oral cancer, even in the absence of these established factors. This indicates the need to identify other possible risk factors. In recent years, viral carcinogens have gained interest in which human papillomaviruses (HPVs) are the most researched risk factor in oral carcinogenesis. Therefore, we conducted this study to evaluate the presence of HPV-16 in potentially malignant and malignant lesions of the oral cavity. **Methods:** The current study comprised 40 patients, and they were divided into two groups: Group A: 20 patients with histopathologically diagnosed oral potentially malignant disorders (leukoplakia/erythroplakia) and Group B: 20 patients with histopathologically diagnosed oral squamous cell carcinoma (OSCC). Tissue samples were collected from all patients and made into formalin-fixed paraffin-embedded (FFPE) blocks. Polymerase chain reaction (PCR) test was carried out to detect the presence of HPV-16. **Results:** In our study, we found that none of the FFPE tissue samples was positive for the HPV-16 genome using the conventional PCR technique. The level of significance ( $P$  value) was set at  $P < 0.05$ . **Conclusion:** The absence of HPV-16 in OSCC could possibly due to variation in the geographical location and anatomical subsites, ethnicity, and host serological response.

**Keywords:** Human papilloma virus 16, oral potentially malignant disorders, oral squamous cell carcinoma, polymerase chain reaction

### Introduction

Head and neck squamous cell carcinoma (HNSCC) causes cancer morbidity worldwide, with 650,000 new cases and 350,000 deaths occurring every year.<sup>[1]</sup> HNSCC encompasses tumors of the oropharynx, oral cavity, larynx, and hypopharynx; these are associated with different risk factors and prognosis.<sup>[1]</sup> Within the head-and-neck region, the oral cavity is the most frequent site of cancer. Smoke or smokeless tobacco and alcohol consumption are the main risk factors in the etiology of oral squamous cell carcinoma (OSCC). The occurrence of OSCC in patients who have never or rarely been exposed to this well-established risk factor paves the way to research the role of viral oncogenes, namely the human papillomaviruses (HPV).<sup>[2-4]</sup>

Papillomaviruses are one of the oldest known viruses, dating back 330 million years. During this long evolution, HPV

have developed into hijackers of human cellular and immune systems in which they replicate and remain silent.<sup>[4]</sup> HPV infection is considered the most common sexually transmitted infection.<sup>[5]</sup> The etiologic role of HPV infection in the development of squamous cell carcinomas (SCCs) of the uterine cervix has been widely demonstrated. There is also strong evidence to support the role of these viruses in the etiopathogenesis of SCC in the oral cavity.<sup>[6]</sup>

In 2007, the International Agency for the Research on Cancer recognized HPV type 16 (HPV-16) as the only carcinogenic type of HPV in sites other than the cervix uteri, including the anus, penis, vagina, vulva, oral cavity, and oropharynx.<sup>[7]</sup> HPV16-associated carcinogenesis is mediated by the expression of the viral E6 and E7 oncoproteins, which inactivate the tumor-suppressor proteins p53 and retinoblastoma: This then disrupts cell cycle regulatory pathways.<sup>[8]</sup> Therefore, the lack of p53 mutations and p16 protein accumulation,<sup>[9-12]</sup> which occur as a result of the loss of transcriptional repression

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during early tumorigenesis are considered to be hallmarks of HPV-related HNSCC. Numerous studies support the involvement of high-risk HPV in the samples of HNSCC. However, considerable heterogeneity exists in the detection rates, and fewer studies have been conducted to determine the frequency of HPV DNA exclusively in SCC of the oral cavity. The aim of this study was to evaluate the presence of high-risk HPV-16 in OSCC.

## Methods

### Patient eligibility criteria

This comparative study was undertaken in 40 consecutive patients with clinically and histologically diagnosed leukoplakia/erythroplakia and OSCC. Patients were divided into two groups: Group A: 20 patients with clinically and histologically diagnosed leukoplakia/erythroplakia and Group B: 20 patients with clinically and histologically diagnosed OSCC. Formalin-fixed tissue samples, embedded in paraffin blocks (FFPE), were collected from all patients. Ethical committee clearance was obtained from the Institutional Ethical Committee. Written informed consent was obtained from all patients who volunteered to participate in the study. The patients for the study were selected on the basis of inclusion and exclusion criteria. The inclusion criteria were as follows: patients of either gender within the age range of 18–80 years, patients with clinically or histologically diagnosed leukoplakia/erythroplakia, and OSCC irrespective of their stage, patients with or without a history of habits (smoking/drinking/tobacco chewing) as per Herrero's definition.<sup>[13]</sup> A smoker is defined as a subject who reported having smoked tobacco daily for at least 1 year. Participants were asked about the duration of smoking, amount, and type of tobacco smoked (cigarettes, cigars, or pipes). A drinker was defined as a subject who reported drinking alcoholic beverages at least once a month. Details were obtained on the type of beverage, amount, and duration were included in the study. The exclusion criteria were as follows: patients with any other oral lesions other than OSCC, patients who have undergone or undergoing radiation therapy and/or chemotherapy to the head and neck region, patients with any other existing concomitant lesions, and patients with any secondary carcinoma.

### Procedure details

The area to be biopsied was selected and prepared using sterile swabs. Local anesthetic (2% lignocaine hydrochloride with adrenaline 1:2,00,000 concentration) was administered to the biopsy target zone. Traction sutures or tissue forceps were used to fix the tissue to be removed. The specimen was obtained by means of a clean and deep cut. The incision included a significant portion of the suspect tissue and also a part of the adjacent normal tissue. The wound margins were subjected to debridement, with control of bleeding, and the lips of the wound were joined with a 3'0 silk suture. The obtained sample washed

with physiological saline<sup>[14]</sup> and was placed in 10% formalin solution until histopathological evaluation.<sup>[2]</sup> The formalin-fixed paraffin-embedded tissue sections were adequate in size and represented the lesion.

### DNA extraction

HPV determination was done by using the conventional polymerase chain reaction (PCR) technique [Figure 1]. For this purpose, first DNA extraction was done from 5 µm-thick sections of paraffin block. Sections were placed in a microfuge tube and 100 µl of resuspension buffer (Tris [Himedia]), EDTA (Merck), triton X-100 (Himedia), and 100 µl of lysis buffer was added. Tubes were boiled at 97°C for 30 min to melt the paraffin and then were centrifuged at 14,000 xg for 10 min. The thin upper layer formed by paraffin was removed. The tube was left to cool, 5 µl of proteinase K (Merck) was added (final concentration of 600 µg/ml), and tubes were incubated at 65°C for 1 h. 200 µl of precipitation solution was added, and the entire solution was transferred to spin columns embedded with collection tubes. 500 µl of wash buffer was added to the spin column assembly. The tubes were centrifuged at 10,000 rpm for 2 min, and flow-through was discarded. The steps were repeated and centrifuged at 10,000 rpm for 2 min to dry the spin cup. The spin cups were placed into 1 ml sterile microcentrifuge tube, and 30 µl of elution buffer was added to the spin cup. The tubes were centrifuged at 10,000 rpm for 5 min. The eluted DNA was stored in -20°C.

### Confirmation of the extracted DNA

To confirm the presence of DNA for gene amplification, human interleukin-1 β gene was amplified with primers whose amplification product is 512 bp. Positive human interleukin1 β gene amplification proved firstly, that the

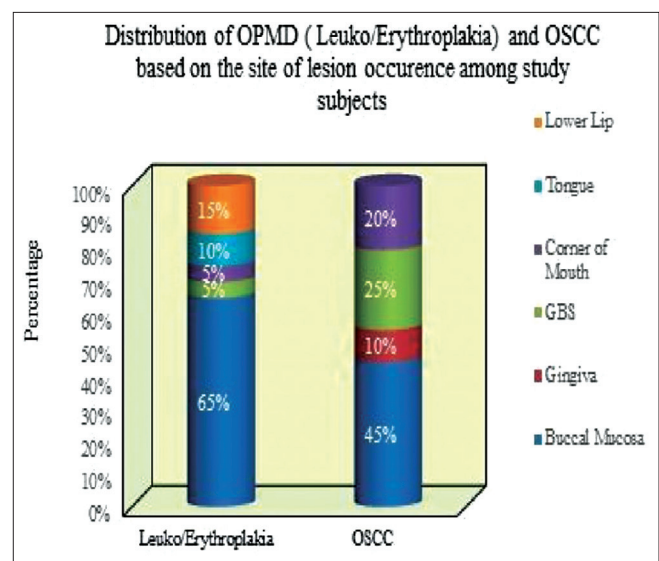


Figure 1: Demonstrates the distribution of oral potentially malignant disorders and oral squamous cell carcinoma based on the site of lesion occurrence among study participants

sample contained enough DNA. Second, it suggested that PCR inhibitors are not present. The reaction mixture was carried out in a total volume of 25 µl, of which 30 ng of synthesized DNA, optimum temperature was confirmed as 60°C for all the primers to give a 512 bp amplicon product. 2X PCR Master Mix (12.5 µl), DNA Template (1 µl), forward primer (10 pM) 2.5 µl, reverse primer (10 pM) 2.5 µl nuclease-free H<sub>2</sub>O 6.5 µl were added into a sterile 0.2 ml PCR tube on ice.

To test for any contamination, a negative control was included in all batches, consisting of the reaction mixture and MilliQ water. Thermocycler (G STORM) parameters were 5 min at 95°C, annealing of 51°C for 2 min with a final extension of 10 min at 72°C for 30 cycles. Products were analyzed by 2% agarose gel electrophoresis and visualized with ethyl bromide staining. In addition, plasmid-containing HPV-16 was used as positive controls and to estimate the sensitivity of the assay. These positive controls were obtained from the cervix which was highly sensitive.

### Human papillomaviruses determination

For the determination of HPV-16 DNA, primers were used. The following are the details of the primer: HPV16 DNA Forward Primer-5'ACCCAGTATAGCTGACAGT3:HPV16 DNA Reverse Primer-5'CTCGTTTATAATGTCTACACA3' A gradient PCR (G Storm) was performed to standardize the optimum annealing temperature of the designed primer using 30 Nano gram (ng) of synthesized DNA in the temperature range of 50°C–60°C. The optimum temperature was confirmed as 60°C for all primers to give a 512 bp amplicon product. 2X PCR Master Mix (12.5 µl), DNA Template (1 µl), forward primer (10 pM) 2.5 µl, reverse primer (10 pM) 2.5 µl nuclease-free H<sub>2</sub>O 6.5 µl were added into a sterile 0.2 ml PCR tube on ice (25 µl reaction volume). Only positive controls amplified HPV DNA; none of the samples in OSCC and oral potentially malignant disorders (OPMD) groups showed HPV DNA.

### Statistical analysis

Statistical analysis was performed using the software SPSS GPower v. 3.1.9.2 for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp. Descriptive statistics were done which included the expression of the study variables with categorical data in terms of frequency and percentage. The effect size to be measured was considered at 80%. The other parameters considered for sample size calculation were the power of the study at 80% and the margin of the error at 0.10%. As per the above-mentioned parameters, the total sample size needed was 40. Hence, each study group comprised 20 samples.

### Results

In the current study, the mean age of the 20 patients in OPMD groups was  $46.4 \pm 13.1$  years. Of these 20

participants, 11 (55%) were men and 9 (45%) were women. In OSCC groups, the mean age of the 20 patients was  $54.4 \pm 9.8$  years. Of these 20 participants, 6 (30%) were men and 14 (70%) were women. The age of patients in OPMD group ranged from 21 to 72 years and in OSCC ranged from 30 to 69 years [Table 1].

Table 2 demonstrates that all the patients in both OPMD and OSCC groups were having tobacco habits (100%), and there is no significant difference with respect to tobacco habits between two groups. The patients in OPMD group were more alcoholic (55%) as compared to OSCC group (30%). However, this difference with respect to alcohol habit was not statistically significant between the two groups ( $P = 0.11$ ) [Table 2].

Figure 1 demonstrates the distribution of participants in OPMD and OSCC based on the site of occurrence of the lesion. Most of the lesions in both the groups were present in buccal mucosa 65% in OPMD and 45% in OSCC followed by the labial mucosa 15% in OPMD and gingivobuccal sulcus (GBS) 25% in OSCC group. In OPMD group, 10% of lesions were present in anterior 2/3<sup>rd</sup> of the tongue and the rest 5% in GBS and 5% in corner of the mouth. However, in OSCC group, 20% was found in the corner of the mouth, and the rest 10% of the lesion was present in the gingiva [Figure 1].

Table 3 demonstrates the distribution of clinical types of OPMD and OSCC among the study participants. 90% ( $n = 18$ ) of the patients from OPMD group had leukoplakia, whereas only 10% ( $n = 2$ ) had erythroplakia. Most of the study participants from OSCC group were having well-differentiated squamous cell carcinoma 70% ( $n = 14$ ) while the remaining 30% ( $n = 6$ ) were having moderately differentiated squamous cell carcinoma [Table 3].

Moreover, in the current study, 45% patients in OPMD group showed dysplastic features, whereas the remaining 55% showed no dysplastic changes. Table 4 demonstrates the distribution of OPMD cases based on the histopathological diagnosis. 36.4% of the cases showed parakeratosis, whereas hyperorthokeratosis was shown by only 9% of cases. Hyperkeratosis with dysplasia was seen in 11.1% of cases, while hyperparakeratosis with mild

**Table 1: Demonstrates the mean age and gender distribution among the two study groups**

Age and gender distribution among two study groups (mean and SD)				
Variable	Category	OPMD	OSCC	P
Age	Mean and SD	46.4 (13.1)	54.4 (9.8)	0.03* <sup>a</sup>
	Range	21-72	30-69	
Gender, <i>n</i> (%)	Men	11 (55)	6 (30)	0.11 <sup>b</sup>
	Women	9 (45)	14 (70)	

OPMD: Oral potentially malignant disorders, OSCC: Oral squamous cell carcinoma, SD: Standard deviation

**Table 2: Demonstrates the distribution of habit characteristics among oral potentially malignant disorders and oral squamous cell carcinoma groups**

Distribution of habit characteristics among OPMD and OSCC groups						
Variables	Category	OPMD, n (%)	OSCC, n (%)	Total, n (%)	$\chi^2$	P
Tobacco	Yes	20 (100)	20 (100)	40 (100.0)	0	0
	No	0 (0)	0 (0)	0 (0.0)		
Alcohol	Yes	11 (55)	6 (30)	17 (42.5)		
	No	9 (45)	14 (70)	23 (57.5)		

OPMD: Oral potentially malignant disorders, OSCC: Oral squamous cell carcinoma

**Table 3: Depicts the distribution of clinical types of oral potentially malignant disorders and oral squamous cell carcinoma among the study groups**

Groups	Clinical diagnosis	n (%)
OPMD	Leukoplakia	18 (90)
	Erythroplakia	2 (10)
OSCC	Well-differentiated OSCC	14 (70)
	Moderately differentiated OSCC	6 (30)

OPMD: Oral potentially malignant disorders, OSCC: Oral squamous cell carcinoma

**Table 4: Depicts the distribution of oral potentially malignant disorders cases based on the histopathological diagnosis**

Variables	Categories	n (%)
Histological diagnosis	Without dysplasia	55
	Hyperkeratosis	3 (27.3)
	Hyperparakeratosis	3 (27.3)
	Hyperorthokeratosis	1 (9.0)
	Parakeratosis	4 (36.4)
	With dysplasia	45
	Leukoplakia	1 (11.1)
	Erythroplakia	2 (22.2)
	HK + mild dysplasia	1 (11.1)
	HPK + mild dysplasia	3 (33.4)
	HPK + moderately dysplasia	2 (22.2)

HPK: Hyperparakeratosis, HK: Hyperkeratosis

dysplasia and hyperparakeratosis with moderate dysplasia was seen in 33.4% and 22.2% cases, respectively [Table 4].

The results of the current study suggested the absence of the expression of HPV-16 genome among OPMD and OSCC groups. This indicates that all patients in both the groups were not at the risk for the development of oral cancer associated with HPV infection.

## Discussion

The main risk factors of oral carcinogenesis are tobacco, smoking, and alcohol consumption. Recent reports have shown the occurrence of oral cancer even in the absence of these causative factors, so further researches were done to investigate the relationship between HPV in oral cancer.<sup>[3,4]</sup> Besides, it has been shown that high-risk genotype 16

is mostly associated with oral cancer.<sup>[14]</sup> HPV16 accounts for 90% of the HPV DNA-positive cases in HNSCC, followed by HPV18 and other high-risk subtypes.<sup>[2]</sup> Genotype 16 has been detected much more frequently in most studies<sup>[15-18]</sup> and some authors have proposed it as a possible etiopathogenic factor in the early stages of oral carcinogenesis.<sup>[19]</sup> Therefore, the current study aimed to detect the presence of HPV-16 in oral potential malignant disorders [OPMDs] and OSCC. However, the presence of HPV-16 was not detected in any of the study groups. This can be attributed to the following reasons: Anatomical subsites, detection method, and geographic location.<sup>[15]</sup> Moreover, studies suggest that the separation of oral cancer from the heterogeneous group of HNSCC is essential to gain valid results of HPV prevalence in oral cancer.<sup>[20]</sup> However, the current study excluded oropharyngeal cancer, and only oral cavity cancers were included in detecting the presence of HPV. Furthermore, a closer inspection of the sub-anatomic sites revealed that almost half of the HPV16-positive oral cancers have been potentially misclassified.<sup>[20]</sup> As per the International Classification of Diseases to Dentistry and Stomatology, third edition (ICD DA) oral cavity and oropharyngeal cancer are different entities and are given specific codes.<sup>[21]</sup> The current study included the lesions of the labial mucosa (C00.3X), gums (C03), dorsal surface of the tongue (C02.0), border of the tongue (C02.1), ventral surface of the tongue (C02.2) anterior two-thirds of the tongue (C02.3), buccal mucosa, and vestibule of the mouth (C06) Oropharyngeal cancer was coded as C10. Oropharyngeal cancers have the highest prevalence in HPV-16 worldwide, but most of the studies do not separate them from the tongue SCC of the oral cavity.<sup>[22]</sup> However, the current study showed the absence of HPV-16, this could be first due to the absence of true positive HPV16 in oral cavity cancer.<sup>[21]</sup> Second due to the constant salivary flow and its cleaning ability along with the mobile nature of the oral cavity shows the lower detection rate of HPV 16.<sup>[23,24]</sup> In contrast, a study conducted by Chowdary *et al.*<sup>[25]</sup> showed the presence of HPV in 55% OSCC cases, especially in those samples that were taken from the posterior-most areas of the oral cavity. A study conducted by D' Costa *et al.*<sup>[26]</sup> also reported HPV-16 positivity in 15% of OSCC, 34% of OPMD, and 15% of participants with normal mucosa. The results suggested that HPV-16 infection may play a crucial role in the early events associated with the development of OPMD.

However, its role in the progression of the disease to OSCC is unclear. The prevalence of HPV and its progression to malignancy would be the highest with the presence of other carcinogenic agents.

According to various studies, the prevalence of HPV in OSCC is 100% in Japan/Malaysia, 82.7% in Taiwan, 21.5% in Jordan 10.6% in the USA, 6.1%, in Europe, 3.1% in Thailand, 2.9% in Bangladesh, and 0% in Brazil. Another study from Latin America to Central Europe also showed the absence of HPV.<sup>[26]</sup> The current study also showed the absence of HPV; this could be probably due to the limited sample size and small geographical areas. However, further studies with larger sample size and border geographical area would validate this particular observation.

Several methods are available for the detection of HPV in OSCC patients. Among them, *in situ* hybridization, immunohistochemistry assays, south blot hybridization, and PCR tests are the most used method of detection.<sup>[2]</sup> A study done by Awan *et al.*<sup>[27]</sup> reported that firstly, that conventional PCR is more sensitive than immunohistochemistry for the detection of HPV. Second, PCR differentiates the HPV subtypes detecting type 16 in majority of patients 57.4% and type 18 in only 2.1%. Third, PCR can be performed in one day, easily regulated and more practical and therefore, considered as highly sensitive.<sup>[27]</sup> Other molecular techniques such as RNA *in situ* hybridization or E6-E7 genes detection of high-risk HPV are more indicative of viral integration and E6 and E7 genes expression. However, in the current study carrying out these molecular techniques within the limited time frame was quite challenging. In the majority of cases, the accurate identification of HPV relies on molecular biology techniques. HPV has a double-stranded DNA genome approximately 8000 base pairs with a well-known gene organization and physical structure. Therefore, the test of choice for the detection of HPV in clinical specimens is based on nucleic probe technology (PCR). PCR-based techniques are highly sensitive, specific, and widely used. In conventional PCR, the thermostable DNA polymerase recognizes and extends a pair of oligonucleotide primers that flank the region of interest. In the final process, they can generate one billion copies of a single double-stranded DNA molecule after 30 cycles of amplification.<sup>[28]</sup> Hence, in the current study, PCR was selected as the method for the detection of HPV 16 in both OPMD and OSCC. However, the results of the current study showed the absence of HPV in both the groups. A study conducted by Akhter *et al.* used PCR test to detect the presence of HPV. However, the results showed the absence of HPV in patients with OSCC.<sup>[24]</sup> Similarly, a study conducted by Deepa R also showed that out of the sixty histopathologically confirmed OSCC samples, none (0%) were positive for HPV-16.<sup>[29]</sup> Therefore, the current study suggests that despite using standard detection techniques, the results showed the absence of HPV 16 in both OPMD and OSCC groups. This could be possibly

due to other factors such as the geographical location, anatomical subsites, social and cultural prohibitions of certain sexual practices such as “oral sex” which is found to be the main cause of the HPV-associated oral carcinoma, and this is believed to be less common in our community compared to Western countries.

Studies suggest that using fresh-frozen tissues (FFPE) are more reliable and accurate for HPV DNA detection than FFPE tissues.<sup>[30]</sup> However, the current study used paraffin-embedded tissue block to perform the PCR test for the HPV detection. This could also be one of the causes for the absence of HPV in both the groups. Further researches with larger sample size using highly sensitive and specific diagnostic modalities should be carried out to eliminate the stumbling block in the expression of HPV-16 in OSCC. Moreover, studies should be carried out to find the association of HPV with the ethnicity, sexual behavior, and the host serological response. This would validate the absence of HPV-16 in both the groups.

## Conclusion

The results of this study showed a complete absence of HPV in OSCC. This shows that the presence of HPV-16 varies concerning to the heterogeneity, ethnicity, anatomic subsites, and host response. Hence, there is a significant relationship between HPV-16 expression and heterogeneity opens the scope of further studies in this area.

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## Conflicts of interest

There are no conflicts of interest.

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