Original Article

# A study to assess expression of human papillomavirus types 16 and 18 in oral squamous cell carcinoma using polymerase chain reaction

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## Abstract

**Objective:** The diverse subset of oral squamous cell carcinoma (OSCC) with different clinical appearance and outcome, independent of traditional risk factors has led to increasing attention in human papillomavirus (HPV) infection.

**Materials and Methods:** The investigation followed a case–control design. Information pertaining to the subjects was retrieved from hospital records. Twenty cases of OSCC and twenty age-matched controls were analyzed to ascertain the prevalence of HPV types 16 and 18. DNA was extracted from the blocks of formalin-fixed paraffin embedded tissues, and HPV-DNA was amplified using HPV type-specific primers by polymerase chain reaction (PCR) method. Data analysis was carried out using Chi-square test.

**Results:** HPV-DNA was detected in 55% of cases (11/20; HPV 16 = 6, HPV 18 = 3 and HPV 16 and 18 = 2) and 30% of controls (6/20; HPV 16 = 3, HPV 18 = 1 and HPV 16 and 18 = 2) indicating higher percentage of HPV presence among OSCC cases. No significant association was found between the presence of HPV and gender, age, site and grade of differentiation of OSCC.

**Conclusion:** Although the presence of HPV was higher in cases compared to controls, none of these differences were statistically significant. HPV 16 and 18 are commonly found in normal oral mucosa mandating the need for distinguishing clinical, subclinical and latent HPV infections.

**Keywords:** Human papillomavirus types 16 and 18, oral squamous cell carcinoma, polymerase chain reaction

## INTRODUCTION

Head and neck cancer is one of the ten most common types of cancer worldwide distressing more than 500,000 individuals every year.[1]

Oral squamous cell carcinoma (OSCC) is most frequent accounting for over 90% of oral cancers. It represents the sixth most frequent malignant tumor worldwide.[2]

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Tobacco, smokeless tobacco products such as gutka, pan masala, betel quid and alcohol are the risk factors for the development of OSCC.[3]

Viruses such as human papillomavirus (HPV), Epstein–Barr virus and herpes simplex virus-1 (HSV-1) are also implicated to play a role in the development of OSCC.[4]

HPVs are small DNA viruses infecting various human epithelial tissues. More than 130 HPV types have been identified and are classified into low- or high-risk groups based on their oncogenic potential.[5]

HPV gives rise to a distinct clinical entity of oropharyngeal squamous-cell carcinomas (OPSCCs) with a considerably better prognosis than HPV-negative OPSCC, often related to tobacco and alcohol consumption.[6]

There is a growing evidence of causal association of high-risk HPV types mainly HPV 16/18 and OSCC. A number of studies have shown that HPV is associated with increased risk of oral cancer, independent of exposure to tobacco and alcohol. This association is valid for HPV 16 and 18 because of its detection in oral dysplastic lesions and oral cancers.[7]

The nature of the relationship between HPVs and OSCC remains unclear owing to difficulties with interpreting studies that have demonstrated prevalence rates ranging from 0% to 100%. The diverse populations and assays with varying degrees of sensitivity for detecting viral DNA also make interpretation difficult.[8]

HPV is a sexually transmitted infection, and findings suggest that the number of lifetime sexual partners is an important risk factor for the development of HPV-associated head and neck SCC. In case–control studies, the odds of HPV-positive malignant disease increased 2-fold in individuals who reported between one and five-lifetime oral sexual partners and 5-fold in those with six or more, compared with those recalling no oral sex.[9]

A wide range of variation has been noticed in HPV positivity rates in cancers at different sites in head and neck region. Highest rates being reported in tonsillar region followed by cancers of tongue and buccal mucosa.[10]

A meta-analysis including 94 studies on HPV presence in oral mucosa showed that oral dysplasia and OSCC are more commonly associated with HPV infection particularly subtypes 16 and 18 compared to that of normal oral mucosa (NOM).[11]

The field of human cancer research has been advanced with the application of highly sensitive molecular biology tools such as polymerase chain reaction (PCR) which permits virus detection soon after infection and even before the onset of disease. The purpose of the current study is to assess the prevalence of HPV in OSCC.

## MATERIALS AND METHODS

### Source of data
In the present study, tissues were collected from clinically suspected patients of OSCC who attended the Department of Oral Pathology and Microbiology. Tissues from retromolar area were collected from patients who underwent surgery for impactions and used as controls after approval from the Institutional Ethics Committee. The study consisted of 40 samples categorized into two groups; twenty cases of OSCC and twenty age-matched controls.

### Methodology
Part of the tissue was processed and sections were stained and examined for routine hematoxylin and eosin to confirm the diagnosis. From remaining part of the histologically proven tissues, DNA extraction was done and subjected to PCR for the evaluation of HPV-positive samples.

### Collection of sample
Specimens were collected from both OSCC patients and controls. After obtaining, tissues were kept in a small ziplock bag for immersing into liquid nitrogen then stored at −20° until use.

### DNA extraction procedure from fresh tissue samples
Tissues collected were subjected to dehydration by the addition of 1 ml of alcohol for 30 min then the mixture is centrifuged, and the supernatant is discarded. Later, the pellet was suspended in 500 µl TE buffer and Vortexed. Then centrifuged at 10,000 rpm for 5 min and supernatant was discarded and washed with fresh TE buffer for 2–3 times. Supernatant was discarded and 50 µl lysis buffer I was added, Vortexed and kept for 5 min. Later, 50 µl Lysis buffer II was added along with 10 µl Proteinase–K (10 mg/ml), vortexed vigorously. Kept in water bath at 60°C for 2 h. Then, kept in boiling water bath for 10 min for enzyme deactivation. The supernatant containing DNA was taken to fresh tube and stored at −20°C. Amplification was done by conventional PCR using HPV 16 and 18 primers [Table 1].

### Polymerase chain reaction procedure
The detection of HPV 16 and HPV 18 was carried out in two separate reactions for each sample. The reaction mixture preparation steps for PCR are as follows:

- Gently vortexed and briefly centrifuge PCR master mix after thawing
- A thin-walled PCR tube is placed on ice, and the following components are added for each 50 µl reaction
- A premixture was prepared and aliquoted into each tube. The premix contains following components in a final volume of 20 µl/aliquot
- The samples are gently vortexed and spinned down
- Then tubes are placed in conventional thermal cycler (Applied Biosystems, USA).

## The polymerase chain reaction conditions were as follows

Initial denaturation was carried out at 95°C for 5 min. Denaturation, annealing and extension were carried out at temperatures of 95°C, 53°C and 72°C, respectively, for 1 min and extension over a period of 2 min. Final extension was done at 72°C for 5 min.

The amplified products were run on 2% agarose gel electrophoresis for detection of HPV 16 and HPV 18 specific bands. The gel for HPV 16 and HPV 18 reactions was run separately. Then, the photo of gel under ultralight light transilluminator was taken, and the bands were recorded using Gel documentation system (Major Science, USA).

Amplicon size of 120 base pair corresponds to HPV 16. Rest other bands were considered as nonspecific [Figure 1]. Amplicon size of 100 base pair corresponds to HPV 18. Rest other bands were considered as nonspecific [Figure 2].

## Statistical analysis

The collected data were entered into the excel sheet, and statistical analysis was done using software, Statistical Package for Social Sciences (SPSS) version 20.0. Comparison of two groups with respect to HPV 16, 18 and 16 and 18 positivity was done by Chi-square test.

## RESULTS

A total of 20 OSCC cases and 20 controls were included in the study. Distribution of age among study and control groups was done at an intervals of ten from 20 to 60 years. Pertaining to gender, controls consisted of 10 males and 10 females, and cases consisted of 12 males and 8 females.

HPV-DNA was detected in 11 out of 20 cases and 6 out of 20 controls indicating a higher percentage of HPV presence among OSCC cases. Statistical analysis was performed using Chi-square test, and the difference was not statistically significant ($P = 0.110$). HPV 16 positive status among cases and controls was 6/20 and 3/20, respectively. HPV 18 positive status was 3/20 among cases and 1/20 among controls. HPV 16 and 18 positivity was noticed in 2 cases and 2 controls. However, this difference was not statistically significant ($P = 0.378$).

No significant association was found between the presence of HPV and gender and age [Table 2], site and grade of differentiation of OSCC [Table 3].

**Table 1: Primer sequences for human papilloma virus 16 and 18**

| HPV type | Primer sequence |
|---|---|
| HPV 16 | Forward primer: 5'- TCA AAA GCC ACT GTG TCC TG-3' |
| | Reverse primer: 5' - CGT GTT CTT GAT GAT CTG CA-3' |
| HPV 18 | Forward primer: 5'- ACC TTA ATG AAA AAC GAC GA-3' |
| | Reverse primer: 5' - CGT CGT TGG AGT CGT TCC TG-3' |

HPV: Human papilloma virus
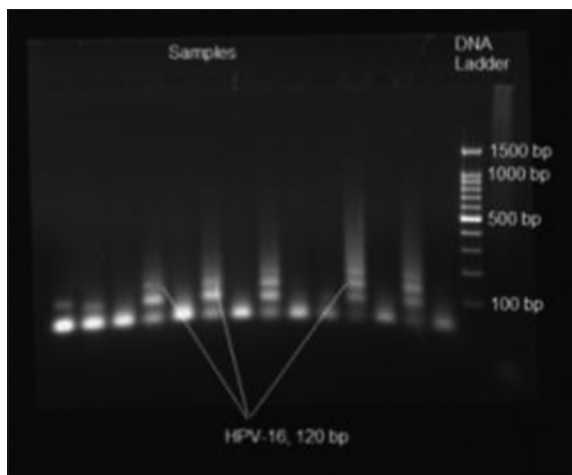


**Figure1:** Human papilloma virus 16 -specific primer mediated polymerase chain reaction of DNA extracted from oral squamous cell carcinomas. Polymerase chain reaction products shown after gel electrophoresis
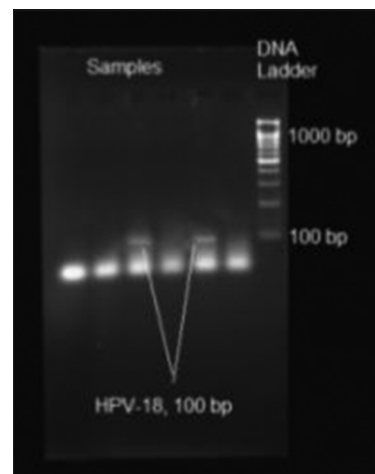


**Figure 2:** Human papilloma virus 18 -specific primer mediated polymerase chain reaction of DNA extracted from Oral squamous cell carcinomas. Polymerase chain reaction products shown after gel electrophoresis

**Table 2: Comparison of human papilloma virus prevalence among cases in relation to gender and age**

| Parameters | Total | HPV 16 | HPV 18 | Both | None | $\chi^2$ | df | P |
|---|---|---|---|---|---|---|---|---|
| Gender | | | | | | | | |
| Males | 12 | 5 | 1 | 1 | 5 | 2.407 | 3 | 0.492** |
| Females | 8 | 1 | 2 | 1 | 4 | | | |
| Age (years) | | | | | | | | |
| 20-30 | 3 | 1 | 1 | 0 | 1 | 8.519 | 9 | 0.483** |
| 31-40 | 2 | 1 | 0 | 0 | 1 | | | |
| 41-50 | 5 | 1 | 0 | 2 | 2 | | | |
| 51-60 | 10 | 3 | 2 | 0 | 5 | | | |

Chi-square test, *P*<0.05 (S), **P*>0.05 (NS). HPV: Human papilloma virus, S: Significant, NS: Not significant

**Table 3: Comparison of human papilloma virus prevalence among cases in relation to site and grade of differentiation**

| Parameters | Total | HPV 16 | HPV 18 | Both | None | $\chi^2$ | df | P |
|---|---|---|---|---|---|---|---|---|
| Site | | | | | | | | |
| Posterior most area | 9 | 1 | 1 | 2 | 5 | 6.617 | 9 | 0.677** |
| Buccal mucosa | 5 | 2 | 1 | 0 | 2 | | | |
| Tongue | 5 | 2 | 1 | 0 | 2 | | | |
| Lower anterior area | 1 | 1 | 0 | 0 | 0 | | | |
| Grade of differentiation | | | | | | | | |
| Well differentiated | 17 | 6 | 2 | 2 | 7 | 8.497 | 6 | 0.204** |
| Moderately | 2 | 0 | 0 | 0 | 2 | | | |
| Basaloid | 1 | 0 | 1 | 0 | 0 | | | |

Chi-square test, *P*<0.05 (S), **P*>0.05 (NS). HPV: Human papilloma virus, S: Significant, NS: Not significant

## DISCUSSION

Squamous cell carcinoma (SCC) is the most frequent oral cavity malignancy accounting for over 90% of oral cancers, representing the sixth most frequent malignant tumor worldwide.[12]

Tobacco and alcohol consumption are implicated in 75% of OSCC, and smoking accounts for 42% of deaths from cancers of oral cavity and heavy alcohol consumption for 16% of the deaths. The rest 25% of OSCC are attributed to HPV infection. Even though at least 15 HPV types are thought to have oncogenic potential, the most prevalent type causing HPV-associated oral squamous cell cancers is HPV 16, which is also implicated in HPV-associated anogenital cancers.[13]

The ethnicity and geographic origin of patients are responsible for differences in HPV prevalence in head and neck SCC (HNSCC). Asiatic countries, in particular, Japan, have the highest worldwide frequency. This high prevalence of HPV in Asiatic patients with oral cancers indicate that viral infection may be an important etiological agent and along with dietary habits and a probable genetic predisposition can cause additional mutations leading to malignancy. The lowest prevalence of HPV-positive HNSCC was noticed in Africa.[14]

In the present study, when gender and viral prevalence are considered, out of 12 male subjects 7 of them exhibited HPV positivity and 4 females were HPV positive out of 8 cases. This is in accordance with the studies conducted by Brandwein *et al*. and Benson *et al*. where they stated that OSCC is most commonly diagnosed among men compared to women.[15,16]

In the current study, HPV was detected in 11 out of 20 cases and 6 out of 20 controls. Results of the present study indicated a higher percentage of HPV prevalence among cases compared to controls, which is in accordance to studies performed by Gan *et al*. where they mentioned that HPV prevalence was higher among cases compared to controls.[13]

Zhu *et al*. did meta-analysis to evaluate the relationship of OSCC with HPV infection in Chinese population and stated that high incidences of HPV infection particularly HPV 16 was found in the samples of Chinese OSCC that elevates the risk of OSCC tumorigenesis. This is in accordance with our current study where a relatively higher percentage of HPV 16 presence was observed.[17]

D' Costa J *et al*. conducted a study to detect HPV 16 and 18 DNA in tissues from patients with oral cancer, potentially malignant lesions (PMLs) and subjects having normal mucosa using PCR. They found HPV 16 positivity in 15% of OSCC, 34% of PMLs and 15% of subjects with NOM indicating that HPV infections are important but may not be sufficient for the progression to malignancies and that synergistic actions with other carcinogenic agents may be required.[18]

Giovannelli *et al*. in 2002 studied the presence of HPV-DNA in various oral mucosal lesions (13 SCCs, 59 PMLs, 49 benign erosive ulcerative lesions) through nested PCR and found in 80% of the HPV-positive controls.[19]

In the present study, HPV positivity (6/20) was also noticed among controls. HPV is commonly found in NOM mandating the need for distinguishing clinical, subclinical and latent HPV infections.

Paz *et al*. have done a study to assess the association of HPV 16 with SCC and found HPV sequences in 25 out of 167 tumors (15%), but HPV was detected most frequently in tumors in Waldeyer's tonsillar ring.[20]

In this study, when site and HPV prevalence was observed, 5 cases out of 9 taken from posterior-most areas of the oral

cavity were positive for HPV. With regard to tissue taken from buccal mucosa, three subjects were HPV positive out of five. While considering lower anterior region, one subject included in the study expressed positivity. Out of five cases selected from tongue region, three of them exhibited positivity indicating HPV predilection for certain sites in head and neck region particularly tonsil and base of the tongue.

Westra WH mentioned that HPV-related oropharyngeal cancers are highly differentiated and not poorly differentiated. A subtype of HNSCC, basaloid SCC presents with aggressive clinical behavior. Within the basaloid subtype, detection of HPV is a highly favorable prognostic factor that helps in identifying a subset of cancers that departs from the highly aggressive behavior associated with this variant.[21]

Benson *et al*. in 2014 mentioned that histologically, HPV-HNSCCs are non-keratinizing with basaloid features. Initially, they were described as poorly differentiated, but on further analysis, they are similar in morphology to the reticulated epithelium of the tonsillar crypts from which they are thought to arise and therefore are more appropriately now described as well differentiated.[16]

In this study on the comparison of grade of differentiation of OSCC with HPV-positive status, 9 well-differentiated OSCC cases were HPV positive out of 17. One basaloid variant of OSCC exhibited HPV 18 positivity while 2 cases of moderately differentiated OSCC were HPV negative. The result of our present study correlated with above-mentioned statement pertaining to grades of OSCC differentiation.

## CONCLUSION

In the present study, expression of HPV was higher among OSCC cases when compared to controls with a relatively higher percentage of HPV 16 positivity. However, the difference was not statistically significant. Most of the cases exhibiting HPV positivity belonged to well-differentiated pattern of OSCC. Although the presence of HPV was higher in cases compared to controls, none of these differences were statistically significant. HPV 16 and 18 are commonly found in NOM mandating the need for distinguishing clinical, subclinical and latent HPV infections. Hence, further studies on larger samples using sensitive detecting techniques such as real-time-PCR provide conclusive results.

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