Original Article

Human Papillomavirus in Oral Squamous Cell Carcinoma: An Institutional Study

Abstract

Background: Human papillomavirus (HPV) is associated with the etiology of oropharyngeal squamous cell carcinomas. Despite the high proportion of oral squamous cell carcinomas (OSCC) in India, there only a few studies on the HPV status. The present study evaluated the HPV status of OSCC and its association with age, site, and tobacco habits. **Methods:** A total of 60 formalin-fixed paraffin-embedded tissues were included in the study, of which 30 were cases with OSCC and 30 formed the control group. The study group consisted of 30 subjects with primary, untreated, histologically confirmed cases of OSCC. The tissues were evaluated for the presence of HPV DNA using conventional polymerase chain reaction. Tissues which tested positive for HPV were further tested for HPV 16 and HPV 18. **Results:** In the study group, 83% were males and 17% were females with an average age of 58 years. The commonest sites of OSCC were the alveolus, alveologingival sulcus, and tongue. All subjects except one had tobacco habit. Among OSCC cases, 16.7% tested positive for HPV and controls were positive in 3.3% samples. HPV 16 was the most common HPV type detected in 60% of HPV-positive OSCC cases, while HPV 18 was present in 20%. Tobacco habits were present in 80% of the HPV-positive cases, while no habit history was reported in 20% of cases. **Conclusion:** Tobacco habits such as smoking and use of smokeless tobacco are found predominantly in OSCC and are the most common etiological factors. The present study found a prevalence of 16.7% of HPV-associated OSCC, with most cases also having tobacco habits. Thus, HPV as an etiological factor in OSCC is confounded by the presence of tobacco-associated risk factors.

Keywords: *Human papillomavirus, oral squamous cell carcinoma, polymerase chain reaction*

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Introduction

Human papillomavirus (HPV) is associated predominantly with oropharyngeal malignancies. However, studies have shown an association between oral squamous cell carcinomas (OSCC) and HPV.[1] Of the various studies conducted in India, the prevalence of HPV differs from region to region with an approximate incidence of 38% in head-and-neck squamous cell carcinoma (HNSCC).[1-3] This may be due to variation in sample collection and diagnostic methods.[2,4] In India, OSCC is commonly associated with the use of tobacco and tobacco-related products. Clinically, it is important to diagnose HPV-positive tumors as they are associated with better prognosis. If the association between HPV and OSCC is proved, there is a potential for prevention using vaccination.[2] Studies have shown that HPV 16 and HPV 18 are the high-risk (HR) subtypes commonly associated with HNSCC.

Our study aimed to determine the HPV status and risk factors in OSCC subjects in the coastal population of Southwest India.

Methods

Sixty formalin-fixed paraffin-embedded tissues (FFPE) were included in the present study, of which thirty included cases with OSCC and 30 samples from healthy individuals formed the control group. Ethical clearance was obtained from the Institutional Ethics Committee.

Demographic data of the subjects were collected. The study group consisted of 30 subjects with primary, untreated, histologically confirmed cases of OSCC. Subjects with recurrent malignancies, nasal, nasopharyngeal, thyroid, and salivary gland malignancy were excluded.

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Methodology

DNA extraction from formalin-fixed paraffin-embedded tissue blocks

The representative FFPE blocks of each subject were sectioned and the tissue was subjected to DNA extraction. An average of 4–5 sections of 10-µm thickness were sectioned using a microtome (Leica RM2125 RTS) and were transferred into a sterile labeled microcentrifuge tube. DNA was extracted using a commercially available kit from Macherey-Nagel (NucleoSpin® DNA FFPE XS, cat no. 740980.50) as per the manufacturer's instructions.

The DNA was quantified with NanoDrop Spectrophotometer (DeNovix, DS-11) and was subjected to quality assessment with agarose gel electrophoresis.

Human papillomavirus detection

DNA extracted from FFPE blocks underwent conventional polymerase chain reaction (PCR), as described in the literature.[4,5] The primers used for the study target the consensus L1 region of the HPV genome and help in detection of any traces of HPV DNA in the sample.

Oligonucleotide primers (sigma) were commercially synthesized and primary PCR was performed with a pair of oligonucleotide primers, PGMY09/PGMY11, that target a 450 bp region in L1 gene. GAPDH gene was the internal control for checking the PCR reaction; HeLa cell line DNA was the positive control and PCR grade water was the negative control [Table 1].

Human papillomavirus type 16 and 18 detection by type-specific polymerase chain reaction

The positive DNA samples were then used to genotype the most common HR subtypes HPV 16 and HPV 18. SiHa cell line DNA and HeLa cell line DNA were used as a positive control for HPV 16 detection and HPV 18 detection, respectively. PCR products underwent electrophoresis on 2% agarose gel with a 100 bp ladder (HiMedia).

Table 1: Primers used In HPV detection by PCR

Primer	Sequence	Product length
PGMY09/11	CGTCCMARRGGAWACTGATC	450
	GCMCAGGGWCATAAYAATGG	
HPV 16	CACAGTTATGCACAGAGCTGC	467
	CATATATTCATGCAATGTAGGTGTA	
HPV 18	CACTTCACTGCAAGACATAGA	322
	GTTGTGAAATCGTCGTTTTTCA	
GAPDH	GAAATCCCATCACCATCTTCCAGG	238
	GAGCCCCAGCCTTCTCCATG	

Statistics

Data were analyzed with SPSS software (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY,USA: IBM Corp.). Descriptive data were presented as frequency and percentages. Chi-square test and Fisher's test were used to test the association between the study variables. $P < 0.05$ was considered statistically significant.

Results

A total of 60 samples were evaluated in the study. Among the 30 subjects with OSCC, 25 (83%) were males and 5 (17%) were females. Subject age varied from 34 years to 75 years with an average of 58 years. The alveolus, alveologingival sulcus, and tongue all had an equal number of cases and formed the most common sites. All subjects in OSCC group had habit history except for one case (3.3%). Among the other subjects with OSCC, 76% were smokeless tobacco users, 13% had the habit of smoking and use of smokeless tobacco, and 3% had alcohol and smoking habit. Well-differentiated cases formed 53%, moderately differentiated cases formed 43%, and poorly differentiated cases formed 3% of the cases.

Human papillomavirus status

Among the 30 cases of OSCC, 16.7% (5) tested positive for HPV. Among the controls, one tested positive with an incidence of 3.3% [Figure 1].

Human papillomavirus association with age

Four of the HPV-positive subjects were in the seventh decade, while one was in the sixth decade of life [Figure 2].

Human papillomavirus association with gender

Among the study group, out of 30 OSCC cases, all 5 HPV-positive cases were males, while one female tested positive among controls.

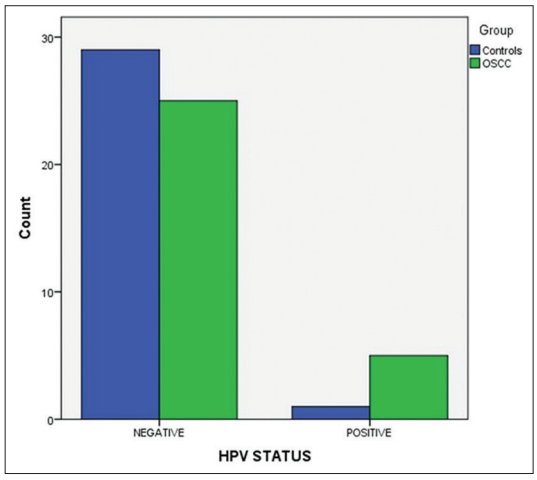

Figure 1: Graph showing the human papillomavirus status of cases and controls

Human papillomavirus association with risk habits

Among the HPV-positive cases, 40% had the habit of smoking and smokeless tobacco and 40% had the habit of smokeless tobacco alone. No habits were reported in 20% [Figure 3].

Human papillomavirus association with oral squamous cell carcinomas site

Two HPV-positive cases had OSCC of alveologingival sulcus, while the buccal mucosa, tongue, and alveolus were the other affected sites [Figure 4].

Human papillomavirus 16 and humanpapilloma virus 18

Three among the five cases of OSCC was positive for HPV 16 (10%), one was HPV 18 positive (3.3%) and one was negative for HPV 16 and 18 (20%). The single case positive for HPV in the control group was positive for HPV 16 and 18. HPV 16 was the most common HPV type in OSCC group with 60% HPV-positive cases. HPV 18 positivity formed 20% of HPV-positive cases in OSCC group. One case (20%) was negative for both HPV 16 and HPV 18. HPV-positive case in the control group was positive for both HPV 16 and HPV 18 [Figures 5 and 6].

Discussion

India accounts for one-third of the world's oral cancer burden, with HNSCC being the commonest cancer in men and the third most common in women.[6] Around 90% of oropharyngeal cancers are caused by HPV; however, the incidence of HPV-positive OSCC is comparatively less. Despite the high proportion of HNSCC in India, there only a few studies on the HPV prevalence in OSCC. HPV-positive carcinomas form a distinct subset with specific clinical and histopathological features. These include younger age group, decreased incidence of tobacco habits, and basaloid morphology on histopathological examination. HPV-positive tumors have been associated with better prognosis when compared to HPV-negative tumors and various studies are currently evaluating modified treatment protocols for HPV-positive cases. The effect of smoking in the prognosis in HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) is unclear. Smokers with HPV-positive tumors have worse prognosis than nonsmokers but better prognosis than OPSCC which tested negative for HPV.[7]

The present study evaluated a total of 30 cases of OSCC. The gender distribution was predominantly male as is seen in OSCC cases worldwide. Maximum cases were seen in the sixth and seventh decades of life. This is in accordance with previously published reports.[8,9]

OSCC in India is commonly associated with tobacco-related habits. India ranks second in the world in both production and consumption of tobacco.[10] Etiological factors in OSCC include tobacco habits such as smoking, betel quid chewing as well as alcohol consumption. The use of gutkha, i.e., processed areca nut with tobacco, is another important

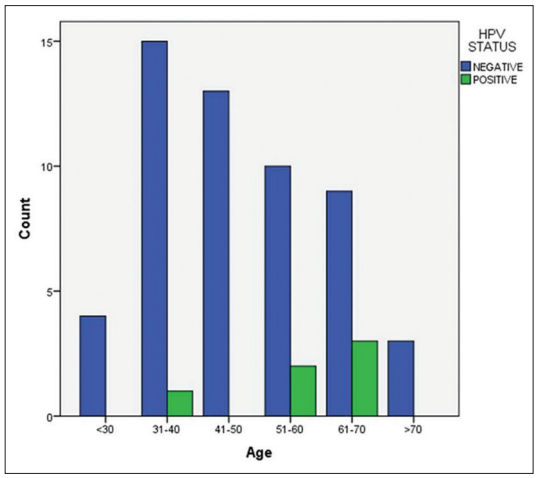

Figure 2: Graph showing the correlation of human papillomavirus status with age in oral squamous cell carcinomas group

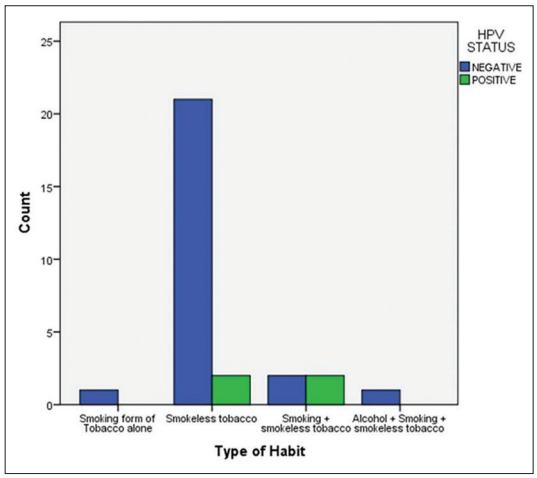

Figure 3: Graph showing the association of human papillomavirus status with tobacco habit

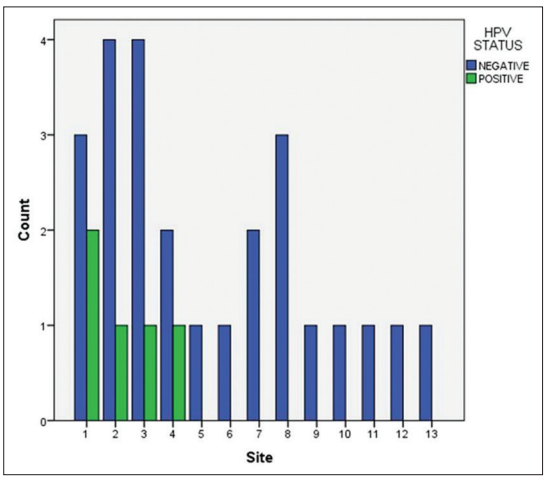

Figure 4: Graph showing the association of oral squamous cell carcinomas site and human papillomavirus status. The alveologingival sulcus (1) had highest cases followed by the alveolus (2), tongue (3), and buccal mucosa (4) with an equal number of cases

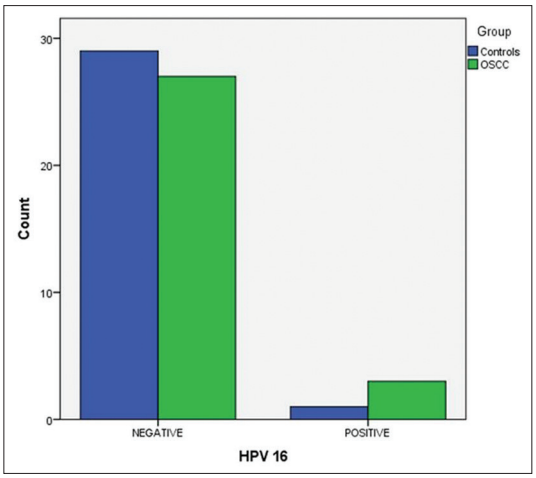

Figure 5: Graph showing the human papillomavirus 16 status of cases and controls

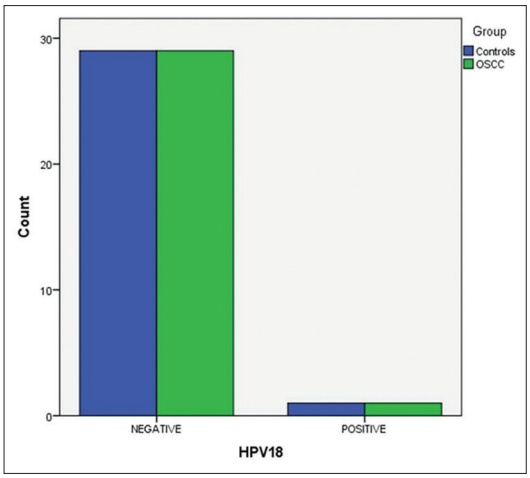

Figure 6: Graph showing the human papillomavirus 18 status of cases and controls

etiological factor.[6,10] The oral mucosa is directly exposed to HPV as well as chemical carcinogens like tobacco and alcohol, probably leading to altered carcinogenesis.[11,12] Majority of the OSCC cases in the present study reported betel quid habit (betel leaf, areca nut, lime, and tobacco), followed by the use of smoking and smokeless tobacco. The HPV-positive cases were associated with the use of both smoking and smokeless tobacco. HPV-positive cases in India are thus often confounded by the presence of tobacco habits.

In the present study, OSCC of the tongue, alveolus and the alveologingival sulcus were the most frequently affected sites, followed by the buccal mucosa. Byakodi *et al*.[13] found the alveolus as the most common site in his study. Sankaranarayanan[14] reported the buccal mucosa as the most common site, followed by the tongue. In India, 60% of oral cancers involve the buccal mucosa, mandibular alveolus, and retromolar trigone and are together termed as cancers of the gingivobuccal complex, the so-called "Indian Oral Cancer." In the West, the tongue and floor of the mouth OSCC is more common.[13] Since chewing or smokeless form of tobacco was the most common habit in the present study, the OSCC incidence was higher in the tongue, alveolus, alveologingival sulcus, and buccal mucosa. Categorization of the OSCC sites was made more difficult in the present study due to OSCC extension to multiple sites. The present study showed no definite site predilection in HPV-positive cases. The alveologingival sulcus was affected in 40% of HPV-positive cases, while the buccal mucosa, tongue, and alveolus were other reported sites with 20% cases each.

The present study found an HPV positivity of 16.7% among 30 cases of OSCC and 3.3% among 30 controls. This data suggest that HPV has a role in OSCC occurrence. Bukhari *et al*.[15] reviewed the published data in the Asian continent and found that the highest HPV prevalence of 10.47% was reported from South Asia, followed by Southeast Asia (5.8%), East Asia (5.7%), and West Asia. Indian studies have shown a wide variation in HPV prevalence in India. Kulkarni *et al*.[16] in a study in Karnataka, India, found an HPV prevalence of 70.6% in the saliva of subjects with OSCC. In contrast, Patel *et al*.[17] reported no HPV-positive cases among 97 patients of OSCC in Gujarat, India. Laprise *et al*.[18] analyzed 350 cases of OSCC from Kerala, India, using Oral CDx biopsy followed by PCR for independent HPV testing in both India and Canada. They reported no HPV-positive cases. Dalakoti *et al*.[19] evaluated 50 subjects with OSCC in Southwest India and found no HPV-positive cases using PCR. Termine *et al*.[3] found an HPV prevalence of 38.1% in a meta-analysis of studies from different geographical areas using varied methods for HPV diagnosis. The geographical variations in HPV occurrence reported in various studies are due to the pattern of global diversity common to all infectious diseases as also the sensitivity and specificity of diagnostic methods that have been used.[2]

Gheit *et al*.[6] evaluated the HPV status in the oral cavity, oropharynx, and larynx/hypopharynx. They found HPV DNA positivity alone in 13.7% of cases, HPV DNA and RNA positivity in 2.7% of cases and 1.1% when p16 results were included. HPV DNA was positive in 11.9% of oral cavity cases with HPV 16 and 18 were the associated HPV types. In Indonesia, Purwanto *et al*.[20] found that 17.9% of OSCC cases were positive for HR HPV and 3.8% were HPV positive among the control population. This was similar to the results of the present study.

HPV infection can be detected either directly on the tumor specimen or by identifying its biomarkers in circulation or saliva.[21-23] HPV DNA detection by PCR, *in situ* hybridization (ISH), HPV E6/E7 mRNA by ISH, and p16 determination are methods used for detection of HPV-positive tumors.[24] PCR is reported to have the highest sensitivity.[24]

In India, HPV-positive OSCC is usually associated with tobacco use. The role of HPV in the etiology of OSCC in India is uncertain. Husain and Neyaz in their review mention that all OSCC cases which were positive for p16 were also associated with tobacco use. HPV-positive OSCC may be associated with p53-related carcinogenesis. Studies have further shown that patient survival was unaffected by HPV, p16, and p53 status.[12]

p16 INK4a expression along with HPV DNA/RNA positivity is believed to be a more reliable indicator of HPV activity. However, recent studies question the reliability of this marker since p16 positive cases have been found to be HPV negative, while HPV-positive cases were p16 negative.[1,6,11] Ursu *et al*.[25] evaluated HNSCC cases in Romania and found that 95.6% of HPV DNA-positive cases were negative for p16. Palve *et al*.[26] reviewed OSCC cases with respect to HPV DNA, HPV RNA, and p16 status to determine their correlation to HPV status and patient survival. They found that HPV DNA and RNA status and p16 status were not predictors of survival. Wai *et al*.[27] mention that p16 is a surrogate marker as well as an important prognostic biomarker for HPV in OPSCC.

There are limitations in the present study. The number of cases evaluated is limited. HPV status was evaluated using PCR alone. Although various studies have mentioned that PCR is the most sensitive for HPV detection, the specificity is low. Studies have shown that HPV DNA detection alone may not be sufficient for viral causality.[6]

Conclusion

In the present study, HPV positivity was analyzed in formalin-fixed paraffin-embedded tissues. HPV was detected in 16.7% of OSCC cases and in 3.3% of controls. HPV 16 was the most common HPV type. Most cases reported the use of tobacco products, both smoking and smokeless tobacco. The results of the present study highlight the presence of HPV-associated OSCC in the population.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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