Evaluation of Human papilloma virus (HPV) -16 and Human papilloma virus (HPV)-18 Presence in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma: A Prospective Study

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ABSTRACT

Aim: The purpose of this study was to examine the clinical and demographic characteristics linked to HPV positivity as well as the prevalence of HPV-16 and HPV-18 in OSCC, OSMF, and healthy controls.

Materials and Methods: This prospective study included 82 subjects in total, split into three groups: OSCC (n=40) patients, OSMF(n=21) patients, and healthy controls(n=21). Histopathological examination was performed in order to confirm the clinical diagnosis and HPV-16 & HPV-18 DNA were extracted and quantified from tissue samples using the polymerase chain reaction (PCR) assay. Additionally, demographic information such as age, gender, tobacco use, and socioeconomic status was recorded. Associations between HPV status and different clinicopathological parameters were assessed using statistical tests, such as χ^2 and t-tests.

Results: HPV-16 was found in 32.5% of OSCC patients, significantly more than the 14.3% of healthy controls ($\chi^2 = 8.41$, $p = 0.003$). Similarly, 20% of OSCC patients had HPV-18, whereas 9.5% of healthy controls did ($\chi^2 = 3.66$, $p = 0.05$). There was no discernible difference in the prevalence of HPV-16 and HPV-18 between OSMF patients and healthy controls. OSCC patients with HPV were younger (mean age 43.77 years) than those without HPV (mean age 53.33 years) ($t = 2.25$, $p = 0.03$), and females were more likely to be HPV-positive ($p = 0.04$). There were no discernible correlations between HPV status and clinical staging, socioeconomic status, or tobacco use.

Conclusion: High-risk HPV, particularly HPV-16, is strongly associated with oral squamous cell carcinoma (OSCC), with a notable prevalence among younger patients and females.

Keywords: *HPV-16, HPV-18, Oral Squamous Cell Carcinoma, Oral Submucous Fibrosis, polymerase chain reaction (PCR)*

INTRODUCTION

Oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSMF) are two significant and related oral health disorders, particularly in regions with high tobacco and areca nut consumption.[1] Inflammation and progressive fibrosis of the submucosal tissues are the characteristic features of OSMF, a chronic, progressive disease that can cause burning, a decreased capacity to open the mouth, and in certain cases, malignant transformation.[2] It is well known that OSMF has the potential to become malignant; research indicates that the progression rate to OSCC can range from 1.5% to 15% over time.[3] In contrast, OSCC makes up more than 90% of all malignant neoplasms in the oral cavity, making it the most prevalent type of oral cancer. OSCC has a high death rate because it is frequently discovered in advanced stages.[4] Therefore, it's crucial to comprehend the elements that lead OSMF to OSCC.

The investigation of how the human papillomavirus (HPV) contributes to the pathophysiology of OSMF and OSCC is still in its early stages. Studies have revealed that high-risk HPV strains, such as HPV-16 and HPV-18, are present in some OSCC patients.

This study intends to assess the presence of high-risk HPV types (HPV-16 and HPV-18) in patients with OSMF and OSCC, given the known role of HPV in OSCC and the growing evidence of its involvement in OSMF. Our specific goal is to determine whether HPV contributes to the development of OSMF into OSCC, as this could have important ramifications for early detection and treatment.

MATERIALS AND METHODS

This prospective study was conducted in the department of Oral & Maxillofacial Pathology, S.C.B Dental College & Hospital, Cuttack in collaboration with department of Zoology, Ravenshaw University, Cuttack. The study period extended from November 2013 to August 2015. Before being included in the study, each participant gave written informed consent, and institutional ethical committee granted ethical clearance (IEC/IRB no-184/ dated 7.9.2015). Three separate subject groups were chosen for the study based on predetermined inclusion and exclusion criteria in order to assess the prevalence of high-risk HPV (HPV-16 and HPV-18) in patients with oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSMF). There were 82 subjects participated in the study. These individuals were divided into three groups. The control group, group A, consisted of 21 healthy people without any clinically noticeable oral lesion. No significant oral pathology was seen in any of these subjects, though some did have alcohol and tobacco use habits. 21 people in Group B had a clinical

diagnosis of oral submucous fibrosis based on criteria described by Sirsat and Pindborg (1967). These criteria included symptoms like a strong burning sensation in the mouth, pallor and blanching of the affected areas, and difficulty opening the mouth because of palpable fibrotic bands.[5] OSMF cases were further classified into subgroups based on clinical stages as described by Ranganathan et al. (2001).[6] Group C comprised of 40 subjects diagnosed with oral squamous cell carcinoma (OSCC) as per the world health organization's clinical guidelines (2005)[7] (Figure 1).

Exclusion criteria for the study included patients with underlying systemic diseases, individuals with other defined oral lesions such as leukoplakia, erythroplakia, or benign tumors, and patients with carcinoma affecting the lip or oropharynx. This ensured the study population represented the specific pathology of interest without confounding factors from other oral conditions.

The data collection process comprised the systematic recording of each participant's clinical and demographic details. The most representative lesion sites for Groups B and C were used for incisional biopsies to obtain tissue samples after consent was obtained. During standard surgical extraction of impacted molars, adjacent normal mucosa samples were taken from healthy individuals for Group A. Two portions of each tissue sample were separated: one was fixed in formalin and prepared for histopathological diagnosis, while the other was immediately preserved in 100% alcohol for molecular analysis.

The criteria outlined by Pindborg (1996), which included the presence of atrophic epithelium, flattening of connective tissue, fibrosis, and subepithelial hyalinization, were used to histopathologically confirm OSMF. Broder's grading system (1920), which categorizes tumors according to the level of cellular differentiation, was used to grade tissue samples for OSCC. Grade I tumors exhibited good differentiation, Grade II tumors were moderately differentiated, Grade III tumors were poorly differentiated, and Grade IV tumors displayed anaplastic development (Figure 2).

HPV-16 and HPV-18 DNA were detected by molecular analysis using traditional polymerase chain reaction (PCR). DNA was extracted using a modified procedure of PCI (Phenol-Choloroform-Isoamylalcohol) method from tissue samples.[8] After being suspended in lysis buffer, tissue samples were subjected to an overnight treatment with proteinase K. Ethanol precipitation was used after phenol-chloroform extraction to extract high molecular weight DNA. DNA concentration and purity were evaluated using 0.8% agarose gel electrophoresis and

spectrophotometry (Figure 3 and 4). Using degenerate primers MY09 and MY11, L1 open reading frame (ORF) of the HPV genome was amplified by PCR, yielding a 450 base pair amplicon. Using specific primers, HPV-16 and HPV-18 were further typed, producing amplicon sizes of 217 bp and 322 bp, respectively. PCR was performed in a final reaction volume of 50 µl using 10 µl of extracted DNA, 5 µl of Qiagen PCR buffer, 1 µl of dNTP mix, 7 µl of $MgCl_2$, 2 µl of each primer, 0.5 U of Taq DNA polymerase and distilled water. The initial thermal cycling condition was denaturation for five minutes at 94°C. 34 cycles of denaturation at 94°C for 30 seconds and annealing at 55°C for 30 seconds came next.

The PCR products were examined using 1.5% agarose gel electrophoresis, followed by ethidium bromide staining and gel documentation system display. Depending on their individual sizes, existence of particular amplicons was verified. Statistical analyses were performed to correlate HPV presence with clinicopathological parameters.

RESULTS

When compared to normal subjects, study found a significant correlation between oral squamous cell carcinoma (OSCC) and HPV-16 status. Among the healthy control group, 3 out of 21 subjects (14.3%) tested positive for HPV-16, while 18 (85.7%) were negative. In contrast, 13 out of 40 OSCC patients (32.5%) were HPV-16 positive, and 27 (67.5%) were negative, with a significant statistical difference (χ^2 = 8.41, p = 0.003), indicating a higher prevalence of HPV-16 in OSCC.(Table 1) When comparing the oral submucous fibrosis (OSMF) group with the OSCC group, 4 out of 21 OSMF patients (19.4%) tested positive for HPV-16, compared to 13 out of 40 (32.5%) in OSCC, which also showed a significant association (χ^2 = 4.11, p = 0.04).**(**Table 2**)** However, no significant difference was observed between the normal group and the OSMF group for HPV-16 status, as 14.3% of normal individuals and 19.04% of OSMF patients were HPV-16 positive (χ^2 = 0.51, p = 0.47). **(**Table 3**)**

Regarding HPV-18 status, a significant association was found between the normal and OSCC groups. Among the normal group, 2 out of 21 subjects (9.5%) were HPV-18 positive, compared to 7 out of 40 OSCC patients (20%) (χ^2 = 3.66, p = 0.05), indicating a higher prevalence of HPV-18 in OSCC.**(**Table 4**)** However, no significant association was found when comparing OSMF and OSCC for HPV-18 status (χ^2 = 0.82, p = 0.36), **(**Table 5**)** nor between the normal and OSMF groups (χ^2 = 0.68, p = 0.40), **(**Table 6**)** suggesting that HPV-18

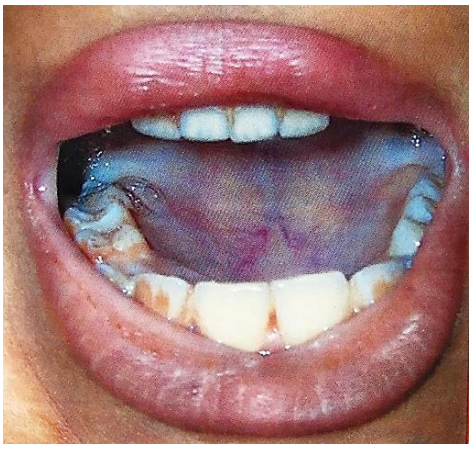
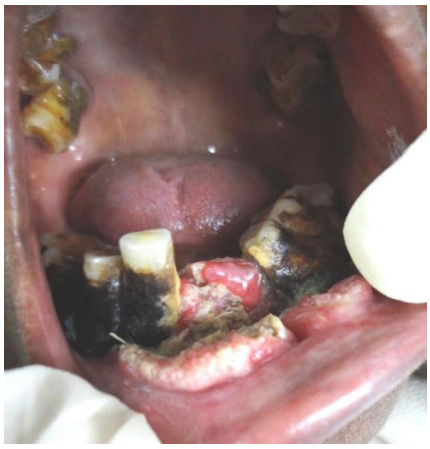
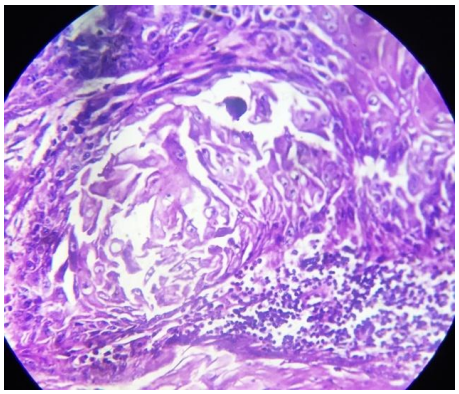
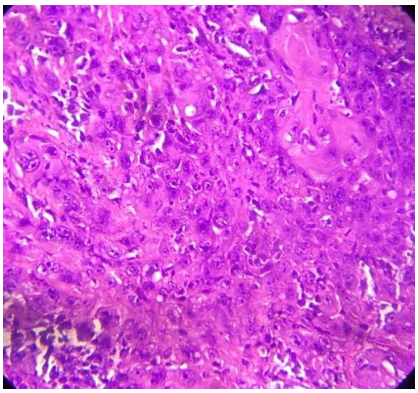
(a)  (b) 

Figure 1: (a) Clinical Photograph of Oral Squamous Cell Carcinoma (b) Clinical Photograph of Oral Submucous Fibrosis.

(a)  (b) 

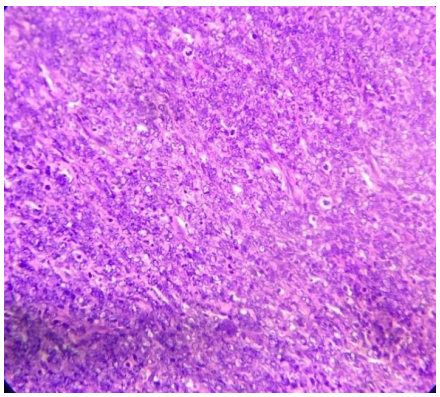
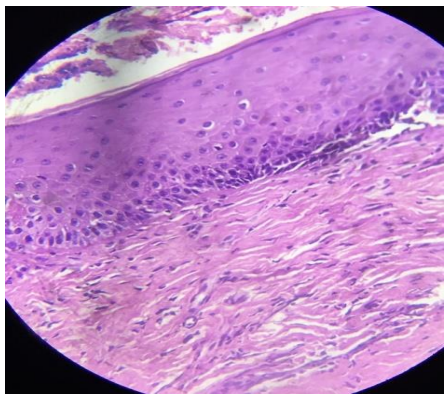
(c)  (d) 

Figure 2: (a) OSCC Grade-1(Well Differentiated Squamous cell Carcinoma) H&E stain 400x (b) Grade 2(Moderately Differentiated Squamous cell Carcinoma) H&E stain 400x (c) Grade-3(Poorly Differentiated Squamous cell carcinoma) H& E stain 400x (d) OSMF H& E stain 400x.

does not play a significant role in differentiating these groups. Demographic analysis revealed a significant association between age and HPV status in both OSCC

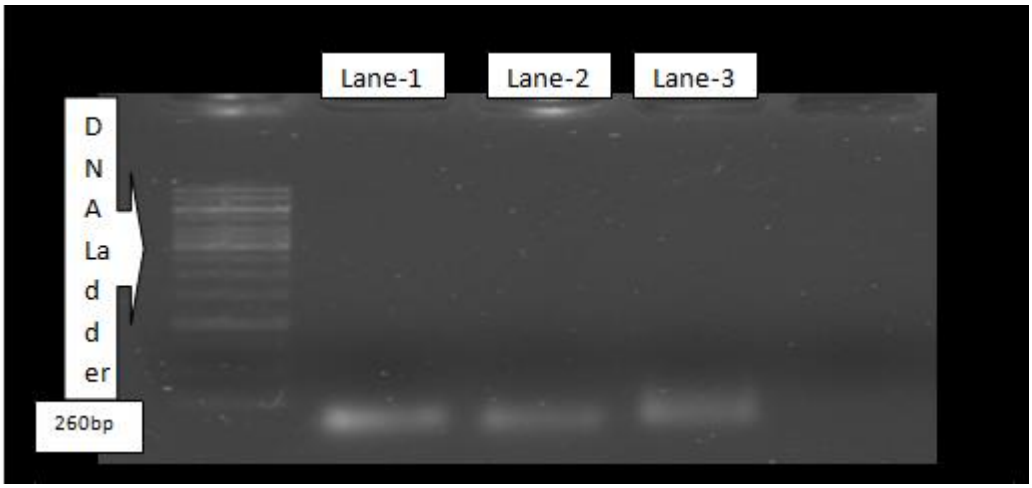


Figure 3: HPV16- Specific primers – mediated PCR of DNA extracted from fresh biopsy specimen of OSCC – Lane 1, OSMF – Lane 2, Normal healthy subjects- Lane 3

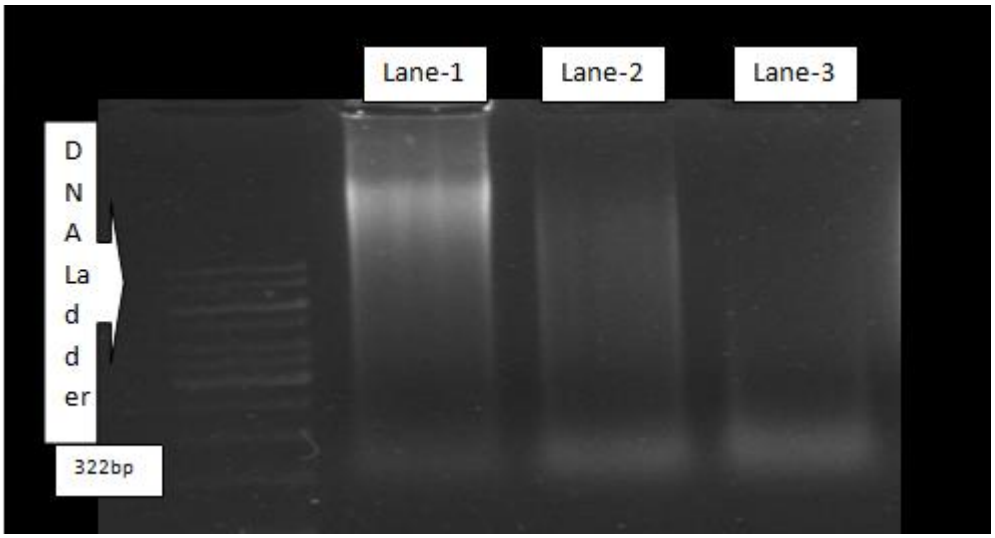


Figure 4: HPV18 - Specific primers – mediated PCR of DNA extracted from fresh biopsy specimen of OSCC – Lane 1, OSMF – Lane 2, Normal healthy subjects- Lane 3.

and OSMF groups. In the OSCC group, HPV-positive patients had a mean age of 43.77 ± 9.6 years, significantly younger than HPV-negative patients, whose mean age was 53.33 ± 13.75 years ($t = 2.25$, $p = 0.03$). Similarly, in the OSMF group, HPV-positive patients were older, with a mean age of 34.0 ± 9.6 years, compared to 26.82 ± 4.46 years in HPV-negative patients ($t = 2.82$, $p = 0.011$). There was also a significant gender-based difference in HPV-16 and HPV-18 status among OSCC patients. For HPV-16, 53.9% of positive cases were female and 46.1% were male, while for HPV-negative patients, 77.8% were male and 22.2% were female ($\chi^2 = 4.0$, $p = 0.04$). A similar pattern was seen for HPV-18, where 62.5% of positive cases were female, and 37.5% were male ($\chi^2 = 4.1$, $p = 0.04$). However, no significant

Table 1: Cross-tabulation of normal and OSCC group according to HPV16 status

Study group	HPV 16		Total	χ^2	*P*
	Present n (%)	Absent n (%)	N(%)		
Normal	3(14.3)	18(85.7)	21 (100)	8.41	0.0038*
OSCC	13(32.5)	27(67.5)	40(100)		

*Significant

Table 2: Cross-tabulation of OSMF and OSCC group according to HPV16 status

Study group	HPV 16		Total	χ^2	*P*
	Present n (%)	Absent n (%)	N(%)		
OSMF	**4(19.4)**	**17(80.96)**	**21**(100)	4.11	0.04
OSCC	**13(32.5)**	**27(67.5)**	**40**(100)		

Table 3: Cross-tabulation of normal and OSMF group according to HPV-16 status

Study group	HPV 16		Total	χ^2	*P*
	Present n (%)	Absent n (%)			
Normal	**3(14.3)**	**18(85.7)**	**21**(100)	0.51	0.47
OSMF	**4(19.04)**	**17(80.96)**	**21**(100)		

Table 4: Cross-tabulation of normal and OSCC group according to HPV18 status

Study group	HPV		Total	χ^2	*P*
	Present n (%)	Absent n(%)			
Normal	**2**(95)	**19**(90.5)	**21**(100)	**3.66**	**0.05**
OSCC	**7**(20)	**33**(80)	**40**(100)		

gender differences were found for HPV status in the OSMF or normal groups. Additionally, no significant associations were observed between HPV status and tobacco habits or socioeconomic status in any of the groups.

No significant association was found between HPV-16 status and the clinical

Table 5: Cross-tabulation of OSMF and OSCC group according to HPV18 status

Study group	HPV 18		Total	χ^2	*P*
	Present n (%)	Absent n (%)			
OSMF	**3**(14.3)	**18**(85.7)	**21**(100)	0.82	0.36
OSCC	**7**(20)	**33**(80)	**40**(100)		

Table 6: Cross-tabulation of OSMF and OSCC group according to HPV18 status

Study group	HPV 18		Total	χ^2	*P*
	Present n (%)	Absent n (%)			
Normal	**2**(9.5)	**19**(90.5)	**21** (100)	0.68	0.40
OSMF	**3**(14.3)	**18**(85.7)	**40**(100)		

staging of OSCC (χ^2 = 1.75, p = 0.62) or HPV-18 status and OSCC staging (χ^2 = 2.5, p = 0.46). Similarly, no association was observed between HPV status and the histologic grading of OSCC (χ^2 = 1.06, p = 0.58 for HPV-16; χ^2 = 0.26, p = 0.87 for HPV-18). In OSMF patients, HPV-16 status was not significantly associated with clinical staging (χ^2 = 0.05, p = 0.82), and neither was HPV-18 status (χ^2 = 0.2, p = 0.64).

DISCUSSION

The current study was a base line study and conducted on eastern Odisha population. This is the first kind of study in the population of Odisha as per published data. The goal of the current study was to ascertain whether high-risk HPV strains, specifically HPV-16 and HPV-18, were present in patients with oral squamous cell carcinoma (OSCC), oral submucous fibrosis (OSMF). Notable findings indicate that HPV-16, and HPV-18 levels were higher in OSCC patients then OSMF and healthy individuals. These results are in line with past studies that demonstrated a strong link between HPV and head and neck cancers, particularly OSCC, which is increasingly being recognized as a subset of cases linked to HPV.

With 32.5% of OSCC patients testing positive for HPV-16 compared to just 14.3% of the healthy controls, this study's major finding was the strong correlation between HPV-16 presence and OSCC. This supports the increasing amount of data indicating that HPV, especially the high-risk HPV-16 strain, is a major contributor

to the development of OSCC. The expression of viral oncoproteins E6 and E7, which deactivate the tumor suppressor proteins p53 and Rb and cause unchecked cell division and malignant transformation, is one way that HPV is known to contribute to carcinogenesis.[9] The idea that HPV may be an independent risk factor for the development of OSCC, even in people without conventional risk factors like alcohol or tobacco use, is supported by the significantly higher prevalence of HPV-16 in OSCC compared to the normal group ($p = 0.003$).

The association between HPV-18 and OSCC was also significant, with 20% of OSCC patients testing positive for the virus compared to 9.5% of healthy controls ($p = 0.05$). This finding implies that both high-risk HPV types may be involved in the pathophysiology of OSCC, even though the role of HPV-18 in OSCC is less well-studied than that of HPV-16.

It's interesting to note that the study found no discernible difference in the prevalence of HPV-16 or HPV-18 between OSMF and healthy people, indicating that HPV may not be a major factor in the pathophysiology of OSMF. The findings of earlier research on the connection between HPV and OSMF have been inconclusive.

The mean age of HPV-positive and HPV-negative individuals differed significantly, and younger OSCC patients were more likely than older patients to test positive for HPV-16 and HPV-18. In the OSCC group, women were more likely than men to be HPV-positive, accounting for 53.9% of HPV-16 positive cases and 62.5% of HPV-18 positive cases, according to the study. This gender disparity in HPV-related OSCC has been reported in other studies as well and may be linked to differences in immune response, hormonal influences, or patterns of viral transmission.[10,11] However, further research is required to understand the gender-based differences in HPV-related OSCC.

In OSCC group, there was no discernible correlation between HPV status and socioeconomic status or tobacco use. This suggests that the presence of HPV in OSCC patients may be independent of these traditional risk factors. HPV-related OSCC is often found in non-smokers and non-drinkers, particularly in younger individuals, further supporting the notion that HPV is a distinct etiological factor in the development of OSCC.[12]

Furthermore, HPV-16 and HPV-18 status did not show a significant correlation with clinical staging or histologic grading in OSCC, nor did HPV status correlate with clinical staging in OSMF. These findings suggest that while HPV may be involved in the initiation of OSCC, it may not directly

influence the tumor's progression or histopathological characteristics.

CONCLUSION

This study concludes that high-risk HPV strains, especially HPV-16, are significantly present in OSCC, confirming the virus involvement in the carcinogenesis of oral cancers. On the other hand, HPV does not seem to be a major contributor to OSMF pathogenesis, indicating that lifestyle and environmental factors may be more important in OSMF than viral oncogenesis. In addition to possible preventive strategies like HPV vaccination in populations at risk of oral cancers, future research could concentrate on examining the mechanisms of HPV integration and its clinical implications.

Conflict of Interest – None

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