ORIGINAL ARTICLE

p16 as an independent marker for detection of high-risk HPV in oral submucous fibrosis and oral squamous cell carcinoma

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ABSTRACT

Background: An alarming increase in incidence of high-risk human papillomavirus (HPV) positive tumors in head and neck squamous cell carcinoma (HNSCC) by 25% and 70% in oropharyngeal HNSCC cannot be ignored. The early oncogenes of HPV, E6, and E7 play a key role in carcinogenesis. HPV associated tumors have a better clinical outcome and a favorable prognosis. The p16 expression has high concordance with other methods of HPV detection, ascertaining p16 as a surrogate marker for HPV. **Objective:** To assess the immunohistochemical expression of p16 in oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC) with and without coexistent OSF as a marker for high-risk HPV detection. **Materials and Methods:** Tissue blocks of 70 cases including normal, OSF, OSCC with and without OSF were subjected to IHC staining with a $p16^{INK4A}$ monoclonal antibody. (Biogenex, San Roman). The p16 expression was noted according to percent positivity and pattern. The data were tabulated, statistically analyzed using the Chi-square test and the *P* value was assessed. **Results:** The percentage of p16 positive cells raised from normal to OSF to OSCC with and without OSF. In addition, a shift from nuclear to cytoplasmic expression from normal to OSCC was noted with a statistical significance ($P < 0.001$). However, no statistical significance was established with any clinicopathologic parameters except age ($P = 0.012$) and habits ($P = 0.023$). **Conclusion:** The presence of HPV using p16 was not detected in OSF but was positive in OSCC. Altered pattern of expression from normal to OSF to OSCC indicates promising use of p16 as a diagnostic marker.

KEY WORDS: High risk, human papillomavirus, immunohistochemistry, oral squamous cell carcinoma, oral submucous fibrosis, p16

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) accounts for 3–5% of all cancers and is the 6th most common cancer worldwide. The incidence of HNSCC, associated with tobacco and alcohol use has fairly descended with the awareness and consequent reduction in the use of commercially available tobacco products. Nevertheless, an increase in the incidence of high-risk human papillomavirus (HPV) positive tumors in HNSCC by 25% and 70% in oropharyngeal HNSCC has been encountered.[1] The early oncogenes of HPV mainly E6 and E7 play a key role in carcinogenesis through inactivation of p53 and retinoblastoma (pRb) to evade host immune surveillance and deregulate cell cycle, thus facilitating DNA damage leading to cellular transformation. HPV associated tumors have been accredited a better clinical outcome and favorable prognosis.[2,3] Hence, HPV detection in HNSCC gains importance. Numerous attempts have been made for the detection of HPV in HNSCC using various methods. Among the several methods for detection of HPV, various studies found p16 expression has high concordance with other methods.[1,4,5] The immunohistochemical (IHC) expression of p16 is convenient as it is employed to tissue blocks and is less tedious than the other methods. p16 is a surrogate marker for HPV infection; however, the significance of p16 discrete from other HPV detection methods is uncertain. In addition, the potential role of HPV in

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potentially malignant disorders (PMD) like oral submucous fibrosis (OSF), which are known to preclude oral squamous cell carcinoma (OSCC) is not well established.

The present study was aimed to assess the IHC expression of p16 in normal mucosa, oral submucous fibrosis and oral squamous cell carcinoma with and without coexistent OSF. To assess the HPV status according to p16 expression in OSF, OSCC with, and without coexistent OSF.

MATERIALS AND METHODS

Formalin-fixed paraffin-embedded (FFPE) tissue blocks of 70 cases including normal mucosa as control (10), histologically proven cases of OSF (30) and OSCC with and without coexistent OSF (30) were retrieved from the archives of Department. Clinicopathological data of all cases were recorded from the patient records and tabulated. Tissue sections of 3 μm were obtained on silane coated slides and subjected to IHC staining with $p16^{ink4a}$ monoclonal antibody (Biogenex, San Roman). The procedure provided by the manufacturer was followed for staining. Brown precipitate in the nucleus/cytoplasm was considered to be a positive p16 expression. Parameters such as percentage positivity, pattern of expression, intensity, and layers of epithelium showing positive staining were assessed. Number of positive cells (x) in an evenly stained area under 40× magnification was counted in each slide. A minimum of 500 cells was counted. Percentage positivity was calculated by the formula x/500 X 100. The pattern of p16 expression was noted as nuclear, cytoplasmic, or both. The intensity was scored as absent, mild, or intense. Expression in different layers of the epithelium was noted for normal and OSF. The data were tabulated and analyzed using the Chi-square test. Ethical clearance was obtained by the institutional review board prior to the study.

Ethical clearance was obtained from the Institutional ethical committee, SDMCDSH (IRB No. 2017/P/OP/54).

RESULTS

Comparison of clinicopathological parameters among groups

The clinicopathological parameters of 4 groups were assessed. Greater number of patients were below 40 years of age in OSF and OSCC with OSF [25/30 (83.3% and 9/15 (60.0%)], while in contrast 9/15 (60%) cases of OSCC without OSF were above the age of 40 years. Statistically, significant correlation was found in the two age groups among the 4 groups ($P = 0.020$). Male predilection was seen in both OSF and OSCC with and without OSF showing a statistical significance ($P = 0.005$). Chewing habit was more prevalent among the groups [56/60 (93.3%)] than smoking, which was statistically significant ($P = 0.05$). The duration of habit was more than 5 years in most of the cases of OSF and OSCC with and without OSF.

Clinical staging for OSF and Tumor node metastases (TNM) staging for OSCC with and without OSF was done. Most cases were in stage III followed by stage II. ($P = 0.143$) Predominant site of involvement for OSCC with and without OSF was buccal mucosa [11/15 (73.3%) and 11/15 (73.3%)] followed by other sites such as lip, alveolus [4/15 (26.7%) and 2/15 (13.3%)], and tongue being the least affected [0 (0%) and 2/15 (13.3%)]. The site distribution among group did not show statistical correlation.

Table 1: Clinicopathological data of all groups

Parameters	*Category*	*NM- 10 n (%)*	*OSF-30 n (%)*	*OSF + OSCC-15 n (%)*	*OSCC-15 n (%)*	*P (<0.05)*
Age (years)	≤40	8 (80.0)	25 (83.3)	9 (60.0)	6 (40.0)	0.020*
	>40	2 (20.0)	5 (16.7)	6 (40.0)	9 (60.0)	
Sex	Male	8 (80.0)	28 (93.3)	14 (93.3)	8 (53.3)	0.005*
	Female	2 (20.0)	2 (6.7)	1 (6.7)	7 (46.7)	
Habits	Chewing	-	30 (100.0)	14 (93.3)	12 (80.0)	0.05*
	Smoking	-	0 (0.00)	1 (6.7)	3 (20.0)	
Duration	≤5 years	-	13 (43.3)	3 (20.0)	6 (40.0)	0.295
	>5 years	-	17 (56.7)	12 (80.0)	9 (60.0)	
Staging	Stage II	-	14 (46.6)	7 (46.7)	3 (20.0)	0.189
	Stage III	-	16 (53.3)	7 (46.7)	12 (80.0)	
Site	Buccal mucosa	-	-	11 (73.3)	11 (73.3)	0.264
	Tongue	-	-	0 (0.00)	2 (13.3)	
	Others	-	-	4 (26.7)	2 (13.3)	
H/P OSF grading	Stage II	-	17 (56.7)	-	-	<0.001**
	Stage III	-	13 (43.3)	-	-	
H/P grading (SCC)	Well	-	-	13 (86.7)	13 (86.7)	1<0.001**
	Moderate	-	-	2 (13.3)	2 (13.3)	
DOI	<5 mm			3 (20.0)	3 (20.0)	0.593
	5-10 mm			12 (80.0)	11 (73.3)	
	>10 mm			0 (0.00)	1 (6.70)	

*- Significant, ** - Highly significant

Histopathological grading of OSF showed the greatest number of cases in stage II [17 (56.7%)] followed by stage III [13 (43.3%)] yielding a statistical significance ($P < 0.001$). Cases of well-differentiated SCC [13/15 (86.7%)] were higher than the moderately differentiated [2/15 (13.3%)], which showed a high statistical significance ($P = 1 < 0.001$).

Depth of invasion (DOI) of the tumor was calculated in OSCC with and without OSF cases and was grouped as <5 mm, 5–10 mm, and >10 mm. DOI was between 5 and 10 mm in 12/15 (80.0%) cases of OSCC with OSF and 11/15 (73.3%) cases of OSCC without OSF ($P = 0.593$) [Table 1].

Comparison of immunohistochemical parameters of p16 among groups

Percentage of positive cells in all cases of Normal mucosa (NM) was 0–30% (100%). In OSF, 25 (83.3%) cases showed 0–30% positivity, whereas 5 (16.7%) showed 61–90% positivity. An increase in percentage positivity was seen in OSCC with and without OSF showing 31–60% positivity in 9 (60.0%) and 8 (53.3%) cases, respectively and 61–90% positivity in 6 (40.0%) and 7 (46.7%), respectively. Highly statistical significance was noted between the 4 groups ($P = <0.001$).

Nuclear expression was predominant in normal cases [8/10 (80.0%)], only 2 (20.0%) cases showed cytoplasmic expression. Cases of OSF showed greater cytoplasmic expression 16 (53.3%) while nuclear expression was also noted [14 (46.7%)] [Figure 1a-c]. In OSCC with and without OSF, cytoplasmic expression was seen in all cases 15 (100%) [Figure 1d-f]. A statistical significance was established among the groups ($P = <0.001$) [Table 2].

HPV presence was interpreted in 6/15 OSCC with OSF and 7/15 cases of OSCC without OSF [Graph 1].

DISCUSSION

OSF is a PMD with an incidence rate between 0.9% and 4.7% reported in China and 0.4% to 10% in India. High incidence of OSF in India is attributed to the highly prevalent habit of areca nut and tobacco chewing among the young Indian population. OSF has a high propensity for malignant transformation with distinct clinicopathological features and a better prognosis.[6,7] The rate of malignant transformation of OSF has been found to range from 3% to 19%.[8]

Table 2: Comparison of IHC expression of p16 among groups

Parameters	*Category*	*NM n (%)*	*OSF n (%)*	*OSF + OSCC n (%)*	*OSCC n (%)*	*P*
% positivity	0-30%	10 (100.0)	25 (83.3)	0 (0.00)	0 (0.00)	<0.001**
	31-60%	0 (0.00)	5 (16.7)	9 (60.0)	8 (53.3)	
	61-90%	0 (0.00)	0 (0.00)	6 (40.0)	7 (46.7)	
	>90%	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
Pattern	Cytoplasm	2 (20.0)	16 (53.3)	15 (100.0)	15 (100.0)	<0.001**
	Nuclear	8 (80.0)	14 (46.7)	0 (0.00)	0 (0.00)	
Intensity	Mild	6 (60.0)	18 (60.0)	4 (26.7)	7 (46.7)	0.177
	Intense	4 (40.0)	12 (40.0)	11 (73.3)	8 (53.3)	
Layers	Basal/parabasal	10 (100.0)	20 (66.7)	-	-	0.035*
	Spinous	0 (0.00)	10 (33.3)	-	-	

*- Significant, ** - Highly significant

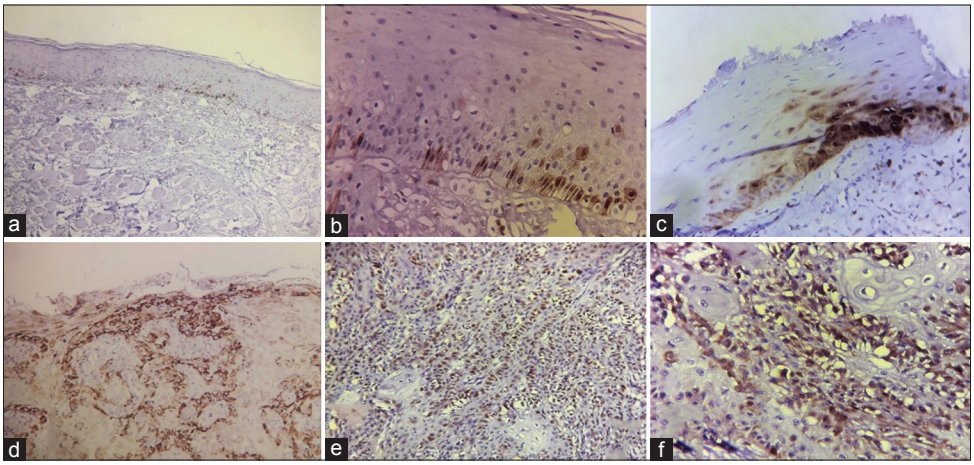
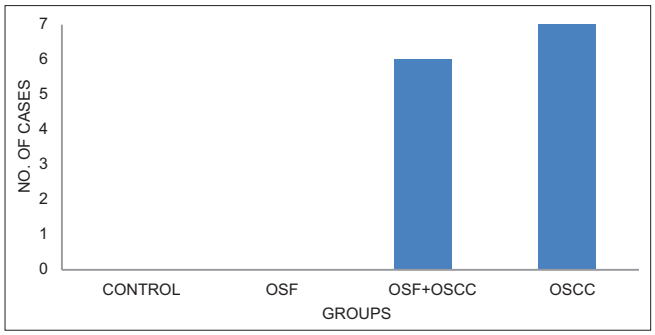


Figure 1: Photomicrographs showing immunohistochemistry with p16. (a) in OSF (10×) (b) cytoplasmic staining in OSF (40×) (c) nuclear staining in OSF (40×) (d) cytoplasmic staining in dysplastic epithelium and tumor cells of moderately differentiated OSCC with OSF (20×) (e and f) cytoplasmic staining in tumor cells of well-differentiated OSCC (10×) and (40×)



Graph 1: HPV positivity in all groups using p16 IHC

The association of HPV as an etiological factor in addition to the habits has been proposed for oral tumorigenesis.[9,10] Various direct and indirect methods have been used to detect the HPV presence over the years. Direct methods such as southern blot assay, polymerase chain reaction (PCR), reverse transcriptase PCR, and *in situ* hybridization (ISH); and indirect methods such as IHC, signal amplification method, and DNA/RNA microarray have been used for detection. In FFPE tissues, HPV can be detected using IHC with p16 and anti-E6-E7 antibodies, HPV DNA.[4,5] A review on the role of HPV in OSCC and oral PMDs from the year 1994 to 2014 done by Gupta *et al.*, showed HPV positivity in oral leukoplakia ascended from 0% (Saghravanian *et al.*) to 45.7%.[11,12] However, to understand the role of HPV in OSF studies is limited. Jalouli *et al.*, has reported HPV positivity in 11/12 (91%) cases.[13] Chen *et al.*, has reported the prevalence of HPV in 52.6% OSF cases.[14]

The relationship between HPV and OSCC was first suggested in 1983 by Syrjanen *et al.*, while the presence of viral DNA was confirmed by means of ISH.[15,16] The prevalence of HPV in oral cancer ranges between 0% (Akhtar *et al.*) and 80% (Elango *et al.*).[17,18] Recently, the incidence of high-risk HPV positive tumors has increased by 25% in HNSCC and by 70% in oropharyngeal HNSCC.[1]

HPV associated tumors are known to have a better prognosis in terms of involvement of a younger age group, higher radiosensitivity of the virus affected tumor cells. This is attributed to the pathogenesis of HPV in oral cancer. HPV viral life cycle is related within the differentiation of its host epithelial cells. The early oncogenes of HPV mainly E6 and E7 play a key role in carcinogenesis through inactivation of p53 and pRb, respectively. The E7 protein targets Rb for degradation, resulting in the release and activation of E2F transcription factors responsible for the expression of S phase genes and hyperproliferation. E7 proteins, thus, disrupt G1- S arrest inducing hyperproliferation.[9]

HPV E6 protein inhibits p53 using the ubiquitin pathway. The E6 protein binds to ubiquitin ligase E6 AP protein to form a complex with p53, leading to the ubiquitylation, degradation of p53, and finally, inhibition of p21. While in HPV negative tumors, p53 mutation or inactivation can be owing to mdm2 protein or other pathways.[19]

In the present study, HPV detection was done by using IHC expression of p16 in control, OSF, and OSCC. The p16 is a tumor suppressor gene that inhibits cyclin-dependent kinase 4A. In normal conditions, it prevents the cells to enter from G1 to S phase of the cell cycle. However, in the presence of transcriptionally active HPV, the E7-E2F complex effectively stops the negative feedback of free pRb on p16, as a result, overexpression of p16 is seen. Thus, p16 is a surrogate marker to detect HPV.[4,9,16]

A distinct staging algorithm for HPV associated oropharyngeal cancer (OPC) has been included in the 8th edition of American Joint Committee on Cancer (AJCC), wherein p16 overexpression is a criterion to designate HR- HPV associated OPC.[20] As per the criteria followed by AJCC, p16 overexpression is determined by intense staining and >75% cut-off. Various studies have shown p16 to have a sensitivity of 74% to 100% and specificity of 46% to 100% when compared with other methods such as PCR and ISH.[16]

In the present study, nuclear expression of p16 was seen (8/10) in control tissues. However, none of the cases showed >30% positive cells. Authors have noted HPV positivity in control ranging from 0% (Sand *et al.*) to 33.3% (Babiker *et al.*).[21,22] Cases of OSF showed both nuclear and cytoplasmic p16 expression with 5/30 (16.7%) cases showing >75% positive cells and fulfilling the criteria for HPV presence. Jalouli *et al.*, and Chen *et al.*, noted 91% and 52.6% HPV positivity in OSF using PCR and nested PCR, respectively.[13,14] Chaudary *et al.*, noted among 208 cases of OSF, 26% showed the prevalence of HPV 16 by PCR and 27.6% by HC-II methods.[23] p16 immunostaining studies in OSF depicting its association with HPV are very meagre.

A shift in the pattern of p16 expression from nuclear to cytoplasmic was noted in OSCC. Cytoplasmic expression was seen in OSCC with and without OSF groups showing a significant difference ($P < 0.001$). 6/15 (40%) OSCC with OSF and 7/15 (46.7%) OSCC without OSF showed >75% positivity and thus HPV presence. Our findings indicated the presence and the possible role of HPV in carcinogenesis. However, Balaram *et al.*, reported 74% HPV presence in OSCC using p16 immunostaining.[24] HPV positivity in 66% and 62% cases of OSCC have also been reported by Kojima *et al.*, and Ostwald *et al.*, respectively using PCR.[25,26] Another study by Patil *et al.*, using p16 immunostaining for detection of HPV in OSCC found 87% cases positive. He also suggested a difference in staining pattern with the grades of OSCC and found diffuse staining in moderately differentiated SCC cases.[27] However, no such findings were noted in our study. This may be attributed to the higher number of cases of well-differentiated SCC than moderate and poor. Authors have also found a high specificity and sensitivity with p16 immunostaining in comparison to ISH.[4] Konig *et al.*, studied HPV presence using tissue microarray and found 16.6% cases to be positive, while HPV protein expression was seen in 58% cases. He, thereby, suggested that HPV plays a role in the alteration of p16 protein leading to malignancy.[28] In addition to IHC analysis of p16, various other methods of HPV

detection have also given similar results. However, 31.4% HPV positivity in OSCC was recorded by Chaudary *et al.*, using HC-II method, whereas 52.7% positivity has been noted by PCR.[23] Although p16 IHC has shown high sensitivity and specificity in the advent of HPV detection,[29,30] contradicting outcome have been encountered by Belobrov *et al.*, who suggested that p16 cannot be used as a marker for HPV in OSCC.[31] This necessitates the establishment of appropriate criteria for interpretation of p16 expression to indicate HPV presence.

Our results showed a shift in p16 expression from nuclear expression in OSF to cytoplasmic expression in OSCC. This increase may be probably owing to the HPV-mediated deregulation of Rb and subsequent inactivation of p16, which results in uncontrolled cell proliferation. However, our results were not confirmed using other parallel methods of HPV detection. Moreover, a larger sample size would substantiate our results.

CONCLUSION

To conclude, presence of HPV was not detected in OSF, whereas OSCC cases revealed a positive correlation with HPV. Thus, indicating the role of HPV in the pathogenesis of OSCC using p16- Rb pathway. We propose that the application of discrete criteria for IHC expression of p16 can be used as a marker for HPV detection in OSCC.

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Conflicts of interest
There are no conflicts of interest.

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