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Original Article

Role of Human Papilloma Virus in Oral Squamous Cell Carcinoma of Patients with Smoking and Non-Smoking Habits Based on Immunoexpression of p16: An Immunohistochemical Study

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Abstract

Introduction: Tobacco when consumed in various forms have resulted in carcinogenic impact in oral cavity. Another risk factor is human papillomavirus, which is also closely associated with benign and malignant oral lesions. Therefore, the present study has been carried out to evaluate the immunohistochemical expression and distribution of p16INK4a antibody in oral squamous cell carcinoma and to correlate the association of human papilloma virus in patients with and without tobacco consumption.

Materials and Methods: As the tongue and buccal mucosa remains the most common site for the patients affected with papilloma virus related tumors and oral squamous cell carcinoma, p16 expression has been evaluated in patients who consume tobacco (with habits) and patients who never consumed tobacco (without habits).

Results: In the present study, out of 60 samples, 73 % are males and 27 % are females and out of 30 samples (patients with tobacco habits) 73.3 % patients have the habit of pan chewing and 26.7 % have the habit of smoking. When the buccal mucosa and tongue of patients affected with oral squamous cell carcinoma were compared, expression of p16 didn't show any significant difference in relation to site. The results showed that p16 expression was observed in all the groups, irrespective of patient's intake of tobacco.

Conclusion: It can be concluded that tobacco is a major risk factor for oral carcinogenesis and human papilloma virus can act as an independent risk factor or synergistic factor with tobacco in the development of oral cancer.

Keywords: Tobacco Chewing, Smoking, Oral Squamous Cell Carcinoma, Buccal Mucosa, Human Papilloma Virus.

1. Introduction

Cancer has become one of the most challenging health issues to have plagued the human race. With the change in lifestyle and habits, there has been an increase in the incidence of cancer across the world. More than 95 % of the carcinomas of the oral cavity are of squamous cell type in nature. According to Willis, "a neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissue and persists in the same manner after cessation of stimuli which evoked the change" [1]. Neoplasm

may be benign or malignant, in which malignant tumors are collectively referred to as cancers. The word cancer has been derived from Latin which means crab, because they tend to adhere to any part that they seize on in an obstinate manner. Malignant tumors can invade and destroy adjacent structures and spread to distant sites to cause death [1]. Squamous cell carcinoma is defined as a "malignant epithelial neoplasm, exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges" [2]. Oral squamous cell carcinomas (OSCC) are characterized by multiphasic and multifactorial etiopathogenesis. Tobacco, alcohol and Human Papilloma Virus (HPV) are the most common risk factors for oral malignancy; other factors such as low vitamin A and C and exposure to ultraviolet light are also included [3]. HPV involvement in oral carcinoma was first proposed in 1983 by Syrjanen et al [4] and then supported by others on basis of the fact that HPV shows epitheliotropism and has the ability to immortalize human oral keratinocytes in vitro [3,5]. High risk HPV type 16 and 18 are declared as human carcinogens by the International Agency of Research on Cancer [1].

The HPV family comprises of more than 100 genotypes, classified in accordance with the type of epithelial cells infected and the ability to affect cellular transformation. Certain types of HPV such as HPV1 infect cutaneous epithelial cells, whereas HPV6, 11, 16, and 18 infect mucosal epithelial cells of the oral cavity, oropharynx, anogenital tract, and uterine cervix respectively [5,6]. Chewing, smoking of tobacco products and consumption of alcoholic beverages have become social habits in India. The prevalence of these habits are more common among men compared to women [7]. These habits have been positively associated with oral lesions such as Oral submucous fibrosis, Leukoplakia and Oral lichen planus which has the potential for malignant transformation [7,8]. Smoking has been considered as a relevant public health problem and are related to more than 50 different pathological alterations. The harmful influence of smoking on oral tissues are due to the fact that it contains some 300 carcinogenic compounds that are converted into reactive metabolites capable of interacting with DNA by the action of oxidative enzymes. Out of these, 60 have been known for carcinogenic action, especially polycyclic aromatic hydrocarbons and tobacco specific nitrosamines found in the tar [9]. Alcohol are associated with cell hyperproliferation (which increases vulnerability to inhaled or ingested carcinogens), thereby producing metabolites with carcinogenic action, such as acetaldehyde, induction of enzymes that activate procarcinogens and reduction of retinoic acid. The consumption of alcohol, especially ethanol, interferes with DNA repair and can have an immunosuppressive effect [10].

Simultaneous exposure to tobacco and alcohol are significantly associated with a higher risk of developing OSCC, because these substances show a synergistic effect. In patients not exposed to the risk factors, the lesions develop primarily in the oral cavity, especially in the tongue, alveolar ridge, gingiva and in individuals who smoke and drink alcohol, the tumors occur mostly in the larynx, hypopharynx, posterior tongue, retro-molar trigone and mouth floor [9]. Therefore, the purpose of the present study is to evaluate the immunohistochemical expression and distribution of p16 in OSCC and to correlate the association of HPV in patients possessing adverse oral habits and patients with no adverse oral habits.

2. Materials and Methods

This study was conducted on the archival retrieved formalin fixed, paraffin embedded tissues obtained from the Department of Oral Pathology and Microbiology. The

study group included histologically diagnosed 30 cases of OSCC present in the buccal mucosa (Group A, n = 15) and tongue (Group B, n = 15) of patients with chewing and smoking habits as well as 30 cases of OSCC found in the buccal mucosa (Group C, n = 15) and tongue (Group D, n = 15) of patients possessing no such adverse oral habits. Two subsequent sections, with 4µ thickness were cut for each case from the formalin fixed, paraffin embedded tissues histologically diagnosed as OSCC. These sections were treated with the immunohistochemical reagent (p16INK4a antibody).

2.1. Paraffin Blocks

The following equipments and reagents were used for the entire study. All the reagents were purchased from Biogenex Life Sciences Private Limited, unless specified otherwise.

Equipments	Reagents
Microtome Hot Water bath Hot Plate Microwave Oven Measuring Jars Coplin Jars Pipettes Timer Absorbent wipes Binocular Light Microscope APES coated microscopic slides Coverslips for microscopic slides	1. Primary antibody: p16INK4a 2. Secondary antibody Kit a-Super Enhancer (Enhances the signal) b-Poly Horseradish Peroxidase (HRP), Anti-rabbit and mouse IgG with enzyme polymer 3. Blocking agents: 3 % Hydrogen peroxide 4. Chromogen: DAB 3,3'–diaminobenzidine substrate 5. 4 % Chromic acid [63 g of Potassium dichromate + 1L of distilled water + 600 ml of sulphuric acid] 6. Deionized water 7. Soap water 8. Acetone 9. Amino Propyl Ethoxy Silane (APES): 144 ml of acetone + 6 ml of APES 10. Buffers: a-Phosphate Buffer Saline (PBS): pH 7.4 b-TRIS EDTA Buffer: pH 9 11. Counter stain (Harris Hematoxylin) 12. Xylene 13. Mounting media: Diestyrene Di-N-Butyl Phthalate in Xylol (DPX)

2.2. Immunohistochemistry Procedure

The following protocol was used for the slide preparation. 1) Slides were kept in 4 % chromic acid overnight. 2) Following this, they were washed in water (5 changes). 3) Additional wash in soap water was performed for 2 hours and 4). Slides were finally air dried.

2.3. APES Coating (144 ml of acetone + 6 ml of APES)

The slides were placed inside the trough along with slide holder for 5 mins. They were blotted for 2 mins and subsequently rinsed in distilled water container for additional 2 mins. In the last step, the slides were dried in a microwave oven at 60° C overnight.

2.4. Tissue Preparation

1. In the first step, formalin fixed paraffin embedded tissues were sectioned at 4 µm and mounted on APES coated slides.
2. Following this, they were placed in incubator (60° C) overnight. Then, the slides were deparaffinised and rehydrated through using the following steps.

Solution	Incubation Time
Xylene	10 mins
Xylene	10 mins
Absolute Alcohol	10 mins
Absolute Alcohol	10 mins
Running Tap Water	10 mins

3. By using EDTA buffer in microwave oven, antigen retrieval was performed as follows.
30° C for 5 mins
50° C for 5 mins
70° C for 5 mins
4. Then the solution was allowed to cool at room temperature. They were washed in running water and then in distilled water.
5. Peroxidase blocking by using 3 % Hydrogen Peroxide for 10 mins at room temperature.
6. Slides were then washed in PBS for 5 mins.
7. Slides were then treated with power block for 10 mins.
8. Slides were incubated with primary antibody (p16INK4a) for one and half an hour
9. Slides were washed with PBS for 5 mins. Slides were incubated with secondary antibody Super enhancer for 30 mins and then washed in PBS for 5 mins. Finally, they were treated with Poly HRP for 30 mins.
10. Excess was wiped off and slides were washed with PBS; 2 changes for 5 mins.
11. Slides were incubated with DAB Substrate Chromogen for 7 to 10 mins.

2.5. Preparation of DAB substrate: 1 drop of DAB + 1ml DAB buffer

12. Slides were rinsed with distilled water for 5 mins
13. Slides were counterstained with 1 dip of Harris Hematoxylin followed by bluing in running tap water.
14. Slides were air dried.
15. Sections were mounted using DPX.

2.6. Positive Control

Control sections that include tissue parts and already have been diagnosed as cervix squamous cell carcinoma. Assessment of antigen expression cells was performed using light microscope at x10 and x40 magnification. The antigen positive cells normally stain brown in colour.

2.7. Analysis of Immunoreactivity of p16INK4a

An arbitrary semi quantitative analysis was carried out to determine the immunohistochemical expression pattern and graded according to the intensity of staining which was assessed when nucleus and cytoplasm exhibited strong positivity.

2.8. Immunoreactivity of p16INK4a

As described by Ruediger et al [11], expression profile was divided into different categories as follows.

Negative (0) : <1% of the cells are positive

Sporadic (1) : Isolated cells were positive, but < 5 %

Focal (2): Small cell clusters, but 25 % of the cells are positive

Diffuse (3): > 25 % of the cells were stained (*weak, moderate and strong staining pattern observed were based on the staining intensity). The results obtained among the 4 groups were statistically analyzed using the software Statistical Package for Social Sciences (SPSS), IBM corp., version 16.

3. Result

This study was performed retrospectively with four groups that consisted of histologically diagnosed 60 cases of OSCC found in the buccal mucosa and tongue of patients with habits of tobacco consumption and patients with no adverse oral habits (Table 1).

Table 1. Selection of Study Samples Based on Site of the Lesion

OSCC Affected Group	No of Cases
Buccal mucosa with tobacco consumption (Group A)	N = 15
Tongue with tobacco consumption (Group B)	N = 15
Buccal mucosa without any oral habits (Group C)	N = 15
Tongue without any oral habits (Group D)	N = 15
Total Samples	N = 60

Out of the total sample size, Group A consisted of 53.3 % males and 46.7 % females, Group B consisted of 100 % males, Group C comprised of 80 % males with 20 % females and Group D comprised of 60 % males with 40 % females. Overall, the distribution observed for males and females were 73.3 % and 26.7 % respectively. Based on their habits, the study subjects representing Group A and B were further divided into 2 types, where 73.3 % and 26.7 % were found to be pan chewers and smokers respectively.

Comparison of p16 Expression between Groups

All the groups were evaluated for the expression of p16. Among the groups, Group A, B and C possessed the maximum value in 'Score 0' whereas in Group D alone maximum value was observed in 'Score 1' (Table 2, Figure 1). In other words, from Group A to C, highest number of cases have witnessed negative expression (Score 0, < 1 % positive cells), while that of in Group D highest number of cases were found to be sporadic (Score 1, positive cells > 1 % and < 5 %). In order to compare the four different groups, Chi Square test was performed between all the groups and found to be statistically not significant (*P* value = 0.441).

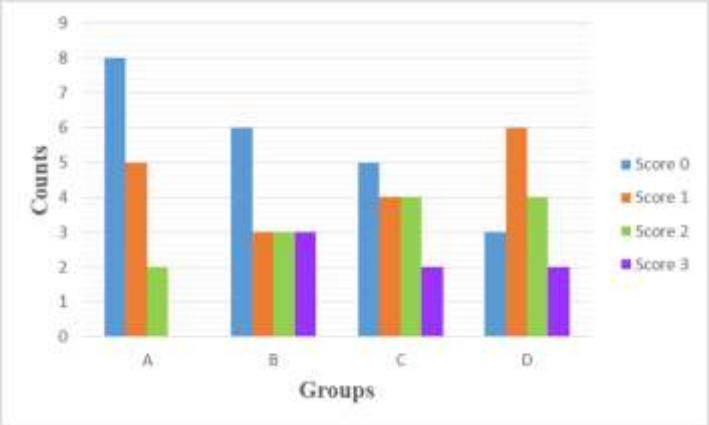


Figure 1. Bar Diagram Representation of p16 Expression in Groups

Table 2. p16 Expression between Groups

S.No	Groups	Scores (Frequency)				*P* Value
		Score 0 (Negative)	Score 1 (Sporadic)	Score 2 (Focal)	Score 3 (Diffuse)	
1.	Buccal mucosa with tobacco consumption (Group A)	8	5	2	0	
2.	Tongue with tobacco consumption (Group B)	6	3	3	3	0.441*
3.	Buccal mucosa without any habits (Group C)	5	4	4	2	
4.	Tongue without any habits (Group D)	3	6	4	2	

*$P > 0.05$ is insignificant

While the study subjects in Group A possessed negative expression (< 1 % positive) in 8 cases, sporadic (< 5 % positive) in 5 cases and focal (25 % positive) staining pattern in 2 cases, among the subjects of Group B, about 6 cases possessed negative expression (< 1 % positive) and 3 cases each possessed sporadic, focal & diffuse staining pattern (Table 2, Figure 1). Similarly, for the study subjects in Group C, about 5 cases possessed Score 0 (negative expression), 4 cases each exhibited Score 1 (sporadic) and Score 2 (focal) and 2 cases possessed Score 3 (diffuse pattern). As for Group D, about 3 cases acquired Score 0 (negative expression), 6 cases acquired Score 1 (sporadic), 4 cases acquired Score 2 (focal, 25 % positive) and 2 cases acquired Score 3 (diffuse pattern) (Table 2, Figure 1). When the Chi Square test was performed for Group A and B combined, it showed that the difference was statistically not significant (*P* = 0.263). Likewise, Chi Square test was also performed for Group C and D combined where the resultant *P* value (0.825) was also found to be statistically insignificant.

negative expression in 8 cases, sporadic in 10 cases, focal in 8 cases and diffuse staining pattern in 4 cases. The calculated *P*

Comparison between Group A & C and Group B & D

Using Chi Square test, the p16 expression of Group A and C (OSCC affected buccal mucosa possessing tobacco consumption and no consumption respectively) were compared (Table 3). The obtained *P* value of 0.325 was found to be statistically not significant. Similarly, the p16 expression of subjects representing Group B and D (OSCC affected tongue with and without tobacco consumption respectively) were also compared (Table 3). In this case too, the resultant *P* value (0.504) was found to be statistically insignificant.

Overall Frequency

Irrespective of the site of lesion, among the 30 samples histologically diagnosed as OSCC (retrieved from patients having tobacco consumption), negative expression was seen in 14 cases whereas sporadic, focal and diffuse staining pattern was observed in 8, 5 and 3 cases respectively. Likewise, the remaining 30 samples diagnosed as OSCC (retrieved from patients without having any oral habits) showed value was found to be statistically insignificant (0.441, Table 4 & Figure 2).

Table 3. p16 Expression between Group A & C and Group B & D

S.No	OSCC Affected Group	Scores (Frequency)				*P* Value
		Score 0 (Negative)	Score 1 (Sporadic)	Score 2 (Focal)	Score 3 (Diffuse)	
1.	Buccal mucosa with tobacco consumption (Group A)	8	5	2	0	0.325*
2.	Buccal mucosa without habits (Group C)	5	4	4	2	
3.	Tongue with tobacco consumption (Group B)	6	3	3	3	0. 504*
4.	Tongue without habits (Group D)	3	6	4	2	

**P* > 0.05 is insignificant

Table 4. p16 Expression between Patients with Tobacco Consumption (Group A & B Combined) and Patients without Oral Habits (Group C & D Combined)

S.No	OSCC Affected Group	Scores (Frequency)				*P* Value
		Score 0 (Negative)	Score 1 (Sporadic)	Score 2 (Focal)	Score 3 (Diffuse)	
1.	Patients with tobacco consumption (Group A & B)	14	8	5	3	0. 441*
2.	Patients without tobacco consumption (Group C & D)	8	10	8	4	

**P* > 0.05 is insignificant

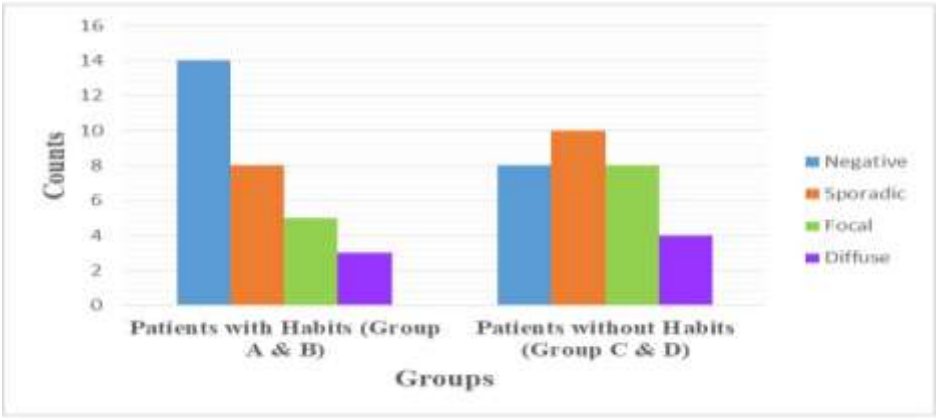
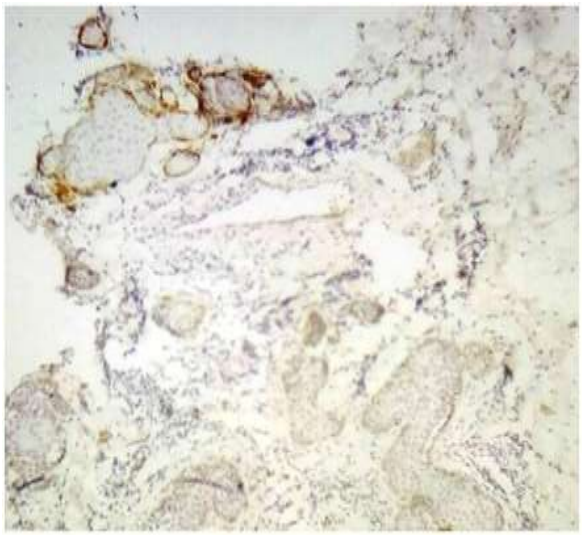
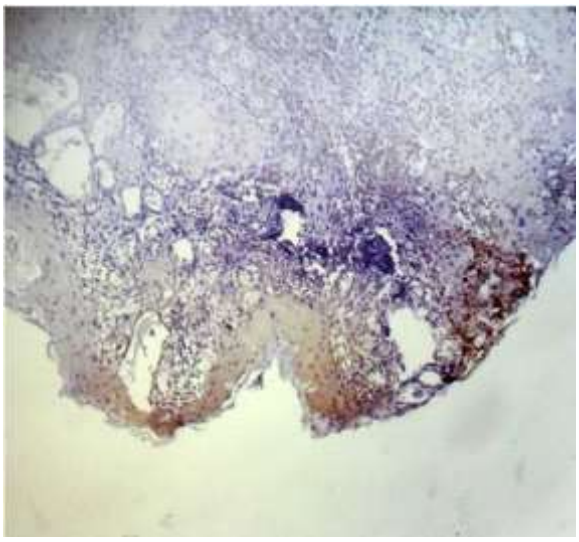


Figure 2. Bar Diagram Representation of p16 Expression between Patients having Tobacco Consumption (with habits) and No Consumption (without habits)

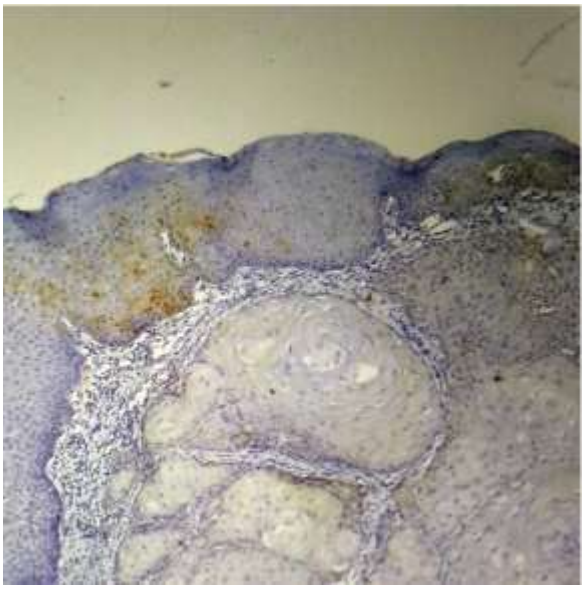


3a

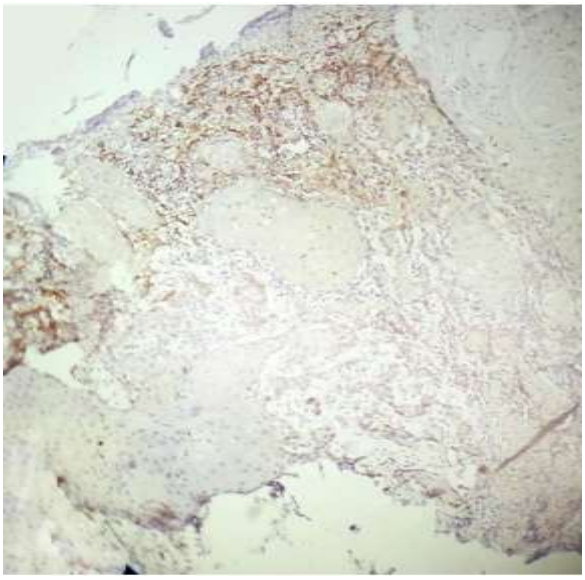


3b

Figure 3a-3b. p16 Expression of OSCC affected Buccal Mucosa and Tongue of Group A and B respectively. Both the Group comprised of patients possessing tobacco consumption.



3c



3d

Figure 3c-3d. p16 Expression of OSCC affected Buccal Mucosa and Tongue of Group C and D respectively. Groups C and D belongs to patients possessing no tobacco consumption. All the four images represent the sporadic expression (Score 1, positive cells > 1 % and < 5 %).

4. Discussion

Oral cancer is one of the most deteriorating health conditions distributed globally with increasing incidence and mortality rate. OSCC has been ranked 12th most common cancer in the world [12]. It has been the most common form of carcinoma affecting the oral cavity and accounts for almost 40 % of all cancers in the Indian subcontinent. Around 300,000 patients are annually estimated to have oral cancer worldwide [13]. Several risk factors are related to this type of cancer with the main being tobacco use, alcohol consumption and infection by high-risk genotypes of Human papilloma virus (HPV) [3]. The relationship between HPV and oral carcinoma was first suggested in 1983 by Syrjanen et al. As in cervical cancers, HPV types 16 and 18 are the cause of malignant transformation. E6 and E7 oncoproteins

play a significant role in causing squamous cell carcinoma. Smoking causes cellular and structural alterations in tonsils, leading to an increased oral acquisition of HPV and are known to suppress the mediators of immune function thus facilitating persistence of HPV infection [14]. It also induces DNA damage which may favor the integration of HPV to human genome at fragile sites or 'hot spots' of DNA breakage [15] thus enhancing the oncogenic potential of virus.

Tobacco/pan masala chewing causes local injury to buccal mucosa and thereby creates an environment for virus infection easily [16] and betel quid along with viral infection causes mutagenic steps in the carcinogenic process [17]. In 2008, Vaccarella et al have reported that tobacco smoking was associated with significant, although moderate, increased risk of HPV infection and it interferes with prevalence of HPV infection [18]. In 2012, Fabina et al have stated that even nonsmoking individuals have a greater probability of developing tumors related to HPV than individuals who smoke [19]. Hence, using this as a parameter, the present study has been done to evaluate the expression of HPV in OSCC and to correlate the association of HPV in patients with and without oral habits by using p16 as an immunohistochemistry marker.

As the tongue remains the most common site for HPV related tumors and buccal mucosa along with tongue for the patients of OSCC, p16 expression was compared and evaluated among these sites comprising of 4 groups (Figure 3a-3d). Possessing the habit of tobacco consumption and no such consumption remained the critical difference between the groups. While the patients of Group A and B had the habit of tobacco smoking and pan chewing, patients of Group C and D were free of nonsmokers as well as non pan chewers. Out of 60 samples, about 73 % were represented with males and 27 % with females. Among the 30 samples that consisted of patients with tobacco consumption (Group A and B), about 73.3 % patients exhibited the habit of pan chewing while the remaining 26.7 % possessed the habit of smoking.

By using a set of histological criteria, histological grade was used for quantifying the degree of differentiation. While the low-grade lesions represent tumours which are well-differentiated, high grade lesions denotes poorly differentiated tumours. In the present study, for all the 4 groups (N = 60) maximum number of lesions were found to be negative expression with 36.6 % (22 cases). Lesions that were stained in sporadic (Score 1) and focal (Score 2) pattern constituted 30 % (18 cases) and 21.6 % (13 cases) respectively. The high-grade lesion exhibiting diffuse pattern (Score 3) was prevalent in 11.6 % (7 cases). Irrespective of the site of lesion, in the present study the expression of p16 did not show any significant difference. When the OSCC affected lesions retrieved from the patients possessing tobacco consumption (Group A and B, P = 0.263) and patients possessing no such consumption (Group C and D, P = 0.825) were compared, the resultant P value was found to be statistically not significant. It was in concordance with the study of Smith et al 2010 where they concluded that no statistical difference was exhibited between p16 expression and tumor site. Similarly, in the clinical studies reported by Anaelle Duray et al, Seyyed Hosse et al and Megha Ralli et al, they have stated that in addition to tobacco and alcohol, HPV also plays a role in the development of oral cancer by acting as a co-carcinogen [20-22].

5. Conclusion

This study was carried out to determine the expression of p16 in squamous cell carcinoma affected patients with and without habits of tobacco consumption. A positive correlation between HPV infection and OSCC was found in the present study. In various OSCC affected groups, the expression of p16 exhibited weak to strong

staining pattern irrespective of patient's intake of tobacco. This can be attributed to the statement that tobacco is a major risk factor in both HPV positive as well as negative cases. Furthermore, HPV can act as an independent risk factor or shows synergistic as well as additive effect with tobacco in causing oral cancer. Based on the present study, p16 immunohistochemical expression can be suggested as a surrogate marker for high risk HPV infection and the development of oral cancer. They may also play a role in early cancer detection, tumor localization and post treatment surveillance. Further studies need to be carried out with larger sample size involving various grades of OSCC to unravel more details of the molecular events involving HPV and tobacco habits that drive the oral carcinogenesis.

Conflict of interest

The authors declare no conflict of interest.

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