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Original Research Article

Assessment of P16INK4A Expression in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma and Its Correlation with the Histological Grade of the Tumour

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Conflict of interest: Nil

Abstract:

Background: The objective of current study is to evaluate the role of HPV-16 in the pathogenesis of oral epithelial dysplasias (OED) and oral squamous cell carcinoma (OSCC) by immunohistochemistry (IHC) and to know whether HPV-16 participates in disruption of the regulation of p16 INK4A suppressor protein in OED and OSCC by IHC.

Methodology: The present prospective study was done at Department of Pathology, ICARE Institute of Medical Sciences and Research and Dr. B.C.Roy Hospital, Haldia between the periods of December 2022 to May 2024 with a sample size of 130 cases after taking Institutional Ethics Committee approval. The following findings were noted down like demographic information, past and family history, history of present illness, and personal history. In clinical examination and type of the lesion (ulcerative, proliferative lesion, ulceroproliferative lesion, white patch and submucosal fibrosis) were noted.

Biopsy samples obtained from patients with oral squamous cell carcinoma and premalignant lesions were fixed in 10% formalin within 30 minutes and for not more than 48 hours (ideal is 12-24 hours). It was then processed and embedded in paraffin wax according to the standard protocols. Thin section of 4 micron were cut and then stained with hematoxylin and eosin. The biopsy specimens were processed in the automated tissue processor for preparation of paraffin blocks as per standard guidelines. The tissue sections were stained routinely with Hematoxylin and Eosin stain as per standard guidelines. Paraffin blocks which were most representative of tumour tissue were chosen for performing immunohistochemistry IHC for p16.

Results: Dysplastic lesions were more common in the age group of 41-50 years (43.6%) while the maximum number of cases (35.1%) S.C.C was in the age group of 51-60 years. Overall male predominance was in both oral dysplastic lesions (84.6%) and OSCC (73.6%). Male: Female ratio was 5.5:1 in dysplastic lesion and 2.8:1 S.C.C. Tongue was the most common site (46.1%) followed by buccal mucosa (43.6%) for dysplastic lesions and the most common site for S.C.C was buccal mucosa (39.5%) followed by tongue (26.4%).Majority of patients with dysplastic lesions presented with leukoplakia (79.5%0 followed by OSMF (10.3%) and maximum number of patients with S.C.C was growth in the oral cavity (60.4%) followed by ulcerative lesion (20.9%) and ulceroproliferative lesion (16.5%). Histopathology revealed maximum number of dysplastic lesions was mild dysplasia (41%) and well differentiated SCC (60%). The p16 positivity rate was 55.38%. Patients in the age group of 21-30 years showed 91.6% positivity. Below 50 years the p16 positivity was 77.7% and above 50 years the positivity was 69.5%.Thus the younger population showed more positivity for p16 than the older population.

Conclusion: Diffuse pattern of 16 expressions was not seen in dysplastic lesion which ultimately helps to differentiate dysplastic lesion from malignancy. P16 proves to be of great help in diagnosing the histological grades in OSCC and dysplastic lesions which ultimately helps to rule out subjective variation in histological diagnosis.

Keywords: Oral cancer, oral epithelial dysplasia, squamous cell carcinoma (SCC), P16INK4A expression, immunohistochemistry, histological grade.

Introduction

Cancer is a disease of cells that escape the control mechanisms of orderly cell growth and acquire the ability to proliferate, invade normal tissues and metastasize. Oral cancer is a major and growing global public health problem [1]. It is one of the most common cancers worldwide and a leading cause of mortality [2]. The term oral cancer is used to describe any malignant neoplasm of oral cavity. In India, oral cancer is among the top three types of cancer with an age standardized incidence rate of 12.6 per 100000 populations [3]. It is more among Indian male approximately 30% of the cases. According to GLOBOCAN 2020, in India there will be 2.1 million new cancer cases by 2040, an increase of 57.5% from the year 2020 [4]. More than 90% of oral malignant tumors are oral squamous cell carcinoma (OSCC) [5]. It is commonly found on floor of mouth, cheek lining, gingiva, tongue but most commonly affects the buccal mucosa among Indian populations. The incidence is progressively increasing in South-East Asian nations [6].

P16INK4A Immunohistochemistry serves as a potential marker for oral mucosal dysplasia and malignant transformation [7-9]. Tumor suppressor p16INK4A or p16 is a member of INK4 family of cyclin dependent kinase inhibitors. The expression of p16 retains Rb-family proteins in a hypophosphorylated state that help in binding of E2F and help to achieve G1 cell-cycle arrest. The losses of expression of p16 are observed in oral premalignant lesion and primary tumour of oral cavity. The inactivation mechanism includes homozygous gene deletion, gene mutation and hypermethylation of upstream CpG island regions [10].

Carcinogenic effect of HPV is characterized by two major virally encoded oncogene E6 that inactivates p53 and E7 which inactivates RB gene. When E7 binds to RB, releasing E2F, causing the cell to enter the S phase, that results in cell cycle disruption, proliferation and malignant transformation leading to over expression of tumour suppressor protein p16INK4A that inhibit phosphorylation of RBE2F complex [11-13]. The p16 expression is now being used as a surrogate marker of HPV infection in squamous cell carcinoma, and thus it is expected that the differences can be made depending on endemicity of HPV infection. The significance of oncogenic HPV infection and its relationship to the diagnosis and prognosis of patient remain an important matter of debate, especially the contradictory result found in the literature [14, 15]. Hence the present study has been undertaken to assess the p16INK4A in oral dysplastic lesion and oral squamous cell carcinoma and also evaluates its role in the diagnosis of OSCC and epithelial dysplastic lesions and its correlation with the histological grading.

Materials & Methods

The present prospective study was done at Department of Pathology, ICARE Institute of Medical Sciences and Research and Dr. B.C.Roy Hospital, Haldia between the periods of December 2022 to May 2024 with a sample size of 130 cases after taking Institutional Ethics Committee approval.

Inclusion criteria

- Patients histologically confirmed to have oral dysplastic lesions
- Patients histologically confirmed to have squamous cell carcinoma

Exclusion criteria

- Patients who are unwilling to provide informed consent for this study
- Patients having non-squamous cell carcinoma
- Patients having any prior chemotherapy or radiotherapy or undergone surgical treatment
- Patients having other systemic disease
- Patients presenting with pregnancy

After obtaining informed written consent in each case, a detailed clinical history was collected from all patients followed by a thorough clinical examination. The findings were recorded in a proforma. The followings were noted down like demographic information, past and family history, history of present illness, and personal history. In clinical examination and type of the lesion (ulcerative, proliferative lesion, ulceroproliferative lesion, white patch and submucosal fibrosis) were noted.

Biopsy samples obtained from patients with oral squamous cell carcinoma and premalignant lesions were fixed in 10% formalin within 30 minutes and for not more than 48 hours (ideal is 12-24 hours). A cross linking fixative 10% neutral buffer formalin (NBF) used in most surgical laboratories is an ideal one. But still formalin is regarded as the satisfactory one. It was then processed and embedded in paraffin wax according to the standard protocols. Thin section of 4 micron were cut and then stained with hematoxylin and eosin. The biopsy specimens were processed in the automated tissue processor for preparation of paraffin blocks as per standard guidelines. The tissue sections were stained routinely with Hematoxylin and Eosin stain as per standard

guidelines. Paraffin blocks which were most representative of tumour tissue were chosen for performing immunohistochemistry IHC for p16. Biogenex ready to use mouse monoclonal antibody was used for this purpose. The basic principle, as with any other special staining method is a sharp localization of target components in the cell and tissue, based on a satisfactory signal-to-noise ratio. Amplifying the signal, while reducing non-specific background staining (noise) is the major strategy to achieve a satisfactory and practically useful result.

Interpretation of Result/Scoring System [16]

Nuclear and /or cytoplasmic staining in cells was considered positive for P16INK4A IHC. The biopsies were scored as positive when more than 5% cells (cut-off) stain positive and was graded as:

- Negative - 0-55 nuclei and/ or cytoplasm positive.
- Sporadic - 5-10% of nuclei and/ or cytoplasm with weak and scattered positivity
- Focal - ≥ 30% of nuclei and / or cytoplasm strongly positive, spreading in one tissue area.
- Diffuse - ≥ 85% of nuclei and / or cytoplasm strongly positive, spreading at several tissue areas.

Biopsies with sporadic pattern were considered to have low IHC overexpression of P16INK4A. Focal distributions were considered as moderate overexpression and diffuse positivity as high overexpression [16].

The result was noted down in tabular form. The numbers of cases in each category were also expressed in the form of percentages. The whole data was analyzed using Statistical Package for Social Sciences (SPSS) version 21 software.

The categorical variables were compered by means of Chi-square test to find out the association between the different variables. A value of $P<0.05$ was taken as statistically significant. Kaplan Meier Survival analysis was used to find out the survival rate.

Results

The present study was carried out in the department of Pathology, ICARE Institute of Medical Sciences and Research, Haldia during the period from December' 2022 to May' 2024. Total 130 cases of histologically diagnosed oral epithelial dysplasia and oral squamous cell carcinoma were subjected to immunohistochemistry with p16, out of which 39 cases were of oral epithelial dysplasia and 91 cases were of oral SCC [Table 1].

Table 1: Age distribution of dysplastic lesions and SCC

Age Group	Dysplastic Lesions (N=39)	SCC (n=91)
21-30	3 (7.7%)	12 (13.2%)
31-40	7 (17.9%)	14 (15.4%)
41-50	17 (43.6%)	19 (20.9%)
51-60	5 (12.8%)	32 (35.1%)
61-70	5 (12.8%)	11 (12.1%)
71-80	2 (5.2%)	3 (3.3%)

Table 1 shows the age of the patients ranging from 21 to 80 years.

Maximus numbers of cases (43.6%) of dysplastic lesions were in the age group 41-50years, while the maximum numbers of cases (35.1%) of S.C.C were in the age group of 51-60 years [Table 1]. Dysplastic lesions showed negative, sporadic and focal pattern of staining. None of the cases showed diffuse pattern of staining. A significant association was seen between p16 expression and age (p=0.000) [Fig. 1].

P16 expression was found to be positive in the age group and the association between age and p16 was significant [Fig. 2].

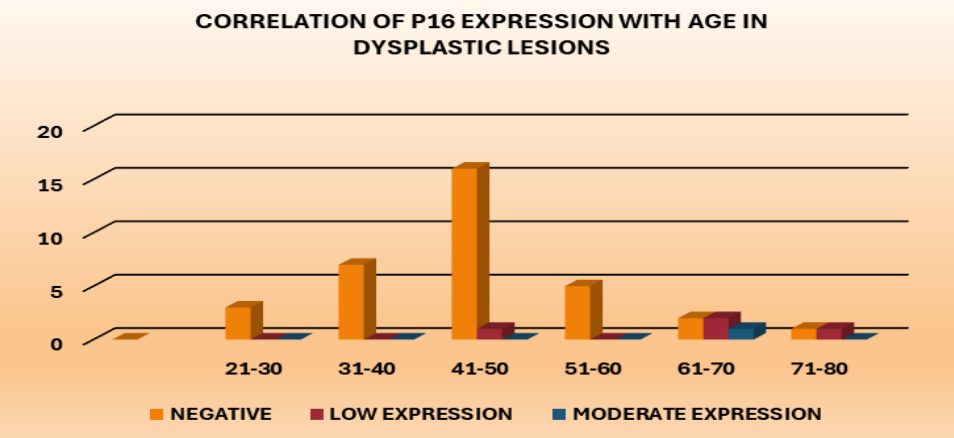

Figure 1: Correlation of P16 expression with age in dysplastic lesions

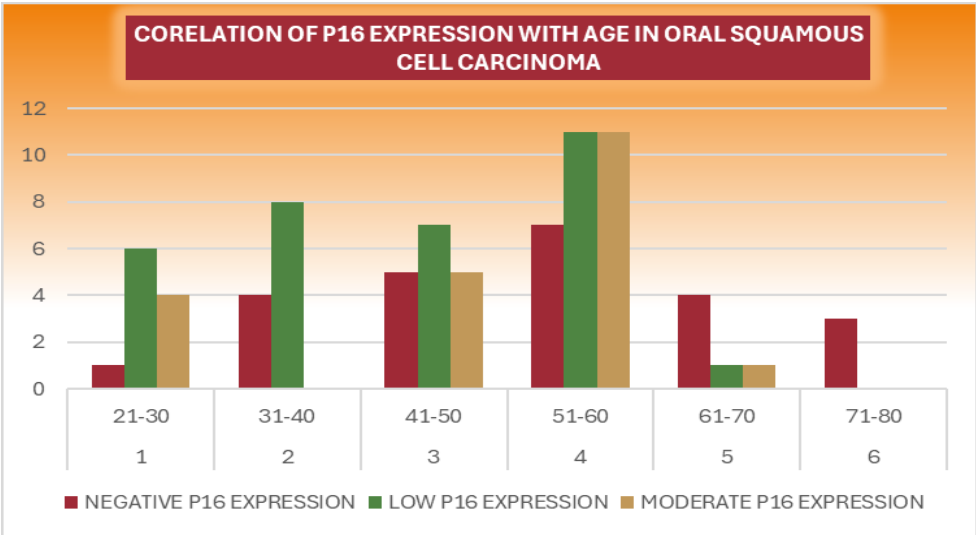

Figure 2: Correlation of P16 expression with age in oral squamous cell carcinoma

Fig. 3 shows there was a male predominance in both oral dysplastic lesions (84.6%) and oral squamous cell carcinoma (73.6%). Male: Female ratio was 5.5:1 in dysplastic lesions and 2.8:1 in SCC.

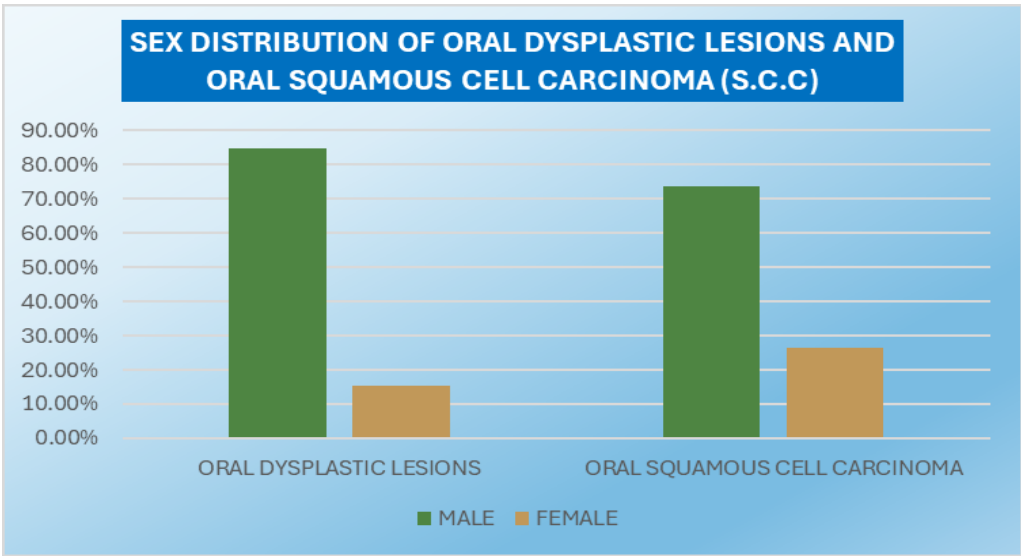

Figure 3: Sex distribution of oral dysplastic lesions and oral squamous cell carcinoma (S.C.C)

Table 2: Correlation of P16 expression with sex in dysplastic lesion

Sex	Negative P16 Expression	Low P16 Expression	Moderate P16 Expression	P-Value
Male	28	04	01	
Female	06	00	00	0.5936

Premalignant lesions showed negative, sporadic and focal pattern of staining. None of the cases showed diffuse pattern of staining [Table 2]. No significant association was seen between p16 expression and sex (P=0.2065) [Table 3].

Table 3: Correlation of P16 expression with sex in S.C.C

Sex	Negative P16 Expression	Low P16 Expression	Moderate P16 Expression	High P16 Expression	P-Value
Male	16	22	19	10	
Female	08	11	02	03	0.2065

Figure 4 shows clinical sites of dysplastic lesions and SCC. Tongue was the most common site (46.1%) followed by buccal mucosa (43.6%) for dysplastic lesions. The most common site for S.C.C was buccal mucosa (39.5%) followed by tongue (26.4%).

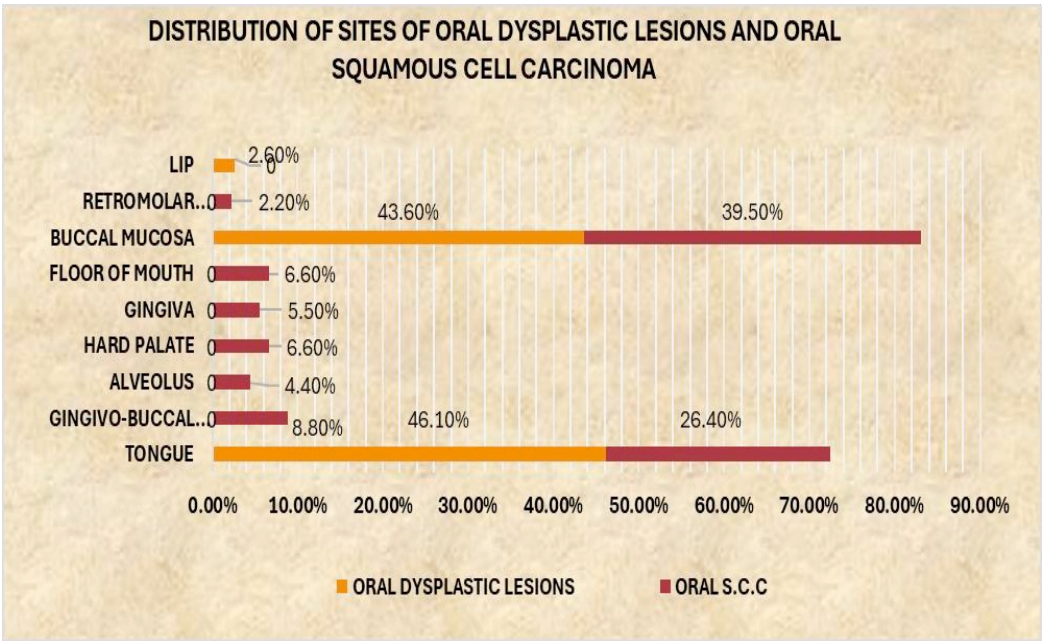

Figure 4: Distribution of sites of oral dysplastic lesions and oral squamous cell carcinoma

Table 4: Correlation of P16 expression with site of lesion in dysplastic lesions

Site	Negative P16 Expression	Low P16 Expression	Moderate P16 Expression	P-Value
Tongue	16	02	00	
Hard Palate	00	01	01	
Floor of the Mouth	01	00	00	0.002
Buccal Mucosa	16	01	00	
Lip	01	00	00	

A significant association was seen between p16 expression and site of lesion (P=0.002) [Table 4].

Table 5: Correlation of P16 expression with site of lesion in oral SCC

Site	Negative P16 Expression	Low P16 Expression	Moderate P16 Expression	High P16 Expression	P-Value
Tongue	08	07	04	05	
Gingivo-Buccal Sulcus	00	08	00	00	
Alveolus	02	00	01	01	
Hard Palate	00	00	05	01	

Gingiva	01	00	04	00	0.000
Floor of the Mouth	05	01	00	00	
Buccal Mucosa	08	17	06	05	
Retromolar trigone	00	00	01	01	

A significant association was seen between p16 expression and the site of lesion (P=0.000) [Table 5].

Table 6: Histological grading of dysplastic lesions

Grade	No of Cases	Percentage
Without Dysplasia	10	25.6%
Mild Dysplasia	16	41%
Moderate Dysplasia	08	20.5%
Severe Dysplasia	05	12.9%

Out of 39 dysplastic lesions 10 cases (25.6%) showed without dysplasia, 16 cases (41%) showed mild dysplasia, 08 cases (20.5%) showed moderate dysplasia and 5 cases (12.9%) showed severe dysplasia [Table 6].

Table 7: Correlation of P16 expression with histological grading in oral dysplastic lesions

Grade	Negative P16 Expression	Low P16 Ex-pression	Moderate P16 Expression	P-Value
Without Dysplasia	10	00	00	0.106
Mild Dysplasia	14	02	00	
Moderate Dysplasia	06	02	00	
Severe Dysplasia	04	00	01	

Table 7 shows grading of oral dysplastic lesion is not significantly associated with p16 expression (P=0.106).All lesions without dysplasia and majority of lesions with mild dysplasia (92.3%) was negative for p16 expression, low (sporadic pattern) expression was seen in moderate dysplasia (25%) and severe dysplastic lesions (20%) shows moderate expression of p16 [Fig. 7-8].

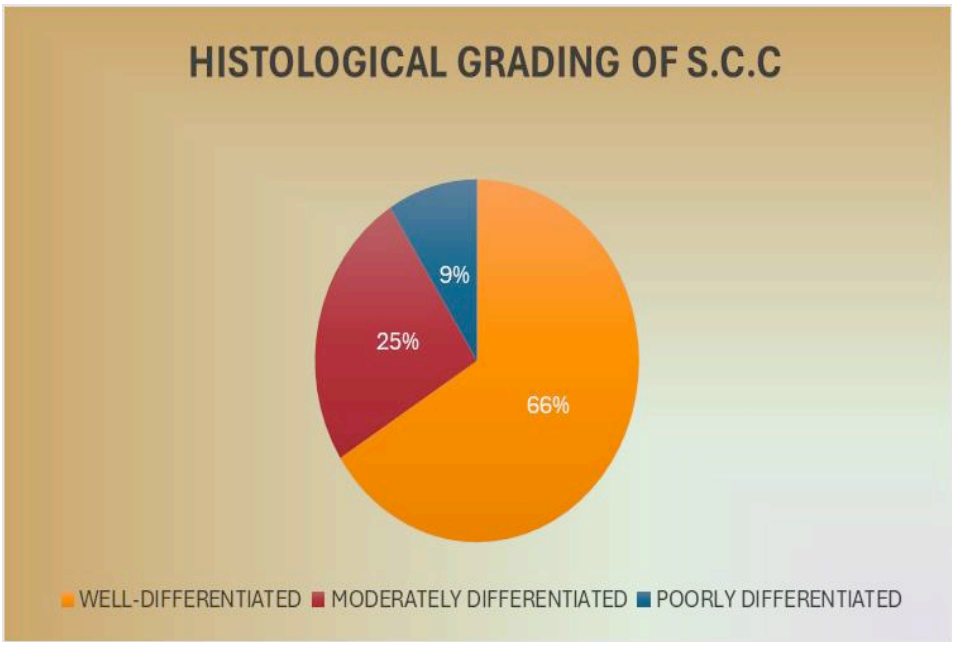

Figure 5: Histological grading of oral squamous cell carcinoma SCC

Majority cases (60%) were well-differentiated SCC followed by 23 (25%) cases of moderately differentiated SCC. Poorly differentiated SCC were least common constituting 9% of cases [Fig. 5].

Table 8: Correlation of P16 expression with histological grading in oral SCC

Grade	Negative P16 Expression	Low P16 Expression	Moderate P16 Expression	High P16 Ex-pression	P-Value
Well-Differentiated	19	31	10	00	
Moderately Differentiated	04	01	10	08	
Poorly Differentiated	01	01	01	05	0.000

Table 8 shows majority of well differentiated tumors (51.6%) showed low (sporadic pattern) p16 expression, moderately differentiated tumors (43.5%) displayed moderate (focal pattern) and high (diffuse pattern) of p16 expression was seen in poorly differentiated tumors (62.5%). A significant association was seen between grade of oral S.C.C and p16 expression (P=0.000) [Fig 6].

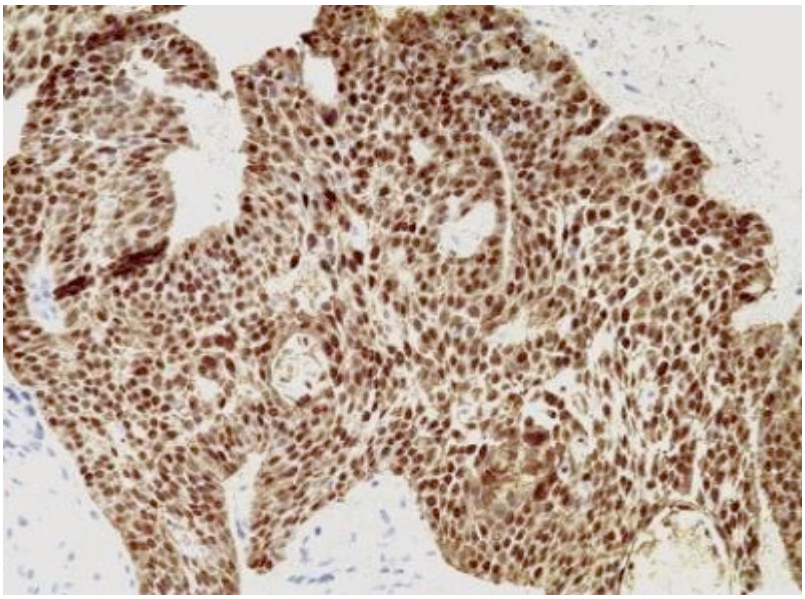

Figure 6: Positive control tissue of cervical squamous cell carcinoma, p16 IHC (X400)

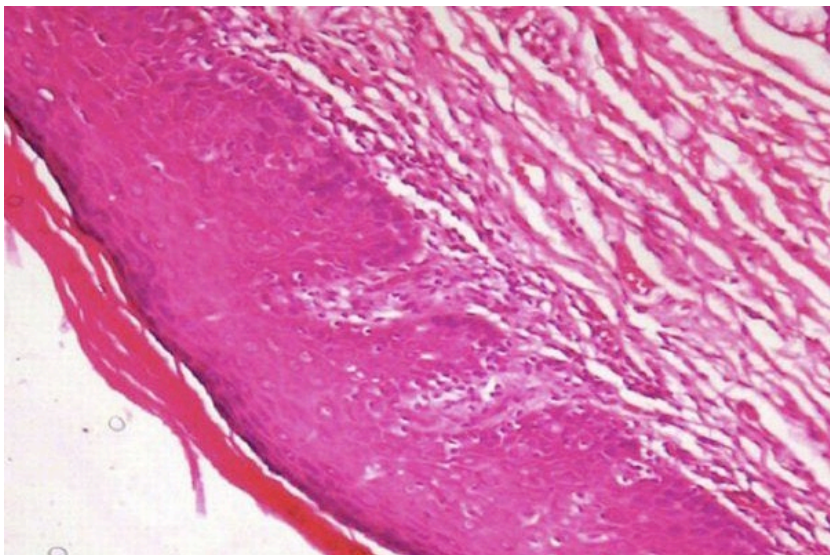

Figure 7: Mild Dysplasia H & E stain (X400)

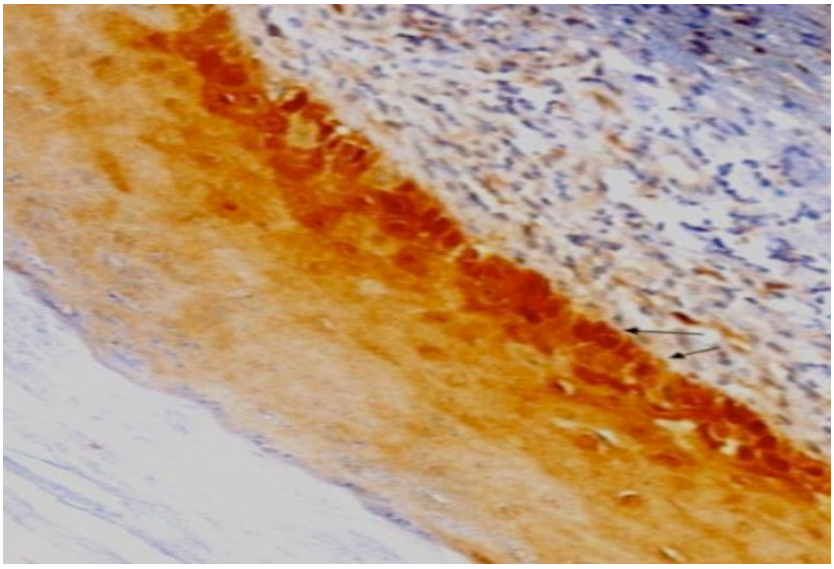

Figure 8: Positive P16 expression in mild dysplasia.

Discussion

The aim of the current study was to further investigate p16 immunohistochemistry as a potential biomarker for dysplastic and oral squamous cell carcinoma using a large series of oral cavity biopsies with the goal of determining its utility for routine clinical practice. The present study was carried out in the department of pathology, ICARE Institute of Medical Sciences and Research, Haldia from December'2022 to May'2024, which includes 39 cases of dysplastic lesion and 91 cases of oral SCC.

In this study the age of patient of oral rage was from 21- 80years. It was observed that maximum cases (35.1%) where in the age group 51-60 years. The youngest patient was 23 years and the oldest was 76 years of age. The male: female was 2.8:1. Age of the patient with dysplastic lesion male: female = 5.5:1). In each group there was overall male predominance. This was comparable to the result obtain by Shenoi R et al., 2012 [17] male: female ratio was 4.18:1 and Singh MP et al., 2015 [18] male: female ratio was 3:1. The most common site of Oral SCC was buccal mucosa followed by tongue, alveolus, gingiva, lower lip, floor of mouth, retromolar trigone and gingivobuccal sulcus. Tongue (46.1%) was most common site followed by buccal mucosa in dysplastic lesions.

Richant et al. in 1986 and Brown et.al in 1970 [19] got similar results. However, Pradyot Prakash et.al (2013) [20] found that tongue was the most common site for S.C.C. and buccal mucosa for oral dysplastic lesion. Maximum number (60.4%) of patient of SCC presented clinically as growth in the oral cavity followed by 20.9% patient presented with an Ulcerative lesion, 16.5% presented with ulceroproliferative lesion and one patient each presented with leukoplakia and non-healing ulcer. In case of oral dysplastic lesion majority of the patient presented with leukoplakia (79.5%) followed by OSMF (10.3%), erythroplakia and leukoerythroplakia.

Majority of SCC patient (63.74%) had tumor of greatest dimension of more than 2 cm. minimum size was 0.7 cm. and maximum size was 4.5cm.The maximum size of lesion in dysplastic cases 1.5 and minimum was 0.8 cm. Majority of the cases (66%) where well differentiated SCC followed by 25% cases of moderately differentiated SCC. Poorly differentiated tumor was least common constituting 9% of the cases. Tissue section of 41% showed mild dysplasia followed by 20.5% moderate dysplasia and 12.9% severe dysplasia while 25.6% showed no dysplasia.

P16 expression:

Out of total 130 cases 72 cases (55.38%) were positive for p16 expression (inclusive of all grades), while 58 cases (44.62%) cases were negative for p16 expression. Out of 91 cases of Oral SCC, 24 cases (26.37%) were negative for p16 expression. Out of 67 positive cases (73.62%) of SCC, 33 cases (36.26%) showed low p16 expression, 21 cases (23%) showed moderate p16 expression and 13 cases (14.28%) showed high expression of p16. Out of 39 cases of dysplastic lesion 4 cases (10.25%) cases showed low p16 expression and 1 case (2.56%) showed moderate expression. In the present study expression of p16 in OSCC was 73.62% which was comparable to Pradyot Prakash et.al [20] (72%) and Fregonesi et al. [21] (69%).

The present study indicates two different subsets of the lesions on the basis of p16 INK4A expression as 73.62% cases of poral SCC and 12.82% cases of leukoplakia were positive for the over expression of the set protein. Correlation of p16 expression was assessed with various clinic pathological variables. In SCC significant association was seen

between age and p16 expression (p= 0.000). The p16 expression was found to be 91.6% positive in the age group of 21-30 years. Below 50 years the p16 expression was 77.7% while above the positivity rate of p16 expression was 69.5%.

No significant association was seen between p16 and tumor site (p=0.702) which was in concordance with the study of Smith et al. [22], which also shows no statistical difference in p16 expression with site of tumour (p=0.081). Histological grades are a mean of quantitating a degree of differentiation by applying a set of histological criteria. In the present study p16 expression had a significant correlation with a histological grade of the tumour (p =0.000).

Majority of the well differentiated tumour showed low expression of p16 (sporadic pattern, moderately differentiated tumour showed focal pattern or moderate expression of p16 and high p16 expression (diffuse pattern) was seen in poorly differentiated tumour. In case of dysplastic lesion, majority of the lesion with mild dysplasia and without dysplasia were negative for p16 expression, sporadic pattern was seen in cases of moderate dysplasia and severe dysplasia showed focal or moderate p16 expression (p= 0.106). Similar to present study, Yuen et.al [23] and Dragomir et al [24] observed no significant association between p16 expression and tumor grades. In contrast to the present study, smith et al (p = 0.02) and Muirhead et. al. (p= 0.001) observed in their study that p16 overexpression was more likely to be detected with higher grade [25].

Bradley et al. in their study concluded that p16 expression is not useful in differentiating dysplastic from non-dysplastic oral lesions although decreased p16 expression with increasing severity of dysplasia was observed [26]. Buajeeb W et al. studied the frequency of p16 expression in10 cases of normal mucosa, 15 cases of OL without dysplasia, 15 cases of OL with dysplasia and 16 cases of OSCC and found positive staining in OL without dysplasia (26.7%) and OSCC (12.5%) with no statistically significant difference in the frequency of p16 expression among OSCC, OL with and without dysplasia, and normal mucosa. This is consistent with our findings as no significant difference was observed in the expression of p16 among normal mucosa, OL and OSCC [27]. In Agrawal GP et al study (2013) histopathologically diagnosed 20 cases of OED and 20 cases of OSCC were selected from amongst the patients attending the OPD of Vasantdada Patil Dental College and Hospital, Sangli. Biopsy tissue sections were then tested for HPV-16 by IHC. HPV-16 positive tissue sections were then again tested by p16 by IHC. Overall 22.5% of cases in our study were found to be positive for HPV 16 which includes 10% of cases of OED and 35% cases of OSCC. Amongst the HPV 16 positive cases, more than 60% of cells were positive for p16INK4A IHC in OED (50%) and OSCC (85.71%). Thus, HPV 16 participates in disruption of the regulation of p16INK4A suppressor protein and can be used as surrogate biomarker for detection of HPV infection in OED and OSCC [28]. These differences could also be attributed to difference in sample size. Distribution of tumor site, different scoring criteria and different types of antibodies were used by different authors.

Limitation of the study

Manual antigen retrieval technique that is used in the present study has its own limitations compared to automated one. Period of the study is too short for this study which may account for discrepancies with other studies that has been observed during the comparison of various parameters. The follow up period is too short, so survival analysis of OSCC cannot be included in this study.

Conclusion

Diffuse pattern of 16 expressions was not seen in dysplastic lesion which ultimately helps to differentiate dysplastic lesion from malignancy. P16 proves to be of great help in diagnosing the histological grades in OSCC and dysplastic lesions which ultimately helps to rule out subjective variation in histological diagnosis.

Ethical approval

Ethics approval has been taken from IEC, IIMSAR, Haldia, and West Bengal.

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