ISSN No:-2456-2165

Expression and Distribution of Galectin-1 and Np-63/ Immuno-Histochemical Markers in Potentially Malignant Oral Disorders with and without Dysplasia

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Abstract:-

Background: The objective of the study was to investigate the immune-histochemical expression of two proteins, galectin-1 and Np-63, in oral malignancy and lesions with and without epithelial dysplasia. The study aimed to compare the expression of these proteins in normal oral epithelium, oral squamous cell carcinoma (OSCC), potentially malignant oral disorders (PMODs) with and without epithelial dysplasia.

Methods: Immuno-histochemical staining for galectins-1 and Np-63 was evaluated in 25 samples of normal oral epithelium, 25 samples of oral squamous cell carcinoma, 25 samples of PMODS with dysplasia and 25 samples of PMODs without dysplasia. Analyzed the data using the Chi-Square test. The result were presented descriptive statistics (frequency and percentage), it was adopted the value of P <0.05 for statistical significance.

Results: No significant difference was found in the inter group comparison of Gal-1 and Np-63 expression, indicating no abrupt change in expression of these markers in epithelium of normal mucosa, PMODs with and without dysplasia and OSCCs.

Conclusion: Regarding the prognosis of different PMODs with and without dysplasia and different grades of OSCCs, no significant difference was found in the expression of both IHC markers. Therefore, the role of these markers as predicting/prognostic markers is questionable.

Keywords:- *OSCC, Epithelial Dysplasia, PMODS, IHC, Immuno-staining, Gal-1& Np-63.*

I. INTRODUCTION

In India, Oral Squamous Cell Carcinoma (OSCC) ranks first amongst male and third in female population in head & neck region related to the use of various forms of tobacco in association with other factors. Despite progress in therapeutic approaches, the 5-years survival rate for OSCC has not improved significantly over the past several decades, and it remains about 50%, making this disease a serious public health problem.[1,2]

Although, tumor-nodes-metastasis (TNM) staging system has proven to be a useful prognostic tool, the biological behavior of individual tumors still remains unpredictable.[1,2]

Potentially Malignant Oral Disorders (PMODs) are lesions or conditions that precede malignancies of the oral cavity with variable transformation rates. PMODs with dysplasia have higher chances for malignant transformation as compared to lesions without epithelial dysplasia.[3]

Most of the OSCCs are preceded by some PMODs for variable duration, which may or may not convert into OSCC.[3] To comment on presence or absence of epithelial dysplasia and severity of dysplasia (mild/moderate/severe/carcinoma in situ), is a subjective matter and has good chances of false reporting. To overcome this problem, one should search for certain methods, which may define the presence or absence of epithelial dysplasia and objectively differentiate between mild, moderate or severe epithelial dysplasia.

Immuno-histochemistry is a powerful tool for studying the cellular and molecular processes that underlie various diseases, including cancer. It can provide valuable insights into the pathogenesis and progression of disease, and can guide the development of new diagnostic and therapeutic approaches.[3]

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Therefore, the present study was undertaken to evaluate the expression Np-63 and Galectin-1 (Gal-1) immuno-histochemical (IHC) markers in OSCC, PMODs with and without dysplasia and in normal mucosa.

II. MATERIALS AND METHODS

A. Setting and Design

The study was retrospective, undertaken using paraffin embedded tissue blocks of diagnosed cases of different histological grades of OSCC, PMODs with or without dysplasia and normal oral mucosa, which were obtained from the archives of Oral Pathology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh with Registration No(Dean/2021/EC/2715)dated23-06-2021.

The sample size was calculated from G*Power software version 3.1.9.7. As summing large differences in the expression of IHC markers among different study groups (effect size= 0.4), keeping power at 90% (P) and alpha error at 0.05. The sample size calculated was 24 per group, which was adjusted to 25 per group. In total 100 cases were included in the study and divided into 4 groups:

Group I: 25 cases of OSCC of different grades.
Group II: 25 cases of PMODs with dysplasia.
Group III: 25 cases PMODs without dysplasia.
Group IV: 25 cases of normal oral mucosa (NOM) as controls (who underwent minor oral surgical procedures for other purposes).

However, cases with previous history of malignancy and under treatment cases (surgery, chemotherapy, radiotherapy), cases with metastatic tumors in the jaws, cases that were under medication for oral epithelial dysplasia were excluded from the study.

Four sections (3-4 micron) each from 100 paraffin-embedded tissue blocks were obtained. Two of these sections were subjected to IHC staining using Gal-1 and Np63 tumor markers, while the other two sections were stained with hematoxylin and eosin (H&E) using a standard protocol. The IHC staining procedure was performed according to the Bio Genex IHC protocol.

B. Evaluation of the Np-63 and Gal-1 immunostaining

The assessment of Np-63 and Gal-1 positive cells was carried out using a compound binocular light microscope at 10x, 20x, and 40x magnifications. The presence of a brown colored end product at the site of the target antigen (i.e., membrane /cytoplasm or nucleus) indicated positive immune-reactivity. The internal positive controls included erythrocytes and vascular endothelium, which demonstrated strong Np-63 positivity (Figure1-3).

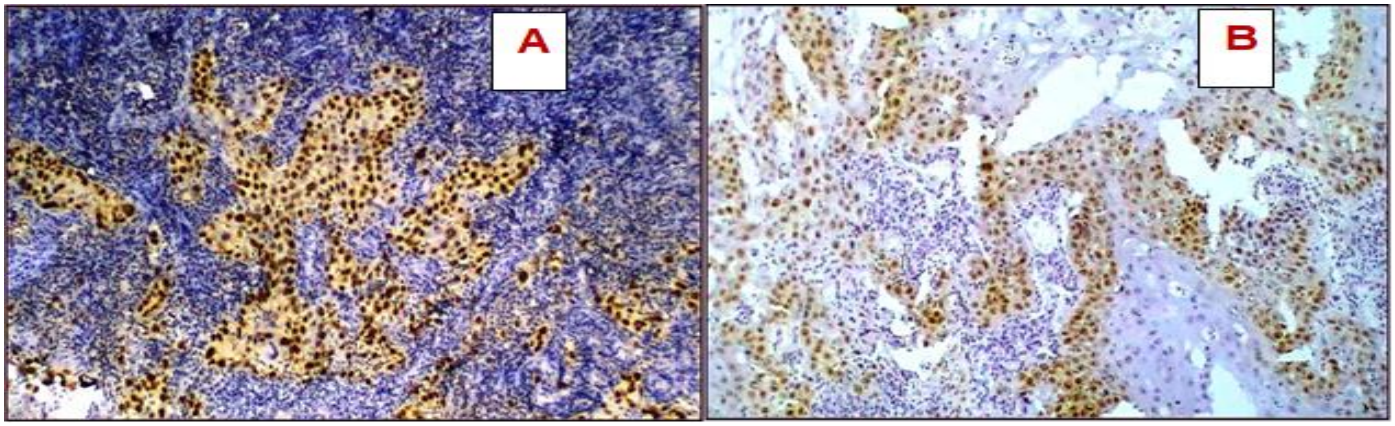

Fig. 1: Showing positive expression of Gal-1 (A) and Np-63(B) in Oral Squamous Cell Carcinoma (Group I) (10x)

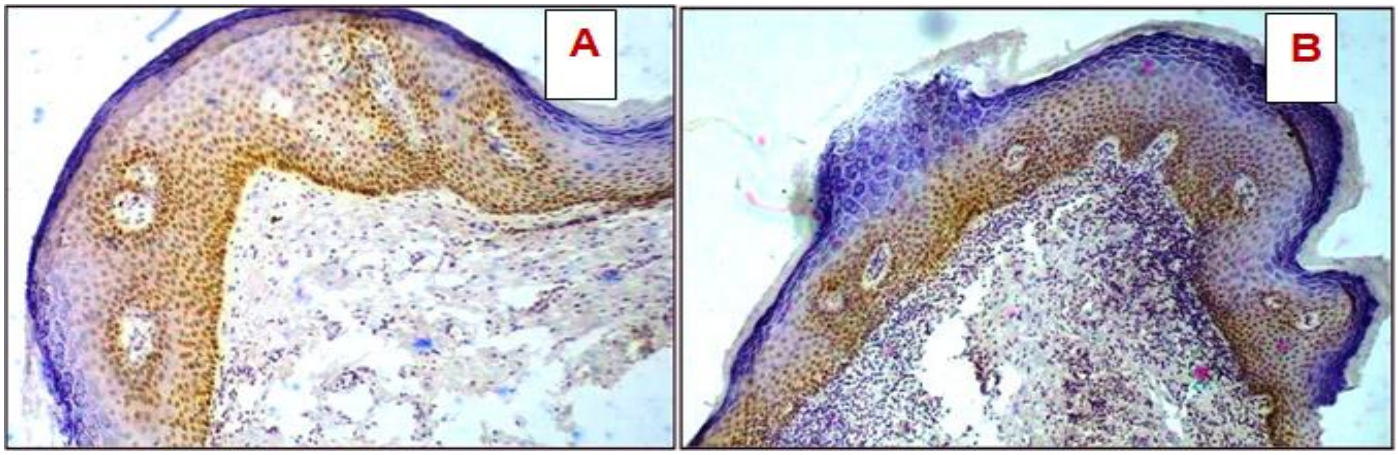

Fig. 2: Showing positive expression of Gal-1 (A) and Np-63 (B) in PMOD's With Dysplasia(Group II) (10x)

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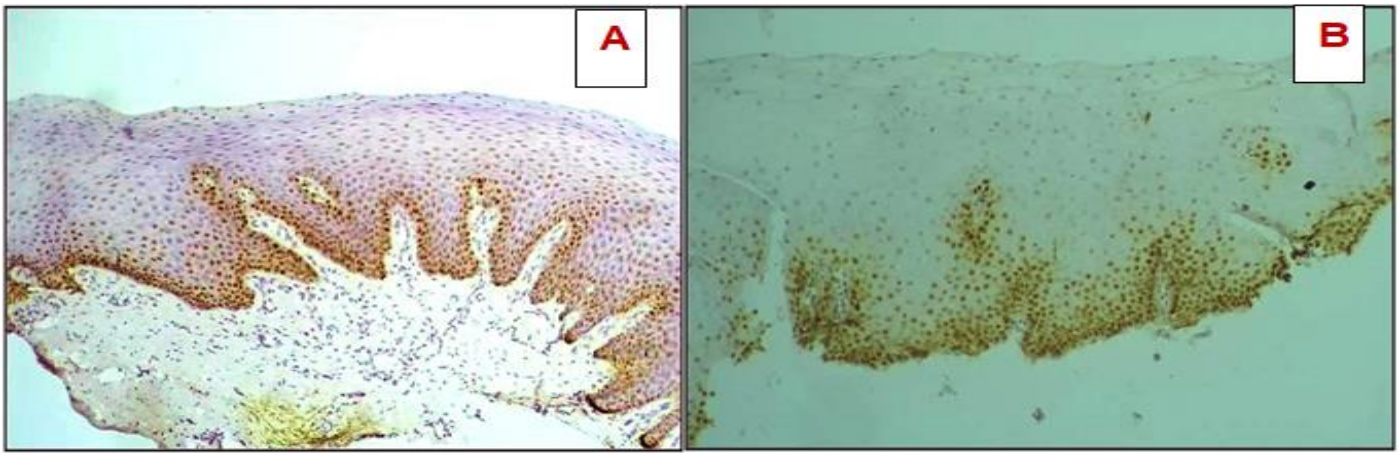

Fig. 3: Showing positive expression of Gal-1 (A) and Np-63 (B) in PMOD's Without Dysplasia (Group III) (10x)

A semi-quantitative analysis was performed for each case, evaluating the percentage of positive cells and the staining intensity for Np-63 and Gal-1 at high power. The level of expression of these markers was assessed in the epithelium (i.e. lower, middle, and upper) according to the criteria adapted from Carvalho et al (2010).[4]

All the cases were analyzed and the results were compared with normal oral mucosa. To eliminate inter observer bias, the IHC stained slides were evaluated by two independent observers.

C. ScoringforNp-63andGal-1

Immuno-reactive Score (IRS) of each case was calculated by the system proposed by **Fedchenko (2014) *et al*** [5], which gives IRS a range of 0-12 as a product of multiplication between positive cells proportion (0-4) and staining intensity score (0-3). The scores were adapted to calculate the expression of IHC markers Np63 and Gal-1**(Table-1).**

Table 1: Immuno-reactive scores (IRS) criteria for analyzing IHC staining

Percentage of positive cells	X Intensity of staining	IRS(0-12)	IRS-classification
0=No positive cells	0=no color reaction	0-1=negative	Negative
1=<10%ofpositivecells	1=mild reaction	2-3	Weak expression
2=10-50%positivecells	2=moderate reaction	4-8	Mild expression
3=51-80%ofpositivecells	3=intense reaction	9-12	Strong expression
4=>80%positivecells			

D. Statistical Tests Used

The data was analyzed using SPSS software version 23. Level of significance was kept at 5%. Results were presented using descriptive statistics (frequency & percentage). Distribution of each IHC marker among different study groups was compared using Chi-square test. Immuno-reactive scores (IRS) among different study groups were also compared using Chi square test.

III. RESULTS

The distribution of Gal-1 in oral epithelium varied amongst different groups. Gal-1 was expressed in the upper one-third of the epithelium only in Group I (OSCC). In Group I, 15 cases (60%) showed expression in the middle third, 6 cases (24%) showed expression in the upper one third, and only 4 cases (16%) showed expression in the lower one third of the epithelium (Table2).

In contrast, the expression of Gal-1 was absent in the upper one third of epithelium in the other groups. Statistical analysis indicated a significant difference (p=0.001) among the different groups, suggesting that the distribution of Gal-1 in oral epithelium is group-specific (Table 2).

Also expression of Np-63 was found in the upper one third of the epithelium, only in Group I. In 48% (12) of cases in this group, it was expressed in the middle third, and in 32% (8) of cases, it was expressed in the upper one third. Only 20% (5) of cases showed expression in the lower one third of the epithelium. However, in the other groups, Np63 expression was absent in the upper one third of the epithelium. Statistical analysis of the data showed a significant difference in the distribution of Np-63among different groups, specifically in terms of its expression in various layers of the epithelium(epithelial localization).(Table2) (Figure 4).

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Table 2: Distribution (Epithelial Localization) of IHC markers Gal 1and Np63 in different study groups

Group	Marker	Lower	Middle	Upper	Total	P Value
OSCC(Group I)	Gal1	4(16%)	15(60%)	6(24%)	25(100%)	0.694
	Np63	5(20%)	12(48%)	8(32%)	25(100%)	
PMOD's With Dysplasia(Group II)	Gal1	16(64%)	9(36%)	0.00	25(100%)	1.00
	Np63	15(60%)	10(40%)	0.00	25(100%)	
PMOD's Without Dysplasia (Group III)	Gal1	9(36%)	16(64%)	0.00	25(100%)	1.00
	Np63	9(36%)	16(64%)	0.00	25(100%)	
Normal(Group IV)	Gal1	13(52%)	12(48%)	0.00	25(100%)	1.00
	Np63	14(56%)	11(44%)	0.00	25(100%)	

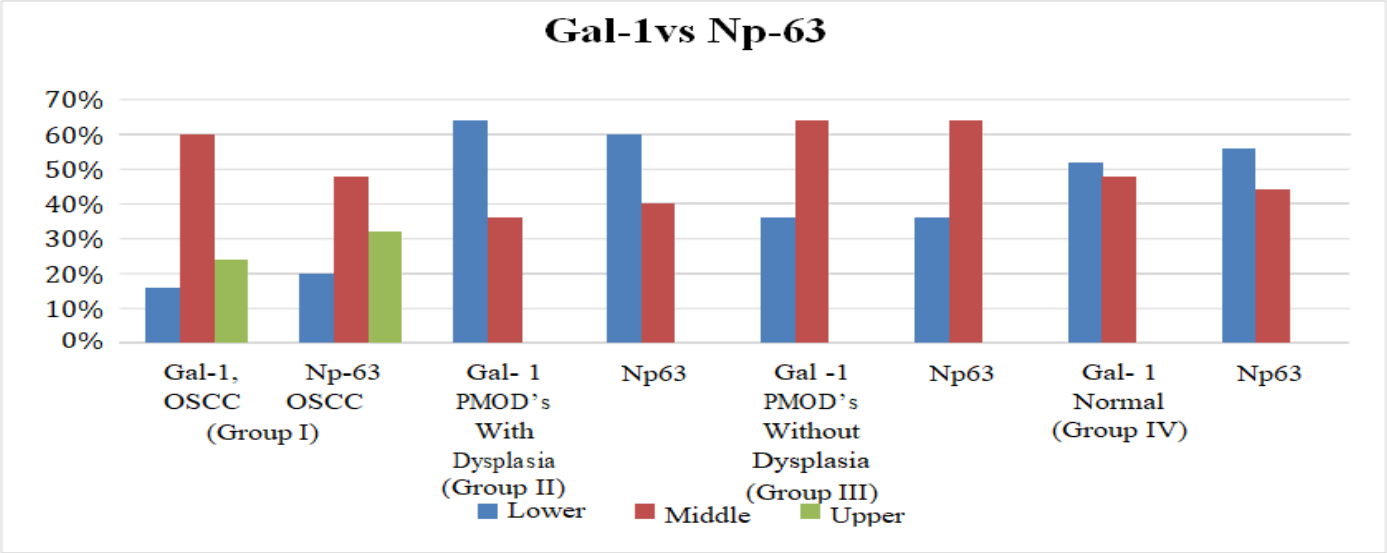

Fig. 4: Distribution (Epithelial Localization) of IHC markers Gal-1 and Np-63 in different study groups

Based on the inter group comparison of epithelial localization of Gal-1, it appears that there is a significant difference in the distribution between study group I and the other study groups (II, III, and IV) (Table 3). The statistical analysis suggested that the differences were significant for Group I vs. Group II, Group I vs. Group III, and Group I vs. Group IV, respectively. (Table3).

Table 3: Inter group comparison of epithelial localization of Gal-1

Group	P Value
OSCC vs PMOD's With Dysplasia (Group I vs Group II)	0.001*
OSCC vs PMOD's Without Dysplasia (Group I vs Group III)	0.019*
OSCC vs Normal (Group I vs Group IV)	0.004*
PMOD's With Dysplasia vs PMOD's Without Dysplasia (Group II vs Group III)	0.089
PMOD's With Dysplasia vs Normal (Group II vs Group IV)	0.567
PMOD's Without Dysplasia vs Normal (Group III vs Group I)	0.393

The inter group comparison of epithelial localization of Np-63 showed high significant difference among inter group distribution between study group I and other groups (study group II, III, and IV). Np-63 is expressed in upper one third of epithelium in Group I only while not in other groups (pvalue≤0.001). (Table4).

Table 4: Inter group comparison of epithelial localization of Np-63

Group	P Value
OSCC vs PMOD's With Dysplasia (Group I vs Group II)	0.001*
OSCC vs PMOD's Without Dysplasia (Group I vs Group III)	0.008*
OSCC vs Normal (Group I vs Group IV)	0.002*
PMOD's With Dysplasia vs PMOD's Without Dysplasia (Group II vs GroupIII)	0.156
PMOD's With Dysplasia vs Normal (Group II vs Group IV)	1.000
PMOD's Without Dysplasia vs Normal (Group III vs GroupI)	0.256

IRS was calculated for the distribution of both Gal-1 and Np-63 indifferent study groups. The maximum number of cases showed mild expression of Gal-1 in all groups with no significant difference among different groups. (p=0.062)(Table5) (Figure5). Similarly, maximum number of cases in all groups showed mild expression of Np-63, and there was no significant difference among the different groups (p=0.112) (Table5).

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Table 5: Analysis of IRS of Gal-1vs Np-63 among different study groups

Group	Marker	Strong+ve	Mild+ve	Weak+ve	Total	P Value
OSCC(Group I)	Gal1	9(36%)	16(64%)	0(0%)	25(100%)	1.00
	Np63	9(36%)	16(64%)	0(0%)	25(100%)	
PMOD's With Dysplasia (Group II)	Gal1	3(12%)	22(88%)	0(0%)	25(100%)	0.702
	Np63	5(20%)	20(80%)	0(0%)	25(100%)	
PMOD's Without Dysplasia(Group III)	Gal1	3(12%)	22(88%)	0(0%)	25(100%)	1.00
	Np63	3(12%)	22(88%)	0(0%)	25(100%)	
Normal(Group IV)	Gal1	3(12%)	22(88%)	0(0%)	25(100%)	1.00
	Np63	3(12%)	22(88%)	0(0%)	25(100%)	

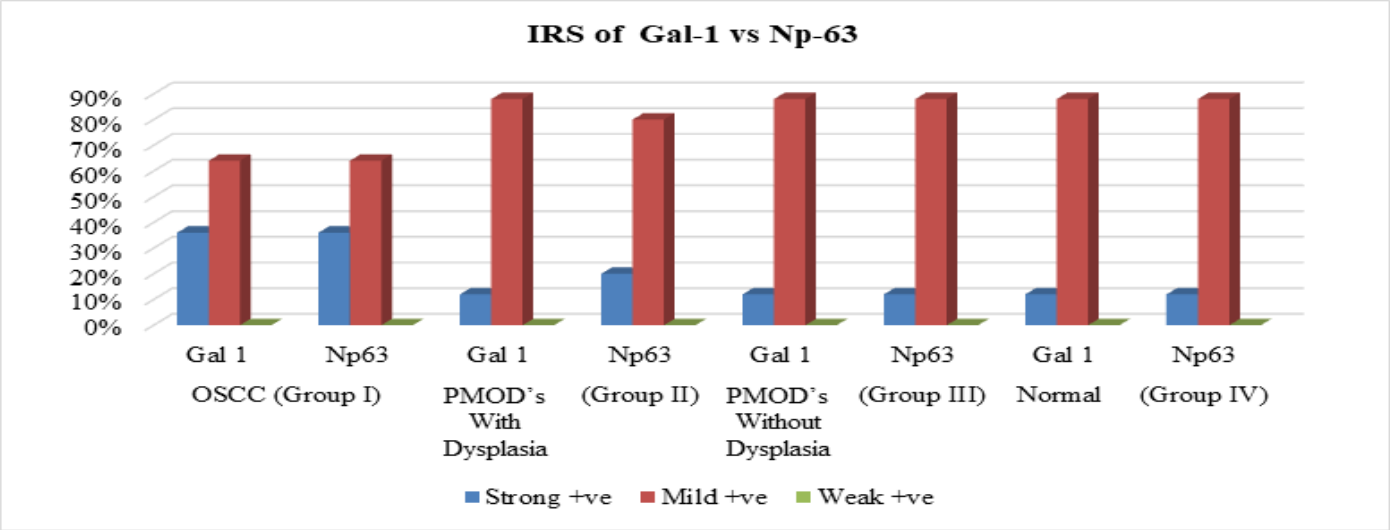

Fig. 5: Analysis of IRS of Gal-1vs Np-63among different study groups

No significant difference was found in the inter group comparison of IRS of Gal-1 and Np-63 expression (Table 6, 7), indicating no abrupt change in expression of these markers in epithelium of normal mucosa, PMODs with and without dysplasia and OSCCs.

Table 6: Inter group comparison of Immuno-reactive Scores of Gal-1

Group	P Value
OSCC vs PMOD's With Dysplasia(Group I vs Group II)	0.095
OSCC vs PMOD's Without Dysplasia(Group I vs Group III)	0.095
OSCC vs Normal (Group I vs GroupIV)	0.095
PMOD's With Dysplasia vs PMOD's Without Dysplasia(Group II vs Group III)	1.00
PMOD's With Dysplasia vs Normal (Group II vs GroupIV)	1.00
PMOD's Without Dysplasia vs Normal (Group III vs GroupI)	1.00

Table 7: Inter group comparison of Immuno-reactive Scores of Np-63

Group	P value
OSCC vs PMOD's With Dysplasia(Group I vs Group II)	0.345
OSCC vs PMOD's Without Dysplasia(Group I vs Group III)	0.095
OSCC vs Normal (Group I vs Group IV)	0.095
PMOD's With Dysplasia vs PMOD's Without Dysplasia(Group II vs Group III)	0.702

IV. DISCUSSION

OSCC is a multi factorial disease. The high prevalence and incidence of OSCC in the Indian subcontinent is likely due to a combination of genetic, environmental, and life style factors.[2]

The aim of the present study is to characterize the distribution and expression of Gal-1 and Np-63 tumor markers in the epithelium of normal oral mucosa, PMOD's with and without dysplasia ad OSCC's. In addition, IRS is calculated to assess the distribution and expression of both Gal-1 and Np-63 in oral epithelium to make it more subjective.

Sundberg J *et al* (2021)[7]studied the expression of Np-63 in various layers of epithelium in recurring and non-recurring oral leukoplakia and observed that there was not statistically significant difference in the expression of Np-63 in various layers of epithelium. The results are in

ISSN No:-2456-2165

consistent with our study

However, in other studies conducted by **Chen *et al* (2005) [8], Kovesi G *et al* (2006)[9] and Ramasubramanian A *et al* (2013)[10],** it was found that there is significant difference in expression of Np-63 in epithelium between PMODs and normal/hyper-plastic oral tissue. Moreover, expression of Np-63 in epithelium increased with severity of dysplasia in PMOD's. **Varun BR *et al* (2014)[11], Patel S.B *et al* (2017)[12]**compared Np-63 expression in epithelium of OLP of various grades of severity and OSCC's and found out increased expression of Np-63 in epithelium with severity of lesions (i.e. poorer grades have higher expression of Np-63 in epithelium)and serves as prognostic marker.

The findings of above studies are in contrast with our findings. It may be due to the subjective nature of analyzing the expression of Np-63 in the epithelium in different studies. We tried to make it more objective and calculated & compared IRS scores along with statistical analysis. In addition, the methodology and selection criteria used might be different in different studies **(Table2-7).**

Hossaka *et al* (2014)[13]observed an expression of Gal-1 in various layers of epithelium of OSCC and normal mucosa and found that there is no statistically significant difference in the expression of Gal-1 in various layers of epithelium.

On the contrary, in other studies conducted by **Carvalho *et al* (2010)[4] and Ding*et al* (2012)[14]**the authors analyzed epithelial expression of Gal-1 in different grades of PMOD's and noted a significant stronger expression of Gal-1 in epithelium in OSCC and PMOD's as compared to normal oral mucosa. They concluded that this protein serve as a potential biomarker to predict the risk for OSSC development in patients with PMOD's.

Zhong L *et al* (2010)[15], WU *et al* (2018)[16]and Salunkhe *et al* (2019)[17]observed statistically significant increase in expression of Gal-1 in epithelium with advancing histological grades of OSCC and concluded that Gal-1 plays an important role in tumor invasion, metastasis as a prognostic marker. Our findings are contrary to these studies results.

V. CONCLUSION

Based on the information provided by statistical analysis, it seems that both Gal-1 and Np-63 were expressed in the upper one third of epithelium in Group 1, indicating high proliferative activity associated with high dysplasia/severity of lesions. However, the expression of both markers in the oral epithelium in different PMODs with and without dysplasia and different grades of OSCCs is similar, suggesting that neither marker is superior to the other in differentiating PMODs with and without dysplasia, as well as differentiating between PMODs and OSCCs.

Furthermore, these IHC markers were not found helpful in differentiating the various histological grades of OSCCs, and the IRS is not providing any valuable information to differentiate between the various histological grades of OSCCs. Regarding the prognosis of different PMODs with and without dysplasia and different grades of OSCCs, no significant difference was found in the expression of both IHC markers. Therefore, the role of these markers as predicting/prognostic markers is questionable.

Further research is needed to confirm these findings and determine whether these markers can be used to predict the outcome of PMODs and OSCCs.

REFERENCES

[1.] Pires FR, Ramos AB, Oliveira JB, Tavares AS, Luz PS, Santos TC. Oral squamous cell carcinoma: clinic-pathological features from 346 cases from a single oral pathology service during an 8-year period. J Appl Oral Sci 2013; 21(5): 460-67.

[2.] Gupta B, Ariyawardana A, Johnson NW. Oral cancer in India continues in epidemic proportions: evidence base and policy initiatives. Int Dent J 2013; 63(1):12-25.

[3.] Mao T, Xiong H, Hu X, Hu Y, Wang C, Yang L et al. A potential biomarker of malignant transformation in oral leukoplakia. Braz Oral Res 2020; 34: e052.

[4.] Marianne de Vasconcelos, Carvalho M, dos Santos Pereira J, Costa AD, de Souza LB, de Almeida Freitas R, da Costa Miguel MC. Alterations in the immune-expression of claudin-1 between different grades of oral epithelial dysplasias. Arcs of orl bio. 2010; 55(4):261-7.

[5.] Fedchenko N, Reifenrath J. Different approaches for interpretation and reporting of immune histo-chemistry analysis results in the bone tissue–a review. Diagnostic pathology. 2014; 9(1):1-2.

[6.] Rivera C. Essentials of oral cancer. Int J clin Exp Pathol 2015; 8(9):11884- 94.

[7.] Sundberg J, Pandey S, Giglio D, Holmberg E, Kjeller G, Kovács A et al. Expression of p53, p63, podoplanin and Ki-67 in recurring versus non-recurring oral leukoplakia. Sci Rep 2021; 11(1): 1-2.

[8.] Chen YK, Hsue SS, Lin LM. Expression of p63 protein and mRNA in oral epithelial dysplasia. J Oral Pathol Med 2005; 34(4):232-39.

[9.] Kövesi G, Szende B. Prognostic value of cyclin D1, p27, and p63 in oral leukoplakia. J Oral Pathol Med 2006; 35(5):274-77.

[10.] Ramasubramanian A, Ramani P, Sherlin HJ, Premkumar P, Natesan A, Thiruvengadam C. Immuno-histochemical evaluation of oral epithelial dysplasia using cyclin-D1, p27 and p63 expression as predictors of malignant transformation. J Nat Sci Bio Med 2013; 4(2): 349.

[11.] Varun BR, Ranganathan K, Rao UK, Joshua E. Immunohistochemical detection of p53 and p63 in oral squamous cell carcinoma, oral leukoplakia, and oral submucous fibrosis. J Investig Clin Dent 2014; 5(3): 214-19.

[12.] Patel SB, Manjunatha BS, Shah V, Soni N, Sutariya R. Immunohistochemical evaluation of p63 and cyclin D1 in oral squamous cell carcinoma and leukoplakia. J Korean Assoc Oral Maxillofac Surg 2017; 43(5): 324-30.
[13.] Hossaka TA, Ribeiro DA, Focchi G, André S, Fernandes M, Carapeto FC, et al. Expression of Galectins 1, 3 and 9 in normal oral epithelium, oral squamous papilloma, and oral squamous cell carcinoma Dent Res J 2014; 11(4): 508.
[14.] Ding YM, Dong JH, Chen LL, Zhang HD. Increased expression of galectin-1 is associated with human oral squamous cell carcinoma development. Oncol Rep 2009; 21(4): 983-89.
[15.] Zhong LP, Wei KJ, Yang X, Pan HY, Ye DX, Wang LZ et al. Over expression of Galectin-1 is negatively correlated with pathologic differentiation grade in oral squamous cell carcinoma. J Cancer Res Clin Oncol 2010; 136(10):1527-35.
[16.] Wu MH, Hong HC, Hong TM, Chiang WF, Jin YT, Chen YL. Targeting galectin-1 in carcinoma-associated fibroblasts inhibits oral squamous cell carcinoma metastasis by downregulating MCP-1/CCL2 expression. Clin Cancer Res 2011; 17(6):1306-16.
[17.] Salunkhe V, Mahajan A, Prakash N, Pradeep GL, Patil R, Gajdhar SK. Galectin-1 expression in oral squamous cell carcinoma: An immunohistochemical study. J Oral Maxillofac Pathol 2020; 24(1): 186.