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Immunohistochemical Expression of P53 in Premalignant and Malignant Lesions in Oral Cavity with the Relevance of Mast Cell Infiltration

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ABSTRACT:

Context: Oral cancer has become a universal health issue and has been distinguished by high morbidity and poor survival rates. It is a challenge for clinician to identify early cancer because of its variegated presentations.

Aim: The aim of the present study was to see the expression of mutant p53 in the oral premalignant and malignant lesions as well as to see the infiltration of mast cells in the stroma.

Methods and Materials: The study was conducted in Sir Salimullah Medical College and Mitford hospital, Dhaka, Bangladesh. It was a cross-sectional analytical study involving 52 cases of oral tumors(premalignant and malignant) over a period of two years. P^{53} was evaluated by immunohistochemical staining and mast cell by Toluidine blue special stain.

Results: The study comprised 46 cases of oral squamous cell carcinoma (OSCC), 5 leukoplakia and 1 oral submucous fibrosis One third (37%) of OSCC cases belonged to grade-II followed by (34.8%) grade-III and grade-I (28.3%). p53 expression was negative in premalignant lesions and showed positive correlation with the increasing grades of oral squamous cell carcinoma. Mast cells showed significant negative correlation with the grades of squamos cell carcinoma and premalignant lesions.

Conclusions: p53 is an effective marker to determine the grades of oral squamous cell carcinoma and Toluidine blue is a cost-effective special stain that indicated negative correlation. Both of them would help in determining grades, predicting survival analysis and planning treatment.

Key-words: Oral cavity, leukoplaqia, squamous cell carcinoma, p53, Mast cells.

INTRODUCTION:

[1]Oral cancer is the sixth most common cancer in the world and has been distinguished by high morbidity and poor survival rates. [2]Worldwide, oral cancer accounts for 2%-4% of all cancer cases. [3]It is established that more of 90% of all oral neoplasms are squamous cell carcinoma.[4] There are multitude of molecular paths and multiple molecular changes, that involve the tumor progression of oral cancers. Beyond prevention, early detection is the most crucial determinant for successful treatment, better prognosis, and survival of the patient. [5] Current methodologies for cancer diagnosis based upon routine histopathological and cytopathological examination alone are insufficient for detecting early tumor progression and molecular transformation rates.[6]Leukoplakia, commonest premalignant lesion, appears as a slightly elevated grayish-white plaque that may be either well defined or may gradually blend into the surrounding normal mucosa.

[7] Loss of function of the p53 tumor suppressor gene plays an important role in the development of cancer.[8]p53 regulates the activity of tumorigenesis, which leads variously to cell cycle arrest, DNA repair, senescence, or apoptosis following cellular injury. [9]Mutant p53 expressed in the nucleus of the tumor cells in OSCC and thus the nuclei stains brown.[10] Mutant p53 rate is significantly higher with higher pathological grades of OSCC than lower pathological grades. As mutant p53 suggests endogenous mutational mechanism, it becomes good predictor of reduced postoperative survival. [11]In addition to tumor cells, tumor stroma consists of various inflammatory cells like lymphocytes, macrophages, neutrophils, plasma cells, mast cells and eosinophils. These

International Journal Dental and Medical Sciences Research
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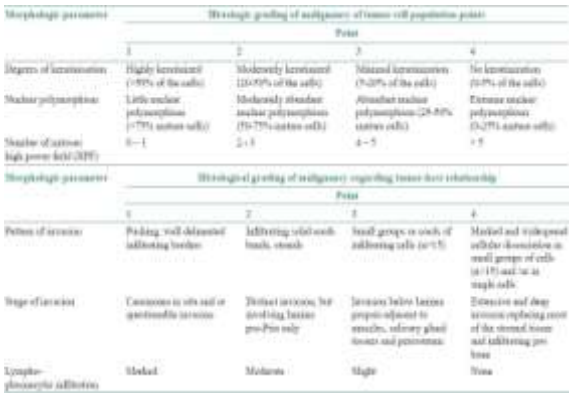
inflammatory cells in the tumor stroma are indicative of host response to tumor cells. [12]Recently, attention has been directed towards tumor associated tissue mast cells and their role in biologic behavior of tumor. [13]Mast cells play antitumoral role by releasing various mediators like cytokines IL-1, IL-4 and IL-6 which induce apoptosis of tumor cells, and TNF–alpha which is cytotoxic to tumor cells. So, in future these could play a role in cancer management.

Subjects and Methods: This is a cross sectional analytical study approved by institutional ethics committee. Total 52 cases were selected for the study. Among them, 46 cases were squamous cell carcinoma and 6 cases were premalignant lesions. All samples were incisional biopsy. Biopsy specimen was preserved in 10% neutral buffered formalin solution. Gross examination of specimen were done and biopsied tissue was processed, embedded in paraffin wax and 3-4µm (micrometer) thick sections were made. After dewaxing the section, one section for haematoxylin and eosin stain and one section for p53 immunostain and one section for toludine blue stain were prepared from each case and examined under light microscope.

Histomorphological study:

Haematoxyline and eosine stained sections of each of the cases were reviewed to confirm the histological diagnosis. Among the recruited cases, six cases were premalignant lesions, and forty six cases were squamous cell carcinoma. Then all squamous cell carcinomas were categorized into well, moderate and poorly differentiated groups according to Anneroth's multifactorial grading system (Table-1). As biopsy specimens were taken in this study, so 'stage of invasion' was omitted.

[14]Table I:Anneroth's multifactorial grading system.



Grade	Score
G-I	5-10
G-II	11-15
G-III	16-20
G-IV	20+

Immunohistochemical study:

After taking 4 µm sections from each case, they were deparaffinized with xylene and rehydrated properly. Then antigen retrieval was done with Dako Target Retrieval solution. Sections were then stained with monoclonal mouse antihuman p53 antibody (Clone DO-7). Immunostaining was done by using Dako Autostainer Plus. For p53 immunostain, positive control was taken from sections of diffuse glioma, grade-II. To validate the stain negative control was taken from sections of normal oral tissue by omitting the primary antibody.

Interpretation of p53 immunostain:

One hundred cells were observed in the areas of hotspots (areas with largest number of p53 positive cells) under high power objective lens (AXIO multiheadmicroscope,x400). [15]Cell showing brown nuclear staining of p53 regardless of intensity were considered to be positive. Cells without nuclear staining or with cytoplasmic staining were considered to be negative.

The proportion of positively stained cells out of all cancer cells (in %), was determined and recorded as follows:

+++ = >50% cells positive,

++ = 26 to 50%cells positive,

+ = 5 to 25% cells positive,
- = <5%cells positive.
[16] In oral premalignant lesions, sections with less than 1% positive cells were considered negative.
Special stain study for mast cells:

One section from each case was deparaffinized and rehydrated with distilled water. Then stained with freshly prepared, 1% Toludine blue for 1-2 minutes, followed by rinsing in distilled water, 3 changes. Special stain was done at the laboratory of department of pathology, Sir Salimullah Medical College and Hospital. For Toludine blue stain, positive control was taken from normal skin tissue.

Interpretation of Special stain for mast cells:

Mast cells cytoplasm contains metachromatic granules. Toluidine blue stains mast cell red purple and the background blue. For determination of mast cell density, the stained sections were screened at low power (10X) to identify the areas of the hot spots (areas with largest number of mast cell). Mast cell was counted at high power (40X) magnification in three randomly chosen fields in the hot spot areas. The mast cell count was expressed as the number of mast cells per high power field (n/hpf).[17] The average figures which were obtained in the hot spot fields, were considered as mean mast cell number for individual case.

Results: Total 52 patients with oral lesions were biopsied and diagnosed histopathologically in the department of Pathology in Sir Salimullah Medical College and Mitford hospital during specified time period. The recruited patients were grouped into 2 groups, namely pre-malignant lesions and malignant lesions. All the malignant lesions comprised squamous cell carcinoma. All cases of squamous cell carcinoma were graded as grade-I, grade-II and grade-III according to Anneroth's Grading system. As grade IV was not found, it was excluded from the study. It was observed that almost one third (30.8%) of patients belonged to age 41-50 years and almost two third (63.5%) patients were male and rest (36.5%) were female. It was observed that 3(23.1%) patients had p53 positive expression in grade I, 14(82.4%) in grade II and 10(62.5%) in grade III. The mast cell count (mean) was 8.79±3.25 in grade-I, 5.61±2.03 in grade-II and 3.69±2.42 % in grade-III. p53 value was expressed in the study population as percentage (%) and mast cell count as mean. The value of Spearman's rank correlation coefficient was -0.644 and p value was 0.001. So it can be concluded that, there was a negative significant ($p<0.05$) correlation between p53% and mast cell count (mean) in the study population.

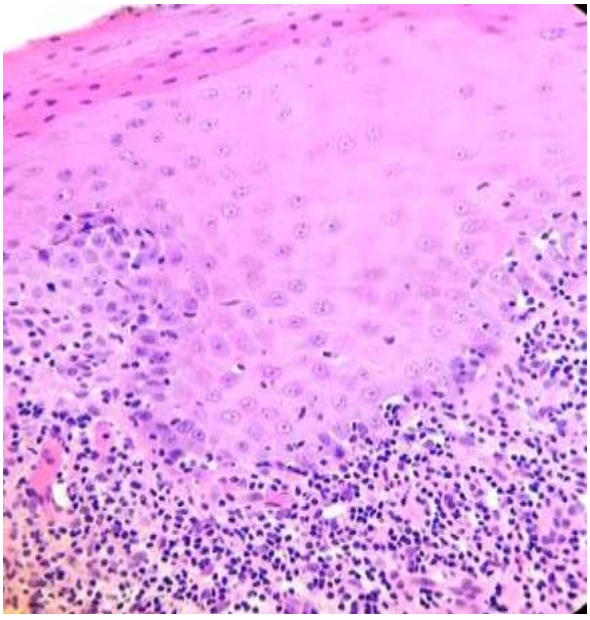


Figure-1: Photomicrograph showing H &E stained sections of leukoplakia (H&E, x400).

International Journal Dental and Medical Sciences Research
Volume 3, Issue 4, July-Aug 2021 pp 756-764 www.ijdmsrjournal.com ISSN: 2582-6018

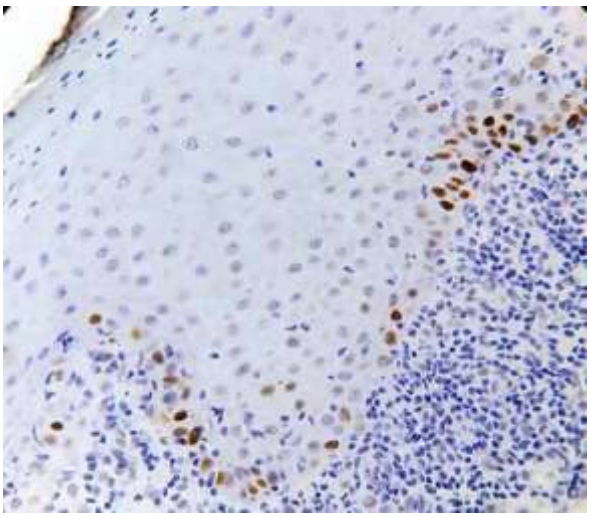


Figure-2: Photomicrograph showing p53 immunostain in leukoplakia (p53, x400).

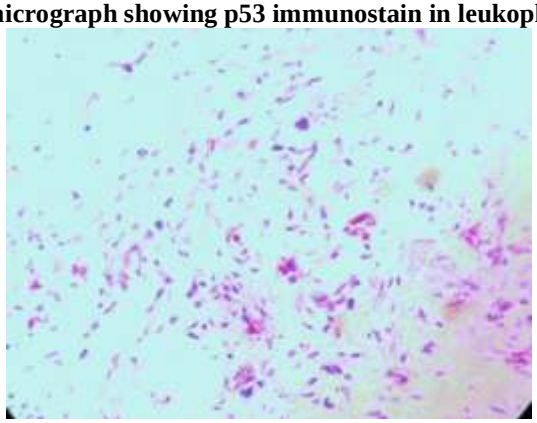


Figure-3: Photomicrograph showing Toluidine blue stained sections of leukoplakia (Toluidine blue, x400).

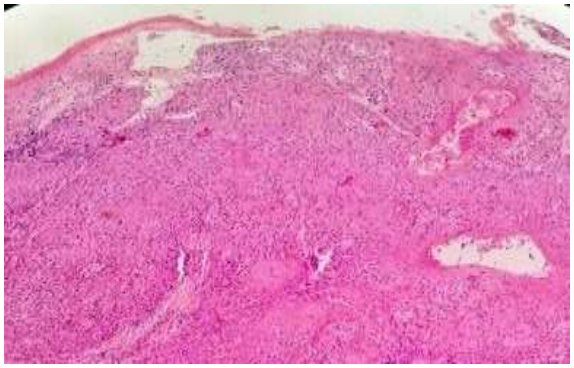


Figure-4: Photomicrograph shows H&E stained section of moderately differentiated squamous cell carcinoma (H&E, x100)

International Journal Dental and Medical Sciences Research
Volume 3, Issue 4, July-Aug 2021 pp 756-764 www.ijdmsrjournal.com ISSN: 2582-6018

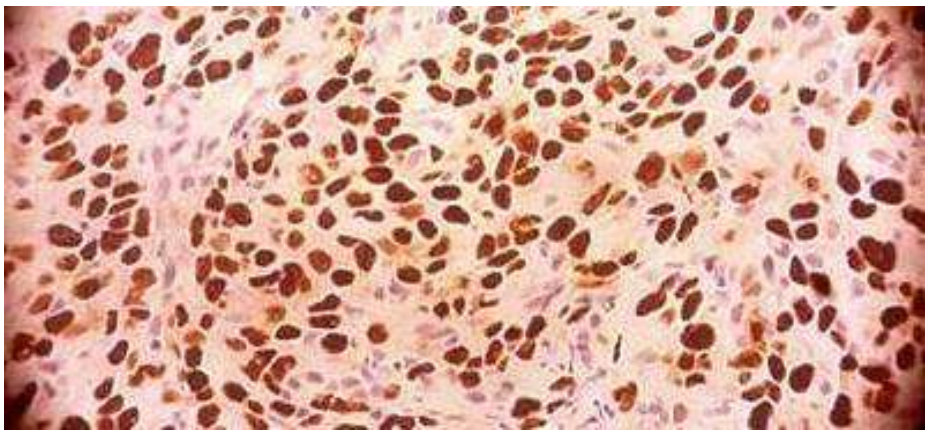


Figure-5: Photomicrograph showing p53 immunostained moderately differentiated squamous cell carcinoma (p53, x400).

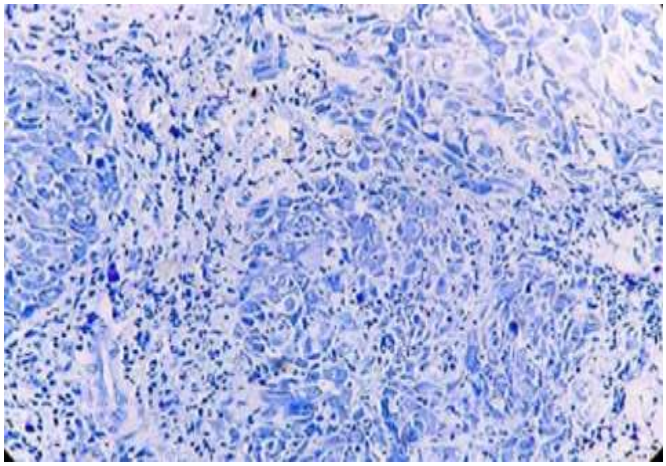


Figure-6: Photomicrograph showing Toluidine blue stained sections of moderately differentiated squamous cell carcinoma (Toluidine blue, x400).

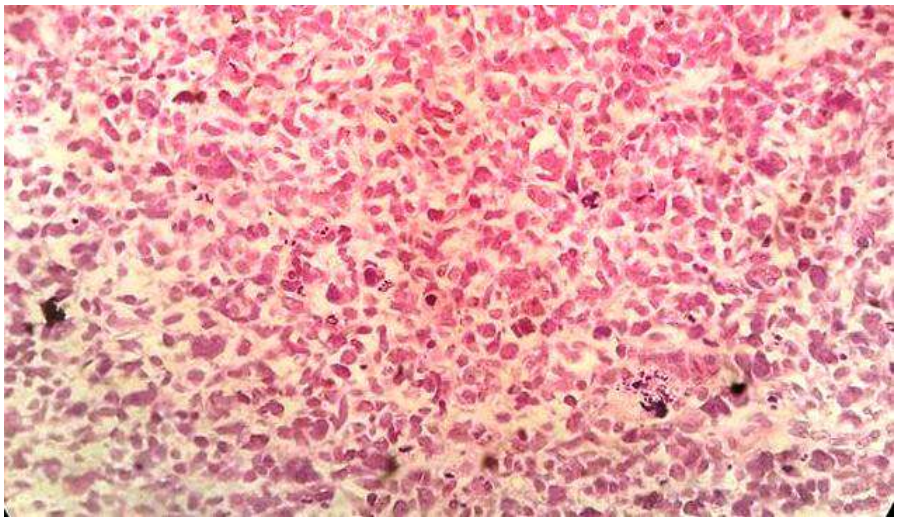


Figure-7: Photomicrograph showing H&E stained poorly differentiated squamous cell carcinoma (H&E, x400).

International Journal Dental and Medical Sciences Research
Volume 3, Issue 4, July-Aug 2021 pp 756-764 www.ijdmsrjournal.com ISSN: 2582-6018

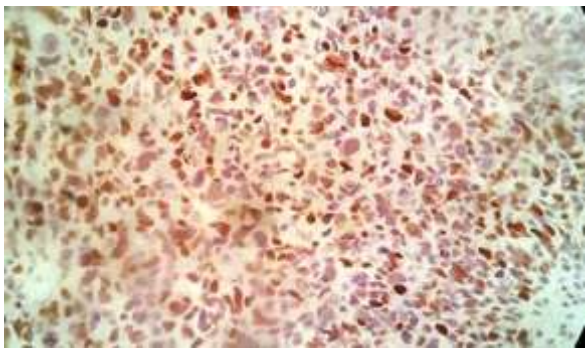


Figure-8: Photomicrograph showing p53 immunostained sections of poorly differentiated squamous cell carcinoma (p53, x400).

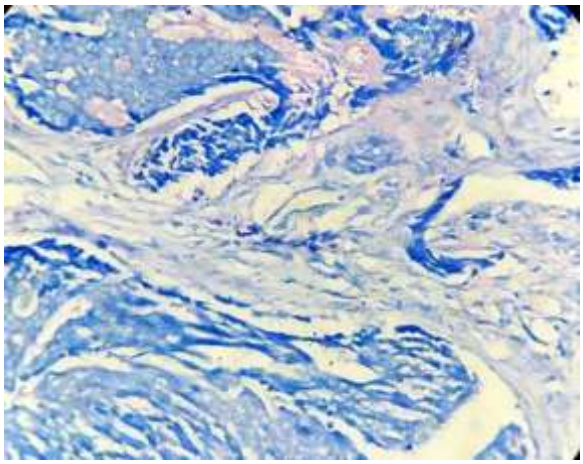


Figure-9:Photomicrograph showing Toluidine blue stained poorly differentiated squamous cell carcinoma. (Toluidine blue, x400).

Discussion: In this prospective and analytical study, total 52 cases were taken. Among them forty-six cases were squamous cell carcinoma and five cases were leukoplakia and one oral submucous fibrosis. No other cases were found during the study period. Premalignant lesions showed no expression of p53 but more infiltration of mast cells. In squamous cell carcinoma, p53 expression had increased with increasing grades and mast cell infiltration had decreased with increasing grades.[4,18]Several previous studies showed that many clinical parameters had been associated with development, local recurrence and death in patients with oral squamous cell carcinoma .

In the present study, it was observed that more than one third (32.6%) of patients belonged to 41-50 years in malignant lesions and (16.7%) belonged to premalignant lesions. The mean age at diagnosis in the present study was 49.73±10.01 years ranged from 35 to 74 years and one third numbers of patients belonged to 41 to 50 years. The mean age was 50.35±10.17 years in malignant lesions and 45.00±7.77 in premalignant lesions. In the present study, it was observed that two third (63.5%) of the patients were male and about one third (36.5%) were female. Among them 60.9% cases of male and 39.1% cases of female had malignant lesions. 83.3% of cases of male and 16.7% cases of female had premalignant lesions. In the present study, It was observed that more than half (55.8%) of patients had tobacco chewing habit, almost two third (65.4%) of patients had betel quid consuming habit and half (53.8%) of patients had habit of smoking.

Regarding site of involvement in the present study it was observed that one fourth (25.0%) of patients had undergone buccal mucosal biopsies followed by 11 (21.2%) tongue biopsies, 7 (13.5%) biopsies from floor of the mouth, 6 (11.5%) from retromolar trigone, 6 (11.5%) from hard palate, 5 (9.6%) from gingival mucosa, 2 (3.8%) from lower lip, 1 (1.9%) from angle of the mouth and 1(1.9%) from right vestibule. Regarding clinical presentation of patients with oral lesions, it was observed that more than one fourth of (28.8%) patients had indurated lump followed by 13(25.0%) cases of exophytic growth and other had plaque type lesions. The present study revealed majority (88.5%) of patients had malignant tumors mostly SCC followed by (11.5%)

International Journal Dental and Medical Sciences Research
Volume 3, Issue 4, July-Aug 2021 pp 756-764 www.ijdmsrjournal.com ISSN: 2582-6018

premalignant lesions. Among the SCC cases, more than one third (37.0%) belonged to grade-II followed by 16(34.8%) grade-III and 13(28.3%) grade-I.

The function of TP53 can be altered by mutation, and this alteration leads to stabilize the protein that can be detected by IHC. In this present study p53 protein was detected by IHC in 52 patients, among them 6 (six) had premalignant lesions and 46 (forty six) had malignant lesions. Aim of present study was to determine any relation between p53 expression and grades of the OSCC. In this present study, among 46 SCC patients 58.7% (27) were p53 positive and 41.3% (19) were p53 negative. It was observed that 3 (23.1%) patients had p53 positive expression in grade I SCC, 14 (82.4%) in grade II SCC and 10 (62.5%) in grade III SCC. It was also observed that more than three fourth (76.9%) patients had negative p53 expression in grade-I, 3(17.6%) in grade-II and 6(37.5%) in grade-III SCC. In this study, the mean p53 was found 9.07±18.22% in grade-I SCC, 47.47±26.66 % in grade-II SCC and 49.28±39.15 % in grade-III SCC. The difference was statistically significant ($p<0.05$) among three groups. It indicates that accumulation of p53 protein is associated with increasing histological grade. [19,20,21] These results correlates with several previous studies.

Table II: Correlation between P53 expression with Grades of the SCC (n=46)

P53	Grades SCC				P value
	Grade-I (n=13)		Grade-II (n=17)	Grade-III (n=16)	
Positive	3	23.1	14	82.4	10 62.5
Negative	10	76.9	3	17.6	6 37.5	0.005[s]

s= significant
p value reached from Chi-square test

Table II shows the correlation between P53 expression with Grades of SCC patients. It was observed that 3(23.1%) patients showed p53 positive expression in grade I, 14(82.4%) in grade II and 10(62.5%) in grade III SCC. The correlation was statistically significant ($p<0.05$) among three groups.

Mast cells are local residents of connective tissue. As this cell contains numerous cytokines, it plays immunoamplifying role, in tumorigenesis of SCC.[19]So it is essential to know the distribution pattern of mast cells in oral premalignant and malignant lesions to understand its role in tumorigenesis of SCC. In the present study the number of mast cells was evaluated in grade-I, grade-II and grade-III SCC. Mean mast cell count per high power field was 8.79±3.25 in grade-I, 5.61±2.03 in grade-II and 3.69±2.42 in grade-III SCC. The differences were found statistically significant ($p<0.05$) among three groups. [12,18]It revealed statistically significant negative correlation between mast cells infiltration and grades of SCC, which correlates with several previous studies.

The mast cell count (Mean) was 5.83±2.23 in malignant tumors and 8.33±1.75 in pre malignanttumors of the oral cavity. ***The difference was statistically significant ($p<0.05$). In this study, p53 value was expressed in the study population as % and mast cell count as mean. It can be concluded that, there was a significant ($p<0.05$) negative correlation between percentage of p53 expression and mast cell count (mean) in the study population. This could be due to progressive increased expression of p53 with increasing grades of OSCC and decreased mast cells with increasing grades of OSCC. [18]It is probable that mediators of mast cells play a key role in cancer immunity by mediating the cross talks between the external antigenic agents and local immunologic factors.

Table III: Association between mast cell count (Mean) with Grades of SCC (n=46)

Grades of SCC			P value
Grade-I (n=13)	Grade-II (n=17)	Grade-III (n=16)	

International Journal Dental and Medical Sciences Research
Volume 3, Issue 4, July-Aug 2021 pp 756-764 www.ijdmsrjournal.com ISSN: 2582-6018

	Mean	±SD	Mean	±SD	Mean	±SD	
Mast cell count (Mean)	8.7925	±3.25	5.6103	±2.03	3.6942	±2.42	0.001[s]
Range (min-max)	4	-15	2	-10	0.66-9		

s= significant
p value reached from ANOVA test

The mast cell count (mean) was 8.79±3.25 in grade-I, 5.61±2.03 in grade-II and 3.69±2.42 % in grade-III SCC. The difference was statistically significant ($p<0.05$) among three groups when compared with mean mast cells counts.

The present study was intended to find out the role of p53 protein, which is associated with TP53 gene mutation, in oral premalignant and malignant lesions. This study also found out the expression of mast cell distribution in oral premalignant and malignant lesions. p53 had significant positive correlation with grades of the oral squamous cell carcinoma. On the other hand, mast cells distribution had a significant negative correlation with grades of oral squamous cell carcinoma. In premalignant lesions p53 expression was negative, whereas numbers of mast cell distribution was high. Hence, it can be concluded that there is a negative correlation present between p53 expression and mast cell infiltration in oral premalignant and malignant lesions mostly squamous cell carcinoma.

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