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## Apoptotic Index and Proliferative Index in Premalignant and Malignant Squamous Cell Lesions of the Oral Cavity

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### Abstract:

**Background:** Oral squamous cell lesions are most commonly diagnosed lesions in India. Both premalignant and malignant lesions are frequently encountered. In this study, we evaluated the role and significance of apoptotic indices (AI) and proliferative indices (PI) in premalignant and malignant squamous cell lesions of the oral cavity.

**Materials and Methods:** A total of 62 histologically proven cases of premalignant and malignant oral squamous cell lesions were analyzed. The biopsies were stained with hematoxylin and eosin and also with monoclonal antibody Ki-67. AI and PI were assessed using a light microscope.

**Results:** AI was found to increase gradually from normal to dysplasia to carcinoma. The highest AI was seen in well-differentiated squamous cell carcinomas (SCCs). PI also was found to increase significantly from normal to dysplasia to carcinoma. The highest PI was seen in poorly differentiated SCC.

**Conclusion:** AI in conjunction with the PI offers an accurate idea as to the nature and course of the lesion and may help to plan timely surgical intervention that results in better clinical prognosis and outcome.

**Key Words:** Apoptosis, Ki-67, proliferation

### Introduction

Oral cancer is one of the most common cancers in third world and developing countries today. It is the fourth most

frequent cancer after lung and stomach in males and fifth most common after cervix and breast in females.<sup>1-4</sup> Oral squamous cell carcinomas (SCCs) are usually preceded by a premalignant lesion mostly presenting clinically as leukoplakia, erythroplakia, oral submucous fibrosis and histologically as hyperplasia, dysplasia or carcinoma *in situ*. Leukoplakia does not necessarily correlates with histological dysplasia. Early diagnosis of these lesions greatly increases the chance of cure and significantly reduces deformity in the patients. In the recent past, histological techniques that identify the parameters such as cell proliferation and cell death have become quite significant. Measuring these parameters may not only help in identifying the individuals who are at a greater risk of developing carcinomas, but they also carry a significant prognostic value and also represent a good model of tumor development.<sup>5,6</sup> Assessment of cell death is performed by counting the apoptotic cells and apoptotic bodies using a light microscope. Since this is relatively an easy method and is feasible under routine circumstances, this technique has been used widely.<sup>6-9</sup> Another useful parameter is the assessment of cellular proliferation. Proliferating cells (cells in G1, S, G2 and M phases of the cell cycle) express Ki-67 antigen, which is a non-histone nuclear protein. These proliferating cells can be visualized by staining with monoclonal Ki-67 antibody. The rate at which a tumor proliferates has long been considered to have a relationship to its clinical course, thus providing an easy means of accurately assessing the growth fraction of normal, dysplastic and neoplastic tissue.<sup>5,10-12</sup>

Hence, the present study was undertaken with the purpose of evaluating the role of apoptotic index (AI) and proliferating index (PI) in premalignant and malignant oral squamous cell lesions.

### Materials and Methods

The study population consisted of 62 (49 males and 13 female patients; aged between 20-79 years) histologically proven cases of premalignant and malignant squamous cell lesions of the oral cavity reporting to the Santosh Medical College, and Santosh Dental College and Hospital, Ghaziabad, Uttar Pradesh, India. Written informed consent was obtained from those, who agreed to participate voluntarily. Ethical clearances were obtained from the institution's ethical committee and review boards. The present study was conducted over a period of 1 year and analyzed. 62 cases with immunological disease, immunocompromised individuals were excluded. Excisional/

incisional biopsy materials were then stained routinely with hematoxylin and eosin (H and E) stain along with immunostaining with Ki-67 antibody. For Ki-67 immunostaining, sections were cut at 4 micron thickness and mounted on poly-L-lysine coated slides. The antibody used was MIB-1 mouse anti-human clone and the detection method was Avidin-Biotin-Complex method. The sections were counterstained with Mayer's hematoxylin. Appropriate positive and negative controls were processed simultaneously.

**Counting and Scoring Technique**

**AI**

Apoptosis characteristically affects single cells. The earliest recognized morphological changes are compaction and segregation of the nuclear chromatin with the formation of sharply delineated, uniformly granular masses that become marginated against the nuclear envelope and condensation of the cytoplasm. While this is occurring, the cytoplasm continues to condense, and apoptotic bodies that contain membrane-enclosed fragments of the nucleus bud from the cell.<sup>5,7,13</sup> Apoptotic bodies are quickly digested by nearby cells. After phagocytosis their digestion, is completed within hours. For calculating AI, the H and E sections were examined using oil immersion lenses (×100). From each section, 10 fields devoid of artefacts were selected. 1000 cells were evaluated for the presence of apoptotic cells and apoptotic bodies. The AI was expressed as a percentage of the total number of non-apoptotic cells counted.<sup>5,6,14</sup>

**Proliferative index (PI)**

The proliferating cells were identified by their expression of Ki-67. Antibodies Ki-67 recognizes the antigen, which is associated with the nuclear component of the cell. Staining pattern of Ki-67 varies with the phase of cell cycle. Mostly it stains the karyoplasm in mixed, granular and speckled form.<sup>15</sup> The immune-stained sections were evaluated for the staining pattern of Ki-67. 3000 cells were evaluated. The PI was determined by counting the total number of Ki-67 positive cells per 3000 cells and expressed as a percentage.<sup>5,16,17</sup>

**Statistical analysis**

Results for each group of tissue (normal, dysplasia and carcinoma) were pooled. An SPSS for Windows computer program (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Student's *t*-test was used for statistical analysis of the results.

**Results**

A total of 62 cases of premalignant and malignant squamous cell lesions of the oral cavity were analyzed. Relevant clinical data in reference to age, sites of lesions and history of tobacco chewing were recorded (Table 1). Of the cases studied 49 were in males, and the remaining 13 were in females. About 43.5% of the cases in our study were in the age group 60-69 years.

Majority of the patients had a history of tobacco chewing. The most common site for all squamous cell lesions was found to be the tongue (40.35%). Histologically, cases were grouped as normal mucosa, leukoplakia, mild dysplasia, moderate dysplasia, severe dysplasia, well-differentiated SCC (WDSCC), moderately differentiated SCC (MDSCC) and poorly differentiated SCC (PDSCC). AI and PI were assessed in all the biopsy specimens (Table 2).

**Discussion**

Epithelial lesions of the oral cavity arise from the mucosal surfaces and are typically squamous cell in origin. They can be divided into the premalignant and malignant conditions. The premalignant squamous lesions associated with the oral cavity are leukoplakia and erythroplakia. Varying degrees of epithelial dysplasias are also encountered in the oral cavity. The most common malignant oral cell lesion is the SCC.<sup>18</sup> The causes for oral SCC are multi-factorial, such as history of tobacco, smoking, increased sunlight exposure and premalignant lesions.<sup>2,4,18</sup>

Apoptosis and cellular proliferation have been known to play an important role in tumor progression and development. The interrelationship and the role of each of these entities in the progression of the tumor are yet to be defined. In this study, we observed that a fairly accurate assessment of apoptotic bodies is possible using a light microscope. Apoptotic bodies were seen in the suprabasal and basal regions of the normal oral mucosa and early dysplastic lesions, but as the severity of the premalignant or malignant lesion increases the apoptosis becomes generalized.<sup>19,20</sup> This observation gives us

**Table 1: Clinical features of squamous cell lesions of the oral cavity (n=62).**

Age in years	Number of cases (%)	Site of lesion	Number of cases (%)
20-29	2 (3.22)	Tongue	25 (40.35)
30-39	3 (4.83)	Lip	15 (24.19)
40-49	4 (6.45)	Buccal mucosa	7 (11.29)
50-59	15 (24.19)	Gingiva	6 (9.67)
60-69	27 (43.54)	Floor of the mouth	4 (6.45)
70-79	11 (17.74)	Tonsils	5 (8.06)

**Table 2: Mean AI and mean PI in premalignant and malignant squamous cell lesions of the oral cavity.**

Type of lesion (Number of cases)	Mean AI (%)	Mean PI (%)
Normal oral mucosa (5)	0.09±0.02	2.38±0.98
Leukoplakia (5)	0.11±0.05	8.28±2.75
Mild dysplasia (13)	0.14±0.09	10.83±3.66
Moderate dysplasia (10)	0.17±0.17	12.79±6.15
Severe dysplasia (5)	0.14±0.04	16.12±4.84
WDSCC (16)	0.35±0.08	35.58±19.40
MDSCC (6)	0.29±0.07	69.16±9.95
PDSCC (2)	0.25±0.07	85.95±9.97

WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, AI: Apoptotic index, PI: Proliferative index

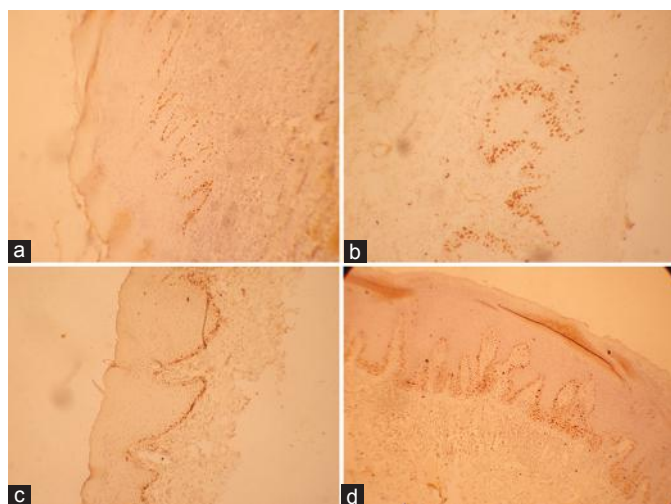
an indication that as the apoptosis became generalized, the severity also increased (Figure 1). The AI was seen to increase gradually from normal mucosa to leukoplakia. A slight drop in AI seen in severe dysplasia when compared to moderate dysplasia was found to be statistically insignificant.<sup>5,6</sup> In the carcinomas, the apoptotic bodies were counted in the substance of the tumor avoiding the apoptotic cells that are present in the surrounding stroma and also those that are seen in the areas of necrosis and inflammation. An increase in AI with the increase in the lesion severity, with a peak value in WDSCC was seen, which showed that increase in apoptosis increased with disease progression. Different studies showed that AI increased gradually up to carcinoma *in situ* but fell again in SCC.<sup>5,6,13,14</sup> In the present study also, AI increased progressively from normal to carcinoma but decreased with decreased differentiation of the tumor.

The PI was assessed by staining the section with monoclonal antibody Ki-67. In the normal oral mucosa, Ki-67 stained nuclei were identified in the basal layers and by and large were restricted to the basal and suprabasal layers. This was because most of the cells in normal oral mucosa remain in Go phase. Only about 20% of the cells are inactive cell cycle and are identified by Ki-67 positive staining. In contrast to normal oral mucosa, the levels of expression of Ki-67 was higher in oral epithelial dysplasia indicating a constant cell cycle re-entry giving rise to a higher level of Ki-67 positivity. This suggests that the most differentiated the epithelium, the lesser the positivity for Ki-67. On the other hand, the more poorly differentiated epithelium all strata are positive for the marker (Figure 2).<sup>17,19</sup>

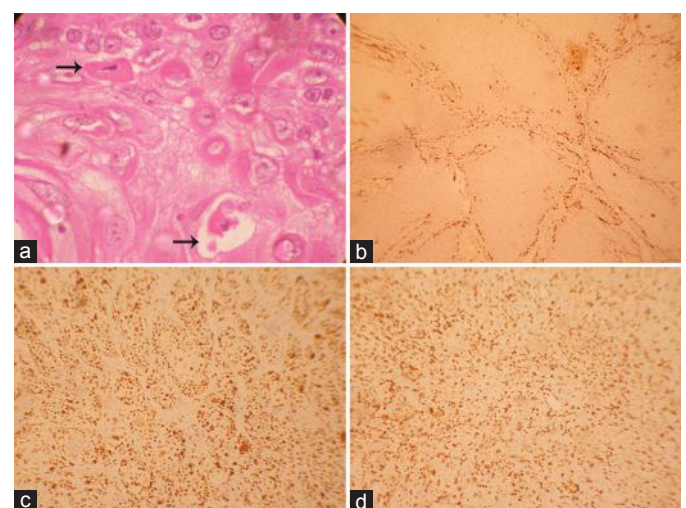
In our study, PI values for normal oral mucosa was  $2.38 \pm 0.98\%$ , leukoplakia showed values corresponding to  $8.28 \pm 2.75\%$ , for mild dysplasias with  $10.83 \pm 3.66\%$ , for moderate dysplasias with  $12.70 \pm 6.15\%$  and for severe dysplasias with

$16.12 \pm 4.84\%$ . The values suggest that there is a gradual increase in the PI as the grades of dysplasia increases from mild to severe. In well-differentiated carcinoma, the PI was found to be  $35.58 \pm 19.40\%$ , which increased to  $69.10 \pm 9.95\%$  in moderately differentiated carcinoma and  $85.95 \pm 9.97\%$  in poorly differentiated carcinoma. We noticed that Ki-67 acts as an excellent marker of cellular proliferation and also helps us to grade dysplasias more accurately. In our study gradual increase in PI was noted from normal epithelium to severe dysplasia but increased many folds as the dysplasia progressed to carcinoma. Larger differences were seen between the various grades of SCC.

The highest PI was noted in PDSCC, this is in accordance with the studies conducted by Macluskey *et al.*<sup>5</sup> and Santos-García *et al.*<sup>19</sup> Hence, we can infer that a high PI suggests a high proliferative state in the tissue. PI showed the largest difference from well-differentiated SCC to MDSCC as also seen by Torres-Rendon *et al.* (Figure 1).<sup>17</sup> In our study, the percentage of Ki-67 positive cells ranged from 2.38% to 85.95% with a mean of 34.42%, which is similar to the study done by Carlos de Vicente *et al.* This suggests that a correlation exists between Ki-67 index and histological grade of the lesion.<sup>20,21</sup> Most of the cases with high PI in our study were from high histological grades corresponding to MDSCC and PDSCC, when compared with normal tissue, the difference in PI was statistically significant ( $P < 0.0001$ ). We compared the mean values of PI of total cases of dysplasia with normal cases and found that the difference proved to be statistically significant ( $P < 0.001$ ). Similarly cases of SCC were compared with the cases of dysplasia, and these values were also found to be statistically significant ( $P < 0.001$ ). Hence, we can conclude that PI increases with the progression of the disease and the results are statistically significant.



**Figure 1:** Ki 67 staining in (a) leukoplakia ( $\times 10$ ); (b) Mild dysplasia ( $\times 10$ ); (c) Moderate dysplasia ( $\times 10$ ); (d) Severe dysplasia ( $\times 40$ )



**Figure 2:** Ki 67 staining in (a) well differentiated squamous cell carcinoma ( $\times 10$ ); (b) moderately differentiated squamous cell carcinoma ( $\times 10$ ); (c) poorly differentiated squamous cell carcinoma ( $\times 10$ ); (d) apoptotic bodies indicated by arrow (haematoxylin and eosin;  $\times 100$ )



This study attempted to determine whether the pattern of expression of Ki-67 is in any way related to epithelial dysplasia. The staining pattern of Ki-67 was in the basal layers in normal epithelium, whereas in severe dysplasias the staining pattern became generalized. Hence, a greater frequency of the suprabasal expression of Ki-67 is related to increasing severity of dysplasia. This finding may help to accurately assess the various grades of dysplasias.

### Conclusion

From this histological evaluation, we can conclude that AI increased with increasing severity of the lesion. Also the topographic location of the apoptotic bodies helps to grade dysplasias better. Decrease in the AI with increasing grades of carcinoma could act as a poor prognostic indicator. Similarly, PI also showed an increased expression as the severity of the lesion increased and could act as a poor prognostic indicator. A positive correlation was seen between Ki-67 index and histological grade of the lesion, increased the suprabasal expression of Ki-67 increased with the severity of dysplasia. This finding helps to accurately assess the various grades of dysplasias. AI and PI together would act as a better marker for the disease progression. Thus, we emphasize that the histopathology report of every premalignant and malignant squamous cell lesions of the oral cavity should include AI and PI along with histomorphology. This would help in timely surgical intervention and result in less deformity and a better outcome prognostically.

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