HISTOPATHOLOGICAL GRADING OF ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH KI-67 - A PROLIFERATIVE MARKER

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ABSTRACT

BACKGROUND

The prognosis of squamous cell carcinoma depends on the size of the lesion, level of local invasion, lymphatic spread, and presence of distant nodal metastases. The behaviour of the squamous cell carcinoma is marked by the degree of cell proliferation and differentiation, histopathological grading and proliferative index which can be derived by measuring Ki-67- an immunohistochemical marker.

MATERIALS AND METHODS

1. All operated cases of oral squamous cell carcinoma diagnosed on histopathology. 2. Conventional Haematoxylin & Eosin Stain. 3. Ki-67 Dako Flex Monoclonal Mouse Anti-Human Ki-67 Ag Clone MIB-1 kit. H & E Stain (Haematoxylin and Eosin stain) and Immunohistochemical staining with Ki-67.

RESULTS

A general increasing trend in the mean Ki-67 LI with higher modified Broder's grade was noted. However, the Ki-67 LI score increases with the increasing grades of oral squamous cell carcinomas. There was a statistically significant difference between these grades.

CONCLUSION

We conclude that the tumor cell proliferation as measured by Ki-67 LI at randomly selected fields has a positive association with the histologic grading in oral squamous cell carcinoma.

KEYWORDS

Cell Proliferation; Human Oral Squamous Cell Carcinoma; Ki-67 Antigen; Immunohistochemistry; Randomly selected fields.

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BACKGROUND

Today Cancer is a global concern**,** rampantly arising with alarming incidence all across the world**;** with approximately 14 million new cases in 2012. Globally it is a leading cause of death. It is expected to increase to 24 million by 2035. The most commonly diagnosed cancers were carcinoma lung (1.82 million), carcinoma of breast (1.67 million) and colorectal carcinoma (1.36 million); the most common causes of cancer death are lung cancer (1.6 million deaths), stomach cancer (723,000 deaths) and liver cancer (745,000 deaths) in 2012.[1]

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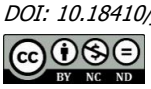


Worldwide oral cancer account for 2-4% of all cancer cases. In 2004-2009 over 3 lakh cases of oral and oropharyngeal cancer cases diagnosed worldwide, and 7000 affected individuals died of cancer.[2] In some regions the prevalence of oral cancer is higher reach in the 10% of all cancers, reaching the around 45 % of all cancer in India.[3]

Carcinomas refers to a malignant neoplasm of epithelial origin or cancer of the external or internal lining of the body. Carcinomas are malignancies of epithelial tissue which account for 80 to 90 percent of all cancer cases. Carcinomas are divided into two major subtypes: squamous cell carcinoma, which originates in the squamous epithelium and adenocarcinoma**,** which develops in gland or an organ.[4]

The incidence of oral squamous cell carcinoma (OSCC) is increasing among young individual age between 18-44 years. The percentage of 5 years survival with OSCC varies from 40-50%. Regardless of the easy access of oral cavity for clinical examination OSCC is usually diagnosed in advance stages.[5]

The greatest risks for oral cancer in the western countries are tobacco and alcohol.[6] Apart from tobacco and

alcohol other risk factors for OSCC are betel quid chewing**,** areca nut**,** narcotics**,** cannabis and other associated factors like impaired ability to repair DNA damage by mutagenes and metabolize carcinogens**,** deficiencies of vitamins of A and C and immune defects.[7]

Despite the steady improvements in treatment modalities, the 5-year survival rate of OSCC is about 55% and it continues to stand poor.[8]

The behaviour of the squamous cell carcinoma is marked by the degree of cell proliferation and differentiation and Ki-67 is most commonly used biomarker for cell proliferation evaluation.[9] Modified Broder's classification for histopathological grading has been used for squamous cell carcinoma and is based on proportion of neoplasm resembling normal squamous epithelium.

Cell proliferation is investigated with immunohistochemical techniques by staining for nuclear antigens related to cell growth and division and searching for them visually under the microscope. Ki-67 is an antigen that corresponds to a nuclear nonhistone protein expressed by cells in the proliferative phases G1, G2, M, and S.[10] Monoclonal antibodies have been developed that detect formalin-resistant epitopes (MIB-1 and MIB-3).[11] In general, there is a good correlation between Ki-67 staining and mitotic count.[12]

The present study is centered on comparing Oral squamous cell carcinoma grade with proliferating indices like Ki-67. The study wants to evaluate the utility of Ki-67 as a proliferative index marker keeping histopathological diagnosis grading as gold standard. The study ultimately would like to assess the added utilization of such immunohistochemical based marker in proliferating malignant squamous cells.

Aims and Objectives

To establish correlation and utility of Ki 67 as a proliferative marker when compared with grades of oral squamous cell carcinoma on histopathology. Objectives: 1. To grade oral squamous cell carcinoma. 2. To assess and analyse Ki-67 proliferative index with squamous cell carcinomas of various grades. 3. Comparison of histopathological grade of oral squamous cell carcinoma with Ki 67 proliferative index.

MATERIALS AND METHODS

This study was conducted in the Histopathology and Immunohistochemistry section of Department of Pathology, Jawaharlal Nehru Medical College and Acharya Vinoba Bhave Rural Hospital, Sawangi (M), Wardha from July 2015 to December 2017. The study is a prospective, cross sectional and analytical study.

A total of 100 cases of oral squamous cell carcinoma were diagnosed during the study period. Incisional biopsy of diagnosed cases for malignancy were studied by the paraffin embedding technique. The routine stain used for tumours was Haematoxylin and Eosin.

A detailed microscopic examination was carried out. During each batch of staining for Ki-67, appropriate positive and negative controls were used. Lymph node was used as a positive control and adipose tissue was used as negative control on slide in which primary antibody was excluded, was used for each batch of slides.

Inclusion Criteria

All the patients attending the OPD and IPD of AVBRH, belonging to all age groups and both the genders, clinically and histopathologically diagnosed for oral Squamous Cell Carcinoma will be included in this study.

Exclusion Criteria

1. Pre-malignant cases
2. Recurrent cases
3. Already treated cases
4. Patient undergoing chemotherapy

In all cases, tumor samples are fixed in 10% buffered formalin, included in paraffin, and stained with H&E according to the following standard procedure. The Haematoxylin and Eosin stain are the most widely used histological stain and all the stained sections of Oral squamous cell carcinomas were then graded as per Modified version of Broder's Classification[13] into Well, Moderately, Poorly differentiated. In well differentiated oral SCC, malignant squamous epithelial cell nests, keratin pearls, and individual cell dyskeratosis seen; moderately differentiated SCC displays nuclear pleomorphism, mitoses and less keratinization; whereas predominant immature cells, with numerous typical and atypical mitoses, minimal keratinization and sometimes necrosis seen in poorly differentiated squamous cell carcinomas.

Histological Grading	Percentage of Differentiation
Well differentiated (Grade I)	75-100% cells are differentiated and 0-25% cells are undifferentiated
Moderately differentiated (Grade II)	50-75% cells are differentiated and 25-50% cells are undifferentiated
Poorly differentiated (Grade III)	>50% cells are undifferentiated

Table 1. Modified Version of Broder's Classification

Materials for Immunohistochemical Staining-Ki-67

Monoclonal mouse anti-human Ki-67/MIB-1 antibody and code IS626 supplied by DAKO (Glostrup, Denmark) and antibody detection was carried out using Avidin-Biotin Complex (ABC) method and lymph node was taken as a positive control and adipose tissue as a negative control.[14]

Ki-67/MIB-1 is a nuclear antibody against proliferating nuclear antigen. Ki-67 positivity is seen as brown discoloration of nuclei in the proliferating cells, the staining intensity by counting 1,000 cells in each sample (magnification, ×400) and assessing the percentage of labelled cells.

The Ki-67 labelling index was calculated using the formula: (Ki67-positive) / (Ki67-positive + Ki67-negative) x 100.[15] Based on the labeling index, the sections were scored from 1 to 3 for ki-67 expression as follows[16]-

Ki-67 Expression	Extent of Proliferation	Percentage of positive Cells
1	High	>50%
2	Moderate	30-50%
3	Low	<30%

Table 2. Expression of Ki-67 Labeling Index

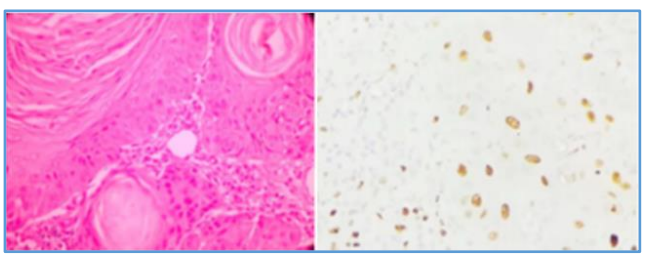


Image 1

Given section stain with a) h & e stain shows sheets of malignant squamous cells along with keratin pearls b) low proliferation (<30%) of ki-67 (brownish nuclear discoloration) in well differentiated squamous cell carcinoma (40x view).

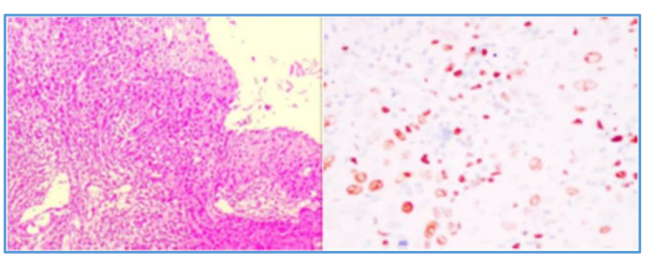


Image 2

Given Section stain with a) H & E stain shows sheets of malignant squamous cells showing areas of extensive nuclear pleomorphism b) Moderate Proliferation (30 to 50%) of Ki-67 (Brownish Nuclear Discoloration) in moderately differentiated squamous cell carcinoma (40x view).

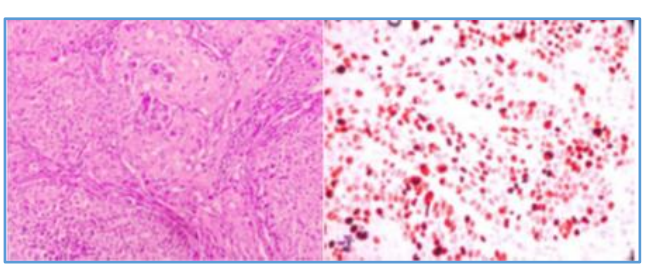


Image 3

Given section stain with a) H & E stain shows sheets of poorly differentiated squamous cells with cells Showing high nuclear pleomorphism and atypical mitosis b) High proliferation (>50%) of Ki-67 (Brownish Nuclear Discoloration) in poorly differentiated squamous cell carcinoma (40x view).

RESULTS

In present study, we found that the according to site wise distribution of the cases of oral squamous cell carcinoma, maximum patients were of tongue (48%), followed by buccal mucosa with alveolus which comprised 14% of cases. Out of 100 cases of Oral squamous cell carcinoma, 31 cases were diagnosed as well differentiated which comprised 31% of total cases, 31 cases were diagnosed as moderately differentiated which comprised 31% of total cases and 38 cases were diagnosed as poorly differentiated which comprised 38% of total cases. According to Ki-67 expression, out of 100 cases 26 cases were showing <30% cellular proliferation, 28 cases showed 30-50% of cellular proliferation, 46 cases were showing >50% cellular proliferation.

The present study provides information (Table 3 & Graph 1) about distribution of patients with Ki-67 labelling index, out of 100 cases, low proliferation (<30%) was seen as 70.97% (22 cases), 6.40% (2 cases), 5.26% (2 cases) respectively in well, moderately and poorly differentiated Oral squamous cell carcinoma; moderate proliferation (30-50%) was seen as 22.58% (7 cases), 61.29% (19 cases), 32.26% (10 cases) respectively in well, moderately and poorly differentiated Oral squamous cell carcinoma; high proliferation (>50%) was seen as 5.26% (2 cases), 5.26% (2 cases), 89.47% (34 cases) respectively in well, moderately and poorly differentiated Oral squamous cell carcinoma. (X2-value 81.98. p-value=0.0001, Significant). The above correlation has been found to be significant.

Table 4 shows mean labeling index of Ki-67 for low proliferation was 27.76 ± 1.47; moderate proliferation was 45.61 ± 4.12; high proliferation was 82.74 ± 3.56. There was a significant (p=0.0001, S) variation in mean Ki-67 labelling index with Well differentiated grade of oral squamous cell carcinoma. Graph 2 shows that in present study maximum cases i.e. 22 cases of well differentiated squamous cell carcinoma belonged to Grade I of Ki-67 (i. e. <30%). When Ki-67 have correlated with well differentiated oral squamous cell carcinoma, its been seen mean Ki-67 felt with <30% (low proliferation).

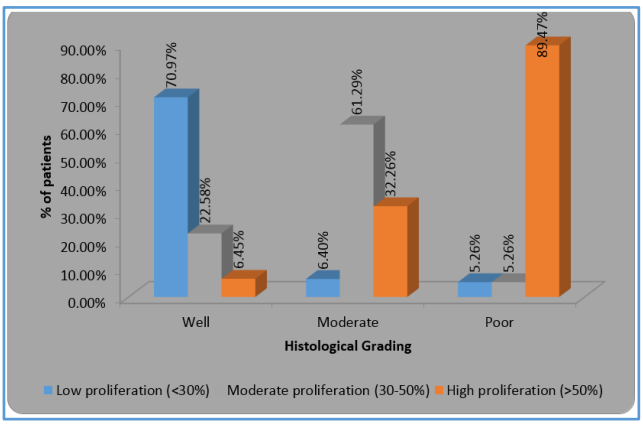
Table 5 shows, mean labeling index of Ki-67 for low proliferation was 27.51 ± 1.42; moderate proliferation was 46.39 ± 3.05; high proliferation was 78.39 ± 11.33. There was a significant (p=0.0001, S) variation in mean Ki-67 labelling index with moderately differentiated grade. Graph 3 shows maximum cases i.e. 19 cases of moderately differentiated squamous cell carcinoma belonged to Grade II of Ki-67 (i.e.30-50%). When Ki-67 have correlated with moderately differentiated oral squamous cell carcinoma, its been seen mean Ki-67 felt between 30-50% (moderate proliferation).

Table 6 shows, mean labeling index of Ki-67 for low proliferation was 29.30 ± 0.12; moderate proliferation was 47.88 ± 1.87; high proliferation was 80.69 ± 9.16. There was a significant (p=0.0001, S) variation in mean Ki-67 labelling index with poorly differentiated grade. Graph 4 shows maximum cases i.e. 34 cases of poorly differentiated squamous cell carcinoma belonged to Grade III of Ki-67 (i.e

>50%). When Ki-67 have correlated with Poorly differentiated oral squamous cell carcinoma, its been seen mean Ki-67 felt with >50% (high proliferation).

Histology Grade	Ki-67 Expression			Total Cases (100)
	Low Proliferation (<30%)	Moderate Proliferation (30-50%)	High Proliferation (>50%)	
Well	22(70.97%)	7(22.58%)	2(6.45%)	31
Moderate	2(6.45%)	19(61.29%)	10(32.26%)	31
Poor	2(5.26%)	2(5.26%)	34(89.47%)	38
Total	26(26%)	28(28%)	46(46%)	100
X2-value	81.98. p-value=0.0001, Significant			

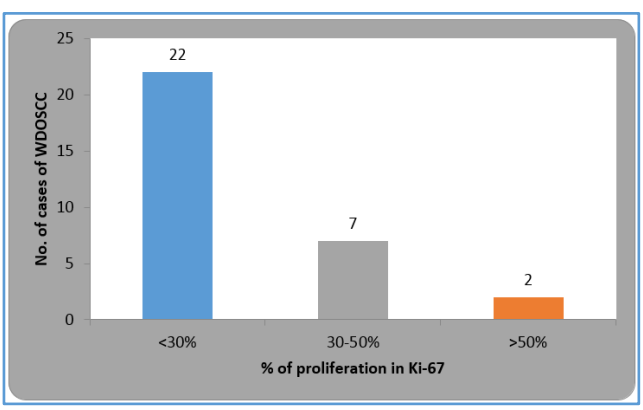
Table 3. Comparison of Ki-67 LI (%) with Histopathological Grades of Oral Squamous Cell Carcinoma



Graph 1. Comparison of Ki-67 LI (%) with Histopathological Grades of Oral Squamous Cell Carcinoma

% of Proliferation	N	Mean	Std. Deviation	F-value	p-value
<30%	22	27.76	1.47	567.70	0.0001, S (<0.05, Significant)
30-50%	7	45.61	4.12		
>50%	2	82.74	3.56		
Total	31	35.34	14.89		

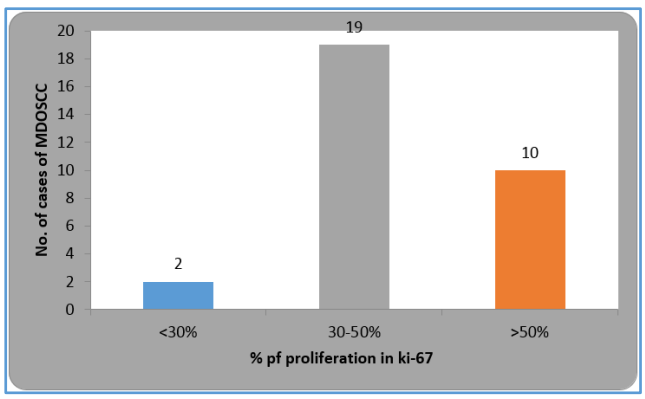
Table 4. Correlation of Ki-67 LI (%) Expression with Well Differentiated Oral Squamous Cell Carcinoma



Graph 2. Correlation of Ki-67 LI (%) Expression with Well Differentiated Oral Squamous Cell Carcinoma

Groups	N	Mean	Std. Deviation	F-value	p-value
<30%	2	27.51	1.42	88.43	0.0001, S(<0.05, Significant)
30-50%	19	46.39	3.05		
>50%	10	78.39	11.33		
Total	31	55.49	17.99		

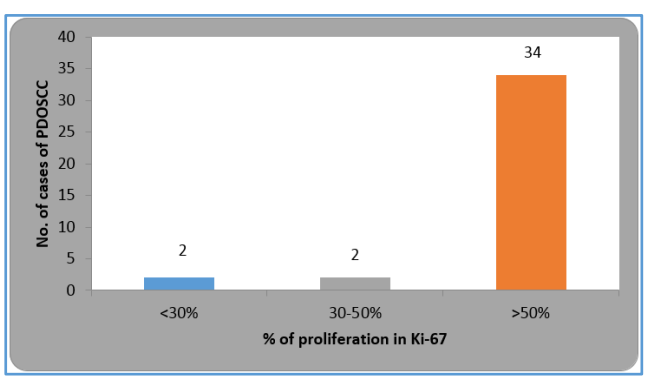
Table 5. Correlation of Ki-67 LI (%) Expression with Moderately Differentiated Oral Squamous Cell Carcinoma



Graph 3. Correlation of Ki-67 LI (%) Expression with Moderately Differentiated Oral Squamous Cell Carcinoma

Groups	N	Mean	Std. Deviation	F-value	p-value
<30%	2	29.30	0.12	42.17	0.0001, S(<0.05, Significant)
30-50%	2	47.88	1.87		
>50%	34	80.69	9.16		
Total	38	76.26	15.99		

Table 6. Correlation of Ki-67 LI (%) Expression with Poorly Differentiated Oral Squamous Cell Carcinoma



Graph 4. Correlation of Ki-67 LI (%) Expression with Poorly Differentiated Oral Squamous Cell Carcinoma

DISCUSSION

Oral malignant neoplasms constitute a significant global health problem with reports signifying that they are the sixth most common malignancies worldwide and are the third most common malignancies in the developing world. Oral squamous cell carcinoma is known for its unpredictable progression and severe damage of the tissues involved.

Conventionally, oral squamous cell carcinoma is evaluated with clinical staging and histological grading system, which are essentially subjective and not efficiently reproducible. Oral squamous cell carcinomas are classified by Broder's grading system, WHO Grading system, Anneroth Grading system, Bryne's Grading system. Various prognostic

markers include site, histological grade, tumor differentiation, nuclear pleomorphism, proliferative activity, Ki-67 expression. Recently Ki-67 labeling index is of significant prognostic importance and therefore has major impact in selecting the appropriate treatment plan.

In the study carried out by Maheshwari et al[16] (2013) maximum cases were of squamous cell carcinoma of tongue which comprise 41.54%, of total cases of oral squamous cell carcinoma which were followed by floor of mouth and other parts of oral cavity. Eman et al (2014) in their study have found most common site of oral squamous cell carcinoma was tongue that is 40% of the cases followed by floor of mouth (17.1%), lip (14.3%) and hard palate (8.6%). Mahima et al[25] (2015) in their study have found that maximum cases of squamous cell carcinoma of tongue which comprise 23.8% followed by buccal mucosa (18.9%); alveolar region (12.38%) and other parts of oral cavity. The findings of the present study are similar with all above studies conducted by Maheshwari et al[16] (2013); Eman et al[18] (2014); Mahima et al[25] (2015) i.e. showing most common site for OSCC being tongue followed by buccal mucosa and alveolar region.

Maheshwari et al[16] (2013) in their study they found that mean Ki-67 LI was 48.52±4.63; 52.23±5.52; 58.55±6.23 in well differentiated, moderately differentiated and poorly differentiated squamous cell carcinomas respectively. The study carried out by Verma et al[17] (2014), mean Ki-67 LI was 67.33±21.14; 75.52±17.34; 81.45 ±12.41 in well differentiated, moderately differentiated and poorly differentiated squamous cell carcinomas respectively; their expressions were found increased linearly, from normal mucosa through various grades of OSCC, statistically significant difference was also seen.

Our study also showed a highly significant correlation between Ki-67 labelling index and Ki-67 expression ($P<0.0001$). Furthermore, the Ki-67 labeling index was found to increase with the advancing grades of oral squamous cell carcinomas. The poorly differentiated oral squamous cell carcinomas showed a significantly higher proliferation than moderately differentiated oral squamous cell carcinomas ($p<0.0001$). Thus, higher Ki-67 labelling index could indicate a poor prognosis, as was demonstrated by Maheshwari et al[16] (2013). Our results supported the cogency of Ki-67 as a potential proliferative marker for oral squamous cell carcinomas.

In the study carried out by Eman M. et al[18] (2014) expression of Ki-67 LI with histological grading was 66.67% in low/moderate and 17.65% in high proliferation with well differentiated; 27.78% in low/moderate and 29.41 in high proliferation with moderately differentiated; 5.56% in low/moderate and 52.94% in high proliferation with poorly differentiated squamous cell carcinomas; statistically significant difference was also seen in their correlation between Ki-67 and tumor grading. The highest Ki-67 expression was found in poorly differentiated squamous cell carcinoma. Also, findings of studies Tumuluri et al[19] (2002); Arul et al[20] (2011); Premalatha et al[21] (2010) were showing concordance with the finding of the present study where increasing trend of mean Ki-67 LI was observed with the increasing histopathological grades of oral squamous cell carcinoma.

Roland NJ et al[22] (1994), concluded that Ki67 index is of no value in predicting the course of squamous cell carcinoma of the head and neck. The study carried out by Massoumeh Zargaran et al[23] (2012) concluded that the evaluation method of expression showed Ki-67 (MIB-1) is not a good immunohistochemical marker to assess invasion status and grade differentiation of OSCC; also, it cannot be used as a diagnostic tool to distinguish between variants of OSCC with similar grade. Both these studies showed discordance with the present study, as they could not find Ki-67 expression in any of the grades.

The study of Smita Shrishail Birajdar et al[24] (2014) showed expression of Ki-67 antigen between well differentiated and poorly differentiated OSCC. But in our study, there was Ki-67 expression in all three grades.

The studies Mahima et al. (2015);[25] Massoumeh Zargaran et al. (2012),[23] were showing highly significant inverse correlation which was found between the Ki-67, the stroma/tumour proportion and the degree of keratinization, this finding was showing discrepancy with the present study where increasing trend of expression (%) of Ki-67 LI was observed with the increasing histopathological grades of oral squamous cell carcinoma, it could be because of some studies show inter observer variability; variation in diagnosis of oral squamous cell carcinomas in grading by pathologists; may be the method of staining was not appropriate, some studies may include patients underwent systemic treatment like surgery, radiotherapy or chemotherapy or partial systemic treatment; the cut off value of Ki-67 expression and antibody types may also different among the included studies which affect the identification of prognostic significance.

CONCLUSION

Our study showed that the tumor cell proliferation as measured by Ki-67 LI has a positive correlation with histopathological Modified Broder's grading in oral squamous cell carcinoma. This finding assures that Ki-67 antigen can be used to determine the tumor behaviour and prognosis of oral squamous cell carcinomas. Further studies considering a greater sample size and with other variants of human Oral squamous cell carcinoma can be done to emphasize the utility of Ki 67 in assessing the biological behaviour of oral squamous cell carcinoma.

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