Original Article

Correlation between Ki-67 Labeling Index and Mitotic Index in Oral Squamous Cell Carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most prevalent type of oral cancer. Counting of mitotic figures as well as the detection of Ki-67 expression by immunohistochemistry have been used to detect cell proliferation in malignant tumors. We correlated Ki-67 expression with mitotic activity and observed any notable relationship between them, to ascertain the role of Ki-67 protein as a tumor proliferative marker in OSCC. **Subjects and Methods:** A cross-sectional study was undertaken at a tertiary care hospital in Western Maharashtra over a period of 2 years. Fifty paraffin blocks of patients diagnosed as OSCC were included in our study, who were classified into well-differentiated squamous cell carcinoma (n = 24), moderately-differentiated squamous cell carcinoma (n = 23), and poorly differentiated squamous cell carcinoma (n = 3). Two slides per case were stained with H and E and Ki-67 marker, then observed under light microscopy to obtain Mitotic Index (MI) and Ki-67 Labeling Index (LI), respectively. Results were analyzed using mean, standard deviation, Chi-square test, and unpaired t-test. **Results:** Hyperplastic epithelium revealed higher Ki-67 LI as compared to normal epithelium. There was a statistically significant (P = 0.045) increase in Ki-67 LI in the proliferating margin with grade of OSCC. Positive linear correlation was noted between Ki-67 and MI in tumor proper (r = 0.186) and proliferating margin (r = 0.337) and was statistically significant in the latter (P = 0.017). **Conclusions:** Ki-67 LI can reliably detect the proliferative potential of cells at the invasive margin of a tumor. MI, on the other hand, can detect the cell proliferation rate of the tumor that is in correlation with the histopathological grade of OSCC.

Keywords: *Ki-67, mitotic count, oral squamous cell carcinoma*

Introduction

Oral cancer is a widely prevalent cancer type emerging as a growing problem in various regions of the world. In India, the incidence of lip and oral cavity cancer is 11.42% with a 5-year prevalence of 19.59/100,000.[1] Despite advancement in research and diagnosis of oral squamous cell carcinoma (OSCC), 5- and 10-year survival rates still remain low.[2] Cell proliferation is considered one of the most important mechanisms in oncogenesis.[3] Any abnormal proliferative changes in the epithelium may be an indicator for the development of neoplasia and malignant transformation. The process of formation of two identical daughter cells from a mother cell by virtue of cellular division is called mitosis. Counting of mitotic figures is the oldest way of assessing proliferation and has been applied as a diagnostic tool in tumor pathology. Numerous tumor grading systems have used mitoses in their classifications.[4] Higher cell proliferation rate in aggressive neoplasms have been detected by assessing mitotic activity. This method has proven to be inexpensive and quick, although there have been studies questioning the reliability of Mitotic Index (MI) estimation in H and E stains.[5]

Gerdes *et al*. observed that Ki-67 marker could be a potentially important tool for estimation of proliferating cells in a neoplastic tissue.[6] He also detected Ki-67 in most human cell lines other than normal resting cells, leading him to conclude that the nuclear antigen detected by Ki-67 is associated with cell proliferation. The role of Ki-67 as a prognostic marker in various neoplasms has been discussed widely in several studies, albeit with conflicting and debatable results between them. Considering these facts, we decided to conduct a study, wherein we would ascertain the role of Ki-67 protein as a

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tumor proliferation marker in OSCC. We also aimed at correlating Ki-67 expression with mitotic activity in OSCC and observe any notable relationship between the two.

Subjects and Methods

A cross-sectional study was undertaken at a tertiary care hospital in Western Maharashtra over a period of 2 years, from September 2016 to August 2018. A total of 50 paraffin blocks of patients diagnosed histologically as OSCC, with satisfactory tissue preservation were included in the study. We excluded patients with major systemic illnesses, disseminated diseases, or history of chemoradiotherapy before the obtainment of samples. The sample size was calculated based on the duration of the study, number of cases of OSCC undergoing hemimandibulectomy every month, and availability of reagents for immunohistochemical staining. Two serial sections, 3–4 μm thick, were cut from each block out of which one was used for staining with H and E while the other for immunostaining with Ki-67 marker. Sections for Ki-67 immunohistochemistry (IHC) were obtained on Poly-L-lysine coated slides. Detection of Ki-67 was done using prediluted rabbit monoclonal antibody provided by BioGenex (Fremont, CA, USA). Super Sensitive™ Polymer-HRP Detection System from BioGenex was used for the staining procedure.

Tumor was graded in accordance with the histopathological classification of malignancy proposed by the World Health Organization in 2005.[7] The acquired slides were examined under a light microscope, and four different areas were noted under ×400 magnification, namely, normal epithelium (NM), hyperplastic epithelium (HP), tumor proper or center of tumor (TP), and proliferating margin of tumor (PM). Ki-67 Labeling Index (LI) was calculated as: number of positive epithelial cells/total number of epithelial cells ×100. In a study by Scurry *et al*., no significant difference existed for Ki-67 positivity in basal cells when lichen sclerosus was compared with squamous cell carcinoma.[8] Thus, we excluded basal cell immunopositivity from Ki-67 LI. The Ki-67 LI was noted in each of these four zones, whereas MI was calculated in TP and PM only. Intensity of Ki-67 immunopositivity was also graded as weak, moderate, or strong depending on the appearance of nuclei as faint brown, brown, and dark brown, respectively. Ki-67 LI was graded as Grade 0 (LI = 0%), Grade 1 (LI = 1%–25%), Grade 2 (LI = 26%–50%), Grade 3 (LI = 51%–75%), and Grade 4 (LI = >75%). MI was calculated as total number of mitotic figures/1000 tumor cells.[5] Identification of mitoses was done in accordance with the criteria of Van Diest *et al*.[3]

Statistical analysis

All parameters were measured using one-way analysis of variance test. The results were statistically analyzed using SPSS Statistics data editor 20, IBM Corporation, NY, US and Microsoft Office 2016. Statistical analysis was done using standard deviation, mean, Chi-square test, and unpaired *t*-test. Results were taken as statistically significant when $P < 0.05$.

Results

The mean age of cases in well-differentiated squamous cell carcinoma (WDSCC), moderately-differentiated squamous cell carcinoma (MDSCC), and poorly-differentiated squamous cell carcinoma (PDSCC) was 54.21 ± 20.64, 51.83 ± 14.76, and 54 ± 13.89, respectively. Males, at 56%, slightly outnumbered females. However, all PDSCC cases ($n = 3$) were seen in females.

The clinical profile of cases showed majority of OSCC in the buccal mucosa ($n = 32$), followed by the tongue ($n = 10$) and gingivobuccal sulcus ($n = 8$). Majority of buccal mucosa cases ($n = 18$) were of WDSCC, while majority of MDSCC was noted in tongue ($n = 6$) and Guillain–Barré syndrome (GBS) ($n = 5$) lesions.

Patients with WDSCC and MDSCC had a mean duration of disease of about 3 months, and the same was 9 months in cases with PDSCC. This relation was statistically significant.

Tumor size was graded on the basis of the American Joint Committee on Cancer tumor-node-metastasis staging of lip and oral cavity cancer, from which we have included only the "T" aspect of the staging.[9] Most cases belonged to T1 category ($n = 22$) followed by T2 ($n = 20$) and T3 ($n = 8$). Majority of WDSCC ($n = 11$) were of small size (≤2 cm), although two cases of small size showed PDSCC. Histological grading was done as WDSCC ($n = 24$), MDSCC ($n = 23$), and PDSCC ($n = 3$) [Figure 1].

MI and KI-67 LI were calculated as shown in Table 1 [Figures 2 and 3]. Distribution of cases based on grading of Ki-67 LI showed Grade 1 in normal ($n = 50$) and hyperplastic ($n = 47$) epithelium. TP ($n = 30$) and PM ($n = 23$) revealed mostly Grade 2 positivity. They had also revealed Grade 3 (TP $n = 3$; PM $n = 15$) and Grade 4 (TP $n = 1$; PM $n = 4$) Ki-67 positivity. Majority of our cases showed weak intensity ($n = 23$), followed by strong intensity ($n = 16$), and moderate intensity ($n = 11$) of Ki-67 immunostaining [Figure 4].

Mean Ki-67 LI was noted in NM as 6.58 ± 4.6, 4.78 ± 3.56, and 8 ± 2 and in HP as 12.96 ± 7.38, 12.52 ± 6.9, and 15 ± 1 in WDSCC, MDSCC, and PDSCC, respectively [Figure 3]. Thus mean Ki-67 LI was highest in PM followed by (in decreasing order) TP, hyperplastic, and normal. Statistically significant relation was noted when comparing MI in both TP ($P < 0.0001$) and PM ($P < 0.0001$) with histopathological grade of tumor, while Ki-67 LI showed statistically significant

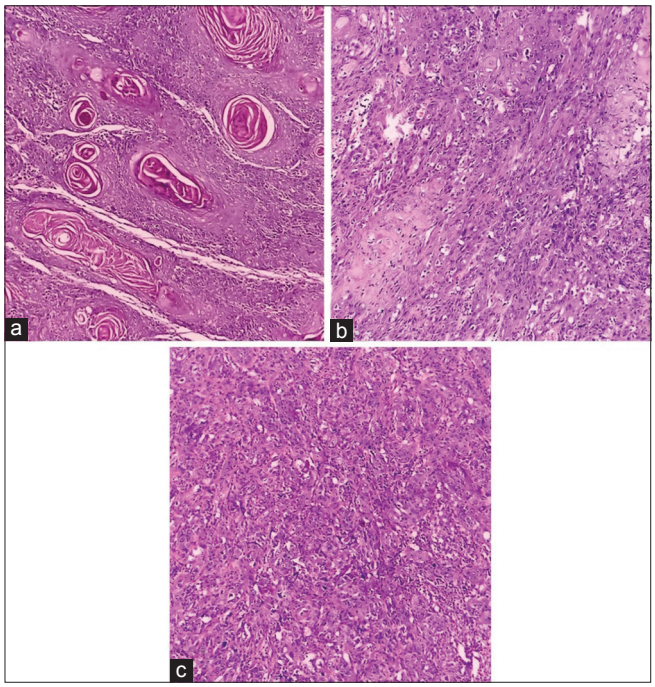

Figure 1: (a) Photomicrograph of well-differentiated squamous cell carcinoma (H and E, ×400). (b) Photomicrograph of moderately-differentiated squamous cell carcinoma (H and E, ×400). (c) Photomicrograph of poorly-differentiated squamous cell carcinoma (H and E, ×400)

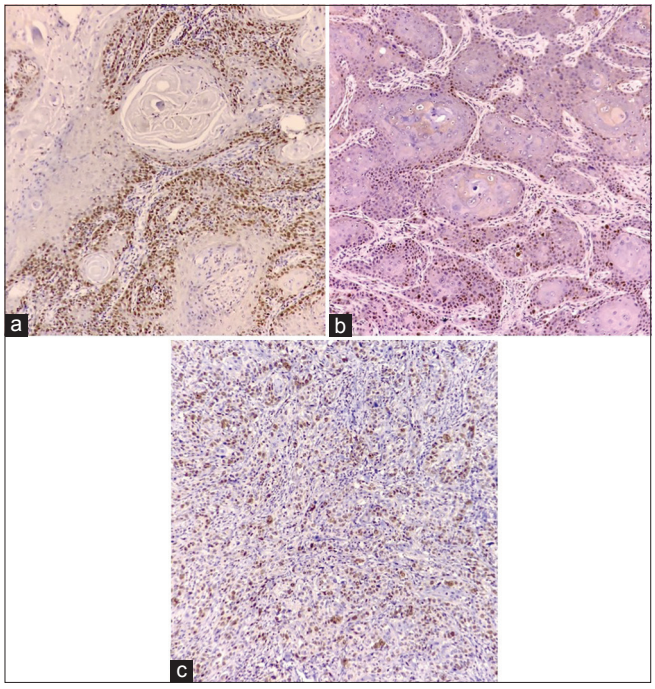

Figure 3: (a) Photomicrograph showing Ki-67 immunostaining in tumor proper of well-differentiated squamous cell carcinoma (Ki-67, ×100). (b) Photomicrograph showing Ki-67 immunostaining in tumor proper of moderately-differentiated squamous cell carcinoma (Ki-67, ×100). (c) Photomicrograph showing Ki-67 immunostaining in tumor proper of poorly-differentiated squamous cell carcinoma (Ki-67, ×100)

relation with grade of tumor only in PM (P = 0.045). Positive linear correlation was noted between Ki-67 and MI in TP (r = 0.186; P = 196) and PM (r = 0.337;

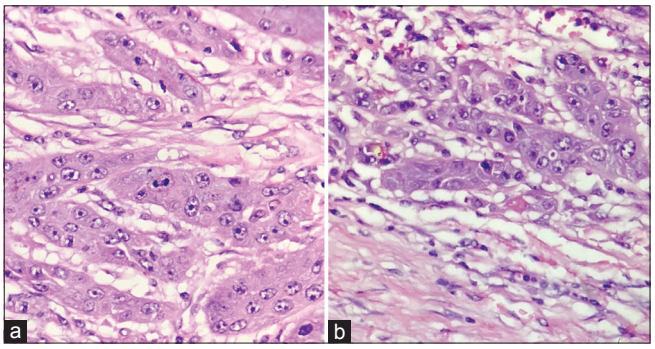

Figure 2: (a) Photomicrograph of oral squamous cell carcinoma showing mitotic figures in tumor proper (H and E, ×400). (b) Photomicrograph of oral squamous cell carcinoma showing mitotic figures in proliferating margin (H and E, ×400)

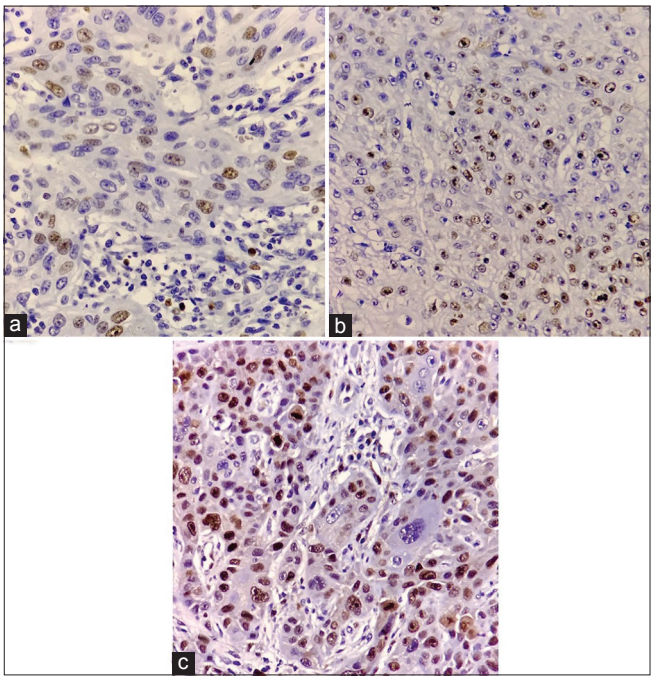

Figure 4: (a) Photomicrograph showing weak intensity of Ki-67 immunostaining in oral squamous cell carcinoma (Ki-67, ×40). (b) Photomicrograph showing moderate intensity of Ki-67 immunostaining in oral squamous cell carcinoma (Ki-67, ×40). (c) Photomicrograph showing strong intensity of Ki-67 immunostaining in oral squamous cell carcinoma (Ki-67, ×40)

P = 0.017) and was statistically significant in the latter [Figures 5 and 6].

Discussion

The mean age of cases was within the 51–60 age group. Johnson *et al*. described that in the Asian population, the fifth and sixth decades of life have been shown to display majority of cases of oral cancer. Worldwide, greater incidence of head-and-neck cancer has been shown in males, especially in the developing countries. This is probably due to greater indulgence by men in habits such as tobacco and alcohol consumption. The trend is, however, changing, as in some parts of the developing world such

Table 1: Comparison of Mitotic Index and Ki-67 Labeling Index in tumor proper and proliferating margin with hyperplastic epithelium diagnosis

HPE Diagnosis	*n*	Mean±SD			
		MI		Ki-67 LI (%)	
		TP	PM	TP	PM
WDSCC	24	7.75±4.089	12.46±5.524	29.96±14.393	40.63±17.282
MDSCC	23	13.48±5.026	17.26±5.964	34.96±12.843	47.09±16.917
PDSCC	3	23.00±7.000	28.00±5.292	33.33±11.372	66.00±10.583
F		18.61	11.62	0.80	3.30
P		<0.0001	<0.0001	0.45	0.045

HPE: Histopathological, MI: Mitotic index, LI: Labeling index, TP: Tumor proper, PM: Proliferating margin, SD: Standard deviation, WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma

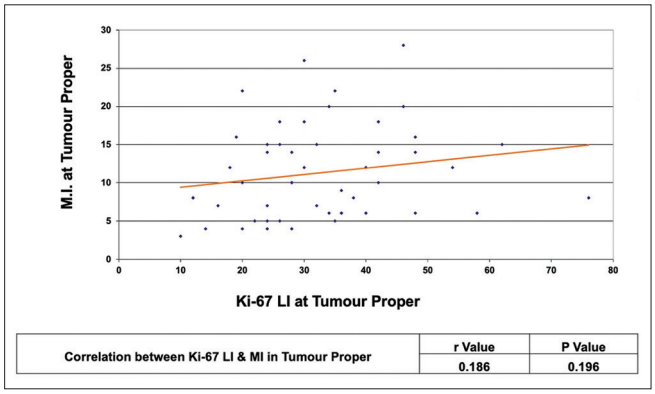

Figure 5: Correlation between Ki-67 Labeling Index and Mitotic Index in tumor proper

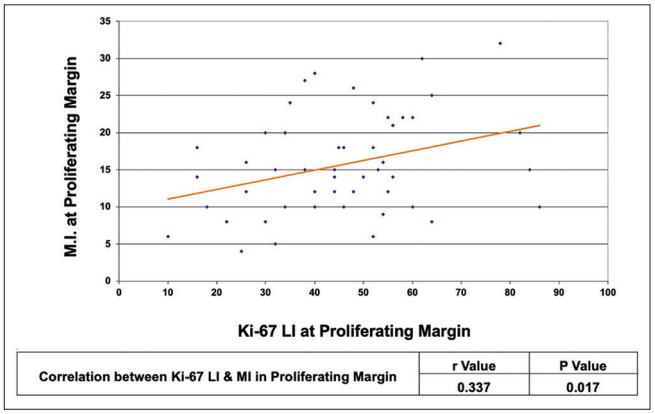

Figure 6: Correlation between Ki-67 Labeling Index and Mitotic Index in proliferating margin

as Brazil and India, females are increasingly indulging in habits similar to men.[10]

In our study, the most common anatomical site of oral cavity involved by OSCC was the buccal mucosa, accounting for over 50% of all our cases. Majority of cases in buccal mucosa were found to be associated with a diagnosis of WDSCC. Tongue and GBS showed higher instances of MDSCC. Ajay *et al*. had also found buccal mucosa to be the most common site of oral cancer in their study,[11] while Padma *et al*. noted majority of cases of OSCC in buccal mucosa to be of well-differentiated type.[12]

Mean duration of complaints by the patients at the time of diagnosis had a statistically significant positive relationship with the histopathological grade of OSCC. This relation could be attributed to the fact that total number of PDSCC cases was low (*n* = 3).

Ki-67 specifically gives nuclear immunopositivity, as it recognizes nuclear antigen which is associated with cell proliferation. We studied Ki-67 immunopositivity on formalin-fixed and paraffin-embedded tissue blocks between 2015 and 2018. We noted weak, moderate, and strong intensity in 23, 11, and 16 cases, respectively. As reported by Grillo *et al*., with decay in antigenicity comes decreased intensity of staining by IHC markers, and Ki-67 is no exception to the same. Cold temperature was shown to be the most favorable method of storage of paraffin blocks. Nuclear and membranous antigens were most affected by antigen decay since they require heat pretreatment for antigen retrieval.[13] Weak immunopositivity noted in our study could thus be attributed to these factors.

LIs were highest for PDSCC in case of both NM (LI = 8%) and HP (LI = 15%). In case of TP, Ki-67 LI was lowest for WDSCC (LI = 29.96%) and highest for MDSCC (LI = 34.96%). A statistically significant increase was noted with grade of OSCC, with highest mean Ki-67 LI being 66% in case of PDSCC. Furthermore, we noted higher mean Ki-67 LI when comparing TP with that of PM. This finding is in accordance with the study by Dissanayake *et al*. which states that in OSCC, more cells in the invasive tumor front are in the proliferative state as compared to the center of the tumors.[14] Tumuluri *et al*. found statistically significant increase of Ki-67 LI with increase in grade of tumor,[15] while Pity and Jalal showed that highest levels of Ki-67 LI were seen in PDSCC.[16]

MI is a measure of mitotic activity in tissues. An increase in the MI with histological grade of OSCC was noted, which was statistically significant [Table 1]. Kapoor *et al*. in his study mentioned that mitotic figures can be abnormally high in OSCC as MI is a measure of the proliferative activity of tissue.[5]

Furthermore, we wanted to find correlation between Ki-67 LI and MI as both of these are measures of cellular proliferation. There was a statistically significant positive linear correlation noted between Ki-67 LI and MI in PM. However, linear correlation between Ki-67 LI and MI was less in TP (r = 0.186; P = 0.196) as compared to PM (r = 0.337; P = 0.017). A study by Rudolph *et al*. similarly correlated Ki-67 LI and MI in various carcinomas and had found statistically significant inverse relationship between the two in squamous cell carcinomas of the upper digestive tract.[17] This discordance of results, thus, may be due to the difference in the site of the squamous cell carcinomas between the two studies.

Not only is the expression of Ki-67 a powerful predictor of grade of OSCC but also shows significant correlation with MI in invasive margin of the tumor. However, there is a tendency of pathologists to favor either of the two criteria and object the other. Whereas, some studies described close concordance in Ki-67 positivity and MI,[18] others found only weak associations,[19] and still others have noted inverse relation between the two in assessing the clinical outcome.[20]

These discrepancies in the relationship between Ki-67 LI and mitotic count/MI stem from reasons best explained in the context of cell cycle. Four distinct phases constitute the cell cycle, namely, the interphase consisting of the G1, S, and G2 phases, followed by the M phase where cell division occurs. These cells can be differentiated from the quiescent cells of resting phase (G0 phase) with the help of Ki-67 marker, as it is negative in cells not in the cell cycle (i.e., G0 phase). This leads to a slew of conclusions as pointed out in the study by Rudolph *et al*.[17] First, M phase constitutes only a small fraction of all cells that may show Ki-67 positivity. Second, since M phase is the shortest phase in the cell cycle, very few mitoses may be noted at any point of time. Third, difference in duration of different phases of cell cycle may lead to substantial variations in the relation between Ki-67 LI and mitotic count/MI. Based on these facts, we can safely assume that variation in types of tumors and degree of differentiation may result in distinct cell cycle kinetics.

Dissanayake *et al*. pointed out that with the help of Ki-67 IHC, we are able to assess the cells that are in proliferative state, but not the cell proliferation rate.[14] This meant that a tumor demonstrating a slow cell cycle may have low rate of cellular proliferation despite a high mean Ki-67 index, while a tumor that featured a short cell turnover could have few cells in cycle leading to a low mean Ki-67 LI. This fact could explain the absence of statistical significance in the association between Ki-67 LI and grade of OSCC in the center of tumor in our study. They also suggested that a method of overcoming this problem is by introducing double labeling of tissues *in vitro*.[21]

It can be aptly said that in OSCC, the nuclear marker Ki-67 is a valid indicator of cellular proliferation at the invasive front or tumor margin, and this has a positive correlation with histopathological grade of malignancy. In addition, we have evaluated the MI in the margin of tumor and correlated with Ki-67 expression and have obtained findings much more significant than with Ki-67 immunopositivity alone. The rapid division of cells in tumor margin leads to higher number of tumor cells in M phase (mitosis phase), and this could have been the reason for higher MI in these areas. With these findings, we can postulate the hypothesis that at the PM of tumor, Ki-67 LI is a measure of the fraction of tumor cells that are in cell proliferation (state of cell proliferation), while MI gives us the cell proliferation rate of tumor cells. The need for a better understanding of the physiological function of Ki-67 has led to growing interest in Ki-67 in recent years.

Conclusions

This study elucidated key findings related to Ki-67 expression and mitotic count in OSCC. While MI can be a cheap and fast method for assessment of cellular proliferation in malignancy, its use assumes greater significance in general practice when combined with Ki-67. Hence, we can state that Ki-67 LI can reliably detect the proliferative potential of cells at the invasive margin of a tumor, while MI can detect the cell proliferation rate of tumor that is in correlation with histopathological grade of OSCC.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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