Original Article

Ki-67 Expression in Oral Potential Malignant and Malignant Lesions and Correlation of Mitotic Index with MIB-1 Labeling Index

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Abstract

Introduction: Oral cancers are the most serious health issues in underdeveloped countries such as India and considered as the main cause of death. Among them, oral squamous cell carcinoma is the most common type (90%) of all malignancies. Various oral potential malignant lesions (OPMLs) can transform into malignancies. This study was conducted to determine the significance of Ki-67 expression in oral potential malignant and malignant lesions (MLs) as well as correlation of mitotic index (MI) with MIB-1 labeling index (LI) in these lesions. **Materials and Methods:** The study was performed on 60 cases in a tertiary care center over a period of 2 years. Ki-67 expression, MI and MIB-1 LI were calculated and correlated. **Results:** In the studied population, there were 49 (81.7%) males and 11 (18.3%) females. The mean age was 46.60 ± 9.94 (23–68 years), with majority of patients in 41–60 years of age group (46/60 cases). Anterior 2/3rd tongue is the most affected site, presented ulcer as the most common lesion. Smoking, tobacco, and betel nutchewing addiction were presented in 72% of the patients. Among 60 cases, 45 (75%) were OPMLs, while 15 (25%) cases were MLs. MI increases in OPMLs and MLs and comparison was significant ($P < 0.01$). MIB-1 LI was significant ($P < 0.01$) on comparison to dysplasia III and MLs. A positive correlation (0.01) was established between MI and MIB-1 LI of OPMLs and MLs. **Conclusion:** Ki-67 expression was found correlated with the progression of disease from OPMLs to MLs. Therefore, it is considered a proliferative marker that corresponds with disease progression. Both proliferative indices (MI and MIB-1 LI) are positively correlated.

Keywords: MIB-1 labeling index, mitotic index, oral potential malignant lesion

Introduction

Oral cancers are a serious health issues in underdeveloped countries such as India and considered as main cause of death.[1] SCC is the most frequent type of oral malignant neoplasm, comprising about 3% of all human malignancies and over 90% of all types of oral carcinomas.[2] Many potentially malignant diseases cause the development of the large number of cancers. Lesions such as leukoplakia (dysplasia), erythroplakia, oral submucous fibrosis, lichen planus, and discoid lupus erythematosus are among OPMDs.[3]

According to the WHO 2005, these lesions are defined as those in which the danger of malignancy is evident at the moment of diagnosis or later on.[4] The shift of oral potential malignant lesions (OPMLs) to malignant lesions (MLs) is complex, and it can be ascribed to a variety of factors, including betel nut chewing, tobacco, smoking, and alcohol consumption.[5]

Most prevalent among all OPMLs is leukoplakia with a specific focus mentioned on the premalignant character of OLP.[6] The amount of dysplastic alterations caused by the aggregation of genetic and molecular abnormalities is directly linked to the proportion of leukoplakia that develops into malignancy.[1]

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As evident, the majority of oral squamous cell carcinoma (OSCC) occurs due to precancerous injury; hence, the risks of malignant expression in these can be reduced if they are recognized in the primary stage. One of the premalignant harmful conditions is OLP, which has the potential to develop into a malignant condition.[7-11] The WHO classified lichen planus as a potentially malignant condition.[12-14] A significant cellular injury occurs in OLP, causing epithelial growth to retain structural integrity. In OLP, the change that happens during the cell cycle allows the lesion to advance toward malignancy.[15]

Histopathological analysis for the existence and severity of epithelial dysplasia is the most useful sign in determining the probability of malignant transformation of these OPMLs. Counting mitotic figures is one of the oldest methods of evaluating cellular proliferation. Identifying mitotic figures under a microscope and calculating their mitotic index (MI) has been used as a investigative device in tumor pathology. Despite various methods for determining cellular proliferation, till date, mitotic count on a well-stained H and E slide under a microscope has remained a popular technique for evaluating MI.[16] Clinical and molecular characteristics should also be considered together with histopathological evaluation.

Many molecular biomarkers had been recommended in the diagnosis and prognostication of OPMDs. Oral cancer precursors could be benefitted from epithelial differentiation, proliferative and genetic markers.[3]

Ki-67 antigen is the most prevalent IHC marker for cellular proliferation. It is a nuclear protein found in actively dividing cells during G2 and M stages of cell cycle. This protein translation at a certain point of cell cycle provides a benefit when used as a mitotic activity biomarker. Ki-67 antigen positivity usually linked to disease progression. It provides important information on the disease's aggressiveness and prognosis.[7, 17]

Considering the use of MI and Ki-67 biomarker in evaluating disease progression, this study is designed to determine the significance of Ki-67 expression in oral potential malignant and MLs, as well as the correlation of MI with MIB-1 LI among these lesions.

Material and Methods

Study design

This is a retrospective study conducted in the department of pathology at TMMC and RC Moradabad.

Study setting

The study was conducted from January 2019 to December 2020 and was approved by Institutional Review Board (IRB/4 (1)/2021).

Sample size

The study comprised 60 cases. In all cases, patients' relevant clinical history and examination findings were gathered.

Method of data collection

All samples were treated and stained with H and E stain as per the standard procedure. The study included all histologically verified OPMLs and MLs.

H- and E-stained slides were subjected to a detailed histological examination. The cases were categorized into two groups:- OPMLs and MLs. Oral submucous fibrosis, lichenoid reaction, lichen planus, mild, moderate, and severe dysplasia were classified as potential MLs. Well-differentiated, moderately differentiated, and poorly differentiated were classified as ML.

Mitotic index calculation

MI was estimated using a 40× objective to examine 1000 tumor cells for the existence of mitoses in H- and E-stained sections.[18] When focusing up and down, mitoses have hairy extensions, no nuclear membrane, and basophilic rather than eosinophilic cytoplasm, as shown in Figure 1. MI was evaluated in each case as the number of mitoses as a proportion of the total number of tumor cells counted.[19]

$$MI = \frac{\text{Number of mitoses per section}}{\text{Total number of tumor cells per section}} \times 100$$

Ki-67 immunostaining method

Ki-67 immunostaining was performed on all the cases. We prepared four micrometer-thin slices. After antigen recovery, endogenous peroxide activity was suppressed with 3% H_2O_2. MIB-1 antibody (Dako Denmark A/S, Glostrup, Denmark), monoclonal mouse anti-human Ki-67 antigen clone was used for MIB-1 immunostaining.

Positive control for MIB-1: With each batch of staining, a histopathological sample of a confirmed case of SCC from buccal mucosa was employed as a positive control.

Negative control for MIB-1: In place of primary antibody, 1% nonimmune serum was used as a negative control, with the rest of the protocols similar to the positive control.

Calculation of MIB-1 labeling index

MIB-1 LI was determined by using immunostained slide with Ki-67 antigen. LI was evaluated by counting the number of positively stained cells (nuclear staining) per 100 oral epithelial

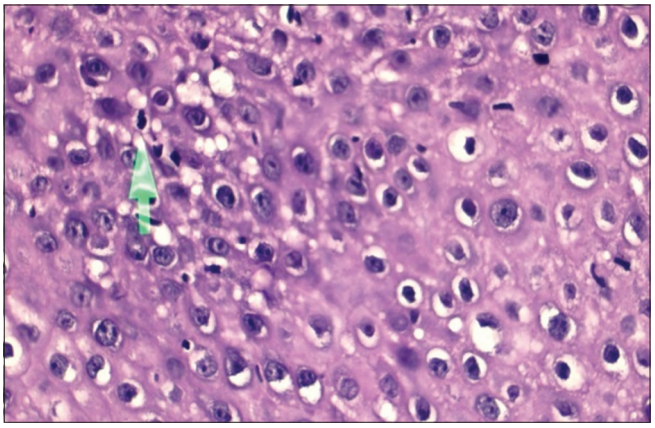

Figure 1: Photomicrograph showing mitosis(green arrow)

cells in different regions under 400× magnification and calculating the average as shown in Figure 2. Positive nuclei were reported as a proportion of the total nuclei counted.[20]

$$LI = \frac{\text{Number of cells showing positive staining}}{\text{Total number of cells}} \times 100$$

Grading of Ki-67 expression

Sections stained for Ki-67 proliferation (seen as nuclear staining) were graded on a scale of 1–3.[21]

- "3+" - High proliferation (>50% positive cells)
- "2+" - Moderate proliferation (30%–50%positive cells)
- "1+" - Low proliferation (10%–30% positive cells).

Statistical analysis

Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS) for Windows (version 25.0). Collected data was analyzed by SPSS (version 25.0) (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) was used to analyze the data. The test employed was the one way analysis of variance (ANOVA). A $P < 0.01$ was considered statistically significant.

Results

This study comprised a total of 60 cases. In the studied population, there were 49 (81.7%) males and 11 (18.3%) females. Our research revealed a significant male predominance, as shown in Table 1.

The patient age varied from 23 to 68 years old, with an average age of the study group was 46.6 ± 9.94. Maximum cases ranged from 41–60 years of age (46 out of 60 cases) as shown in Table 2.

As indicated in Table 3, the majority of lesions appear on the anterior 2/3 of the tongue as an ulcer, followed by growth and patch on buccal mucosa.

According to the findings, smoking, alcohol consumption, tobacco, and betel nutchewing addiction were present in 72% of the patients. Among 60 cases, 45 (75%) cases were classified as OPMLs while 15 (25%) cases as MLs. OPMLs were further subdivided into oral submucous fibrosis (2 case), lichenoid reaction (4 cases), lichen planus (4 cases), dysplasia I (15 cases), dysplasia II (12 cases), dysplasia III (8 cases) and ML as WD SCC-10 cases, MD SCC-3 cases, PD SCC-2 cases, as shown in Table 4.

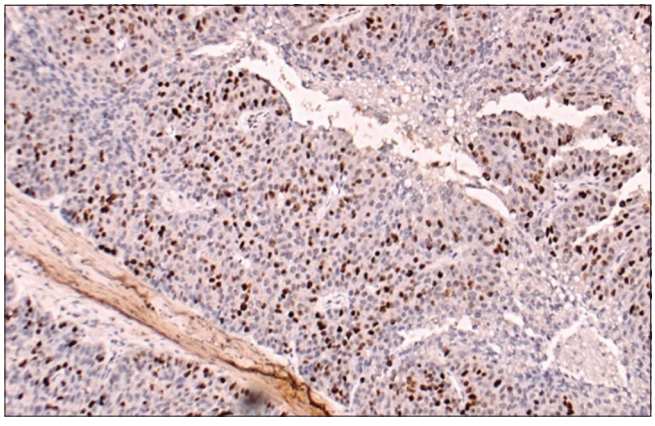

Figure 2: Photomicrograph showing Ki-67 positive cells

In all, 60 cases of OPMLs and MLs MI were assessed on H and E stained sections. MI increases progressively in OPMLs, from OSF to LR, and then to various stages of dysplasia (from Grade I to Grade III), i.e., from Dysplasia I (MI = 1.8% ± 3.0%) to Dysplasia II (MI = 2.29% ± 0.6%) to Dysplasia III (MI = 3.9% ± 1.15%). There was no discernible difference between Dysplasia I and Dysplasia

Table 1: Gender distribution of cases

Gender	Number of cases, *n* (%)
Male	49 (81.7)
Female	11 (18.3)
Total	60 (100)

Table 2: Age distribution of cases

Age group (years)	Number of cases, *n* (%)
1-20	0
21-40	12 (20)
41-60	46 (76.66)
61-80	2 (3.34)
Total	60 (100)

Table 3: Distribution of cases showing clinical findings

Clinical findings	Number of cases, *n* (%)
Ulcer	35 (58.3)
Patch	10 (16.7)
Growth	15 (25)
Total	60 (100)

Table 4: Distribution of histopathological diagnosis with mean mitotic index of cases

Histopathological diagnosis	Number of cases, *n* (%)	Mean MI (%) ±SD	*P*
OPML	45 (75)		
Oral submucous fibrosis	2 (3.3)	0.2±0.0	
Lichenoid reaction	4 (6.7)	0.25±0.05	
Lichen planus	4 (6.7)	0.35±0.05	
Dysplasia I (mild)	15 (25)	1.8±3.08	
Dysplasia II (moderate)	12 (20)	2.29±0.06	0.01
Dysplasia III (severe)	8 (13.3)	3.98±1.15	0.01
Malignant lesions	15 (25)		
WDSCC	10 (16.7)	5.8±0.686	0.01
MDSCC	3 (5)	6.2±0.953	0.01
PDSCC	2 (3.3)	6.5±1.414	0.01

OPML: Oral potential malignant lesions, SD: Standard deviation, MI: Mitotic index, SCC: Squamous cell carcinoma, WDSCC: Well-differentiated SCC, MDSCC: Moderately-differentiated SCC, PDSCC: Poorly differentiated SCC

II. However, dissimilarity among Dysplasia II and Dysplasia III was considerable ($P < 0.01$). On comparison between different subgroups of ML, it was found that MI was increased from WD SCC (MI = 5.8% ± 0.68%) to MD SCC (MI = 6.2% ± 0.95%) to PD SCC (MI = 6.5% ± 1.41%). The variation was considerable ($P < 0.01$). As the lesion progressed from OPMLs to ML MI was increased. On comparing the two (OPMLs and MLs), the dissimilarity was found to be considerable ($P < 0.01$), as shown in Table 4.

All immunostained sections of OPMLs and MLs, MIB-labeling index (LI) were determined. Ki-67 positivity was restricted to the lower one-third of the epithelium in several lesions such as OSF, LR, LP, and dysplasia. However, in Dysplasia III, Ki-67 positivity was found across the epithelium. As indicated in Table 5, increasing Ki-67 positivity was found from OPMLs to MLs.

Ki-67 immunoreaction analysis revealed high proliferation in 16 cases (26.66%), moderate proliferation in 14 cases (23.33%), and low proliferation in 30 cases (50%). Low-to-moderate proliferation was seen in all cases of OPMLs while high proliferation in one case of severe dysplasia and in all malignant cases, as shown in Table 5.

Mean LI was 20.68% ± 3.85% in dysplasia I, 30.12% ± 4.4% in dysplasia II, and 45.68% ± 5.5% in dysplasia III. The variation found was considerable ($P < 0.01$). On comparison of dysplasia III to overall ML and comparing overall OPMLs to that of ML the dissimilarity was considerable ($P < 0.01$) as shown in Table 6.

Correlation between MI and MIB-1 LI was performed by Pearson correlation (2-tailed test), as shown in Table 7. A significant correlation was found at 0.01 level (2 tailed test) and thus establish a positive correlation between MI and MIB-1 LI in all the lesions.

Discussion

Oral cancer is still common and is responsible for several deaths in Western and Asian countries. Every year, over 300,000 new cases of OSCC were recognized globally. According to the WHO, oral cancer is the eighth most frequent malignancy worldwide.[22]

Majority of cases in the studied population were the elderly, with a mean age of 46.6 ± 9.94 and a male preponderance (81.7% males), which correlates with the results of Buch *et al*.[23] and Johnson *et al*.[24] who described that the population of developing countries in Asia has a majority of cases in this age group, with a male preponderance due to men's greater indulgence in activities such as tobacco chewing and alcohol consumption. However, developing countries, particularly Brazil and India, are witnessing a shift in trends as their female population is adopting male-like behaviors.[24]

72% of cases of our studied population were exposed to potential hazards such as smoking, alcohol consumption, tobacco, and betel nut chewing which corresponds with the results of Subapriya *et al*.[25] and Waranakulasuriya *et al*.[26] These factors cause structural changes in cell DNA that, if unnoticed, lead to cancer. Majority of cases 35 (58.3%), present as an ulcer in the oral cavity, followed by growth in 15 cases (25%) and white patches in the oral mucosa, which differs from the findings of Maheshwari *et al*.,[22] who found growth in the oral cavity as most common presentation, followed by white patch and ulceration.

Epithelial dysplasia is distinguished by a change in the number of cells and tissue observed under a microscope, indicating a change in epithelial cell differentiation and an enhancement in suprabasal proliferative activity. According to Pindborg *et al* (1997),[48] Burkhardt and Maerker (1978),[49] and Kramer *et al* (1978),[50] epithelial dysplasia is a significant factor for the progression of malignancy among premalignant lesions owing to the aggregation of genetic and molecular changes. Although epithelial dysplasia does not always progress to cancer and may even reverse in some cases, according to research by Mincer *et al* (1972),[51] Silverman *et al* (1976),[52] Bánóczy and Csiba (1976)[53] and Gupta *et al.* (1980).[54] According to Burkhardt and Maerker, 34%, 43%, and 48% of mild, moderate, and severe dysplasia evolve into cancer, respectively.[49] Our study

Table 5: Expression of Ki-67 in OMPLs and malignant lesions

Histopathological diagnosis	Number of cases	Low expression	Moderate expression	High expression
OPML	45			
Oral submucous fibrosis	2	2		
Lichenoid reaction	4	4		
Lichen planus	4	4		
Dysplasia I (mild)	15	14	1	
Dysplasia II (moderate)	12	6	6	
Dysplasia III (severe)	8		7	1
ML	15			
WDSCC	10			10
MDSCC	3			3
PDSCC	2			2
Total	60	30	14	16

ML: Malignant lesions, OPMLs: Oral potential malignant lesions, SCC: Squamous cell carcinoma, WDSCC: Well differentiated SCC, MDSCC: Moderately differentiated SCC, PDSCC: Poorly differentiated SCC

Table 6: MIB-1 labeling index in OMPLs and malignant lesions

Histopathological diagnosis	Number of cases, *n* (%)	Mean MIB-1 LI (%)±SD	*P*
OPML	45 (75)		
Oral submucous fibrosis	2 (3.3)	4.85±0.49	
Lichenoid reaction	4 (6.7)	5.97±1.60	
Lichen planus	4 (6.7)	11.87±3.47	
Dysplasia I (mild)	15 (25)	20.68±3.85	0.01
Dysplasia II (moderate)	12 (20)	30.12±4.4	0.01
Dysplasia III (severe)	8 (13.3)	45.68±5.5	0.01
ML	15 (25)		
WDSCC	10 (16.7)	55.10±6.1	0.01
MDSCC	3 (5)	62.66±8.02	0.01
PDSCC	2 (3.3)	66.00±8.4	0.01

ML: Malignant lesions, OPML: Oral potential Malignant Lesions, SD: Standard deviation, SCC: Squamous cell carcinoma, WDSCC: Well differentiated SCC, MDSCC: Moderately differentiated SCC, PDSCC: Poorly differentiated SCC, LI: Labeling index

Table 7: Correlation of mitotic index and MIB-1 labeling index

LI	Mean±SD	Pearson correlation (two-tailes test)
MI	0.030150±0.188	Correlation significant (<0.01)
MIB-1 LI	0.3315±0.18812	

MI: Mitotic index, SD: Standard deviation, LI: Labeling index

comprised of (15 cases) mild dysplasia, (12 cases), moderate dysplasia, and (8 cases) severe dysplasia.

Increased proliferation is considered an early indicator of disordered development and is associated with more progressive lesions. The location of proliferating cells in the tissue might disclose more regarding the regulatory system that becomes aberrant throughout the cascade of events of tumorigenesis.[27]

The present study focuses on the proliferative activity of OPMs and MLs by evaluating MI, Ki-67 expression, and its LI. Both used as an indicator of diving cell; however, Ki-67 is thought to be more valid and easier marker due to nominal background staining and this nonhistone protein translate throughout the cell cycle apart from G0 phase.[28] Ki-67 is mainly used as a reference for determining the extent of proliferation. High cell proliferation was found to be a hallmark of tumor development, with predictive and prognostic values.[29]

This study revealed proliferative cells at aberrant places throughout the dysplastic epithelium shown in Figure 2. These findings corroborated the findings of Liu *et al*.,[27] who discovered that the superficial and basal layers revealed the most distinct variations among healthy and diseased tissues.

We attempted to assess Ki-67 expression in Oral potential malignant lesions (OPMLs) and malignant lesions (MLs) in this research and discovered that OPMLs showed expression dependent on their rate of proliferation and the results were considerable ($P < 0.01$) in terms of the average number of positive nuclei per millimeter of length. KI-67 expression was raised in various degrees of epithelial dysplasia (from mild to severe). Gonzalez-Moles *et al*., Kumar *et al*., and Kushner *et al*. all reported comparable findings.[30-32]

Ki-67 expression was minimal in the basal and parabasal layers of the epithelium in mild dysplasia. There was no considerable difference observed among mild-to-moderate dysplasia that is consistent with the results of Dwivedi *et al*.[33] and Birajdar *et al*.[7] It may be concluded that the prognosis of mild dysplasia is extremely difficult to predict since it is a nonviolent lesion with proliferative activity comparable to other potential MLs. However, Ki-67 expression was identified in 2/3rds and the full thickness of epithelium in moderate and severe dysplasia, respectively, that is comparable with the results of Gonzalez-Moles *et al*.[30] and Kumar *et al*.[31] Accordingly, in the future, the rate of carcinogenic alteration will be faster in moderate to severe dysplastic lesions. Hence, Ki-67 protein will be used as a predictive tool in the detection of malignant alteration in all forms of dysplasia.

Enhanced proliferative activity in the parabasal layers of dysplastic epithelium is mostly connected to the loss of heterozygozity in 3p, 9p, and 17p, which continues to be a premalignant sign and raises the possibility of emerging numerous tumors.[34]

Malignant transformation rate might be affected by the severity of dysplasia. High-grade dysplastic lesions might be 4–5 times more liable to acquire malignant transformation than mild dysplastic lesions.[35] We discovered that Ki-67 expression increased and demonstrated a significant difference ($P < 0.01$) with increasing histological grades, i.e., from well to poorly differentiated SCC, which is comparable to Takkem *et al*.[36] and Tumuluri *et al*.[37]

Moreover, we tried to assess the malignant transformation rate in OPMLs and MLs by using the Ki-67 LI. The presence and severity of dysplasia is the most crucial indicator, whether a lesion is developing toward malignancy. As a result, it is critical to identify markers for these alterations. Ki-67 LI in the current study was considerably raised in moderate and severe dysplasia and showed statistical significance ($P < 0.01$) when compared to mild dysplasia, LP, LR, and SMF because these are chronic immunologic mucocutaneous diseases, which correlates with the results of Beevi *et al*.,[38] Dwivedi *et al*.[33] and Takeda *et al*.[39] However, Neppelberg's research[40] showed the intensity of Ki-67 immunostain in the epithelium was positive (+) in 77.3% samples of OLP and (++) in 18.2% samples of OLP and Zargaran *et al*.[41] discovered that Ki-67 expression was similar in OLP and epithelial dysplasia, but much greater in Oral cellular carcinoma which concludes that the risk of OLP progressing to cancer is comparable with that of epithelial dysplasia. Thus, frequent checks were advised.

Among ML Ki-67 LI in this study was greatest in PDSCC (66.00 ± 8.4) compared to MDSCC (62.66 ± 8.02) and WDSCC (55.10 ± 6.1). A statistical significance ($P < 0.01$)

was found with Ki-67 LI in different grades of malignancy, that is comparable with the results of Buch A *et al*., Tumuluri *et al.* and Dissanayake *et al.*[23,37,42]

MI is a measure of tissue activity. The study showed statistical significance ($P < 0.01$) for a rise in MI with a rising degree of dysplasia (from moderate to severe), and the same results were observed with increasing histological grades in MLs, which corroborate the findings of Kapoor *et al*.[43]

Furthermore, we attempted to find out correlation among MI and MIB-1 LI as a measure of cell proliferation activity. We found considerable positive linear correlation among MI and MIB-1 LI that is comparable with the results of Buch *et al*.[23] Rudolph *et al*.,[44] on the other hand, found a statistically significant inverse connection between the two indices in upper digestive tract SCC. This difference in results might be attributed to dissimilarity in the site of lesions in the two studies.

Ki-67 is not only a strong predictor of OPMLs and MLs, but also has a strong relation with MI. Pathologists, on the other hand, have a tendency to prefer one of the two criteria while opposing the other. While some studies reported a good correlation among MI and MIB-1 LI[45] others report a poor correlation[46] and yet others reported an inverse relationship between the two when evaluating clinical outcomes.[47]

Conclusion

The study concluded that Ki-67 expression was considerably raised in MLs in contrast to OPMLs, suggesting that Ki-67 is a simple cell proliferation marker that corresponds with disease progression. It can be utilized as a prognostic indicator in the early detection and prediction of malignant transformation. The study established a positive correlation among MI and MIB-1 LI. Because MI is a low-cost and rapid approach for assessing cellular proliferation, its application becomes more significant and practically applicable, when coupled with Ki-67. As a result, we conclude that MIB-1 LI accurately identifies proliferative potential whereas MI can detect the proliferation rate of cells in the lesion in connection to histopathological grade. Thus a combined assessment of both indexes can play an essential part in the early detection of malignant potential lesions and appropriate management could be done at the earliest.

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Conflicts of interest

There are no conflicts of interest.

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