A study of Ki-67 expression and its clinicopathological determinants in nondysplastic oral leukoplakia

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Abstract

Context: Oral cancer is the third most prevalent malignancy in India. Leukoplakia is its most common precursor lesion. **Aims:** This study aimed at evaluation of the Ki-67 expression and thereby detection of the dysplastic potential in histopathologically nondysplastic oral leukoplakia (OL). Secondarily, another purpose was to correlate various clinicopathological factors with the labeling indices (LIs) of Ki-67 in those cases as well. **Settings and Design:** In total, 97 OL cases were examined. Relevant clinical and demographic information was retrieved from the pro forma, prefilled by the patients themselves during their first visit. **Subjects and Methods:** Ki-67 immunohistochemical staining was performed on paraffin-embedded tissue samples. Its LIs were calculated and correlated with different clinicopathological parameters using statistical software SPSS version 16.0. **Results:** 58.8% (57 cases) lesions exhibited a Ki-67 positivity of ≤5%, and 25.8% (25 cases) lesions exhibited it in the range of 6%–25%. Only 15 (15.4%) patches were stained positively between 26% and 60%. Patients' age beyond 50 years, nonhomogeneous leukoplakia, and tobacco addiction were the significant risk factors for high Ki-67 scores ($P < 0.05$). **Conclusions:** Ki-67 is an essential immunohistochemical marker for epithelial dysplasia in OL, especially when the conventional histopathology fails to appreciate the same. In this purpose, Ki-67 labeling on a routine basis delivers the most convenient results for patients aged above 50 years, and/or addicted to tobacco products, and/or suffering from nonhomogeneous patches.

Keywords: Dysplasia, histopathology, immunohistochemistry, Ki-67, oral leukoplakia

Introduction

Oral cancer represents ~3% of all malignancies in the Western nations, whereas among Indians, it accounts for >30% of all cancers.[1] Leukoplakia is the most common potentially malignant precursor lesion of the oral mucosa. It is defined as "a clinical white patch that cannot be characterized clinically or pathologically as any other disease."[2] Worldwide, its malignant transformation rate (MTR) varies between 0.1% and 17.5%. In India, its prevalence and MTR ranges at 0.2%–5.2% and 0.13%–10%, respectively.[3]

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Multiple clinical, pathological, and molecular factors have been indicated as potential predictors of malignant transformation, as well as in the prognostication of oral leukoplakia (OL). Overall, the conventional histopathological evaluation of epithelial dysplasia remains the most popularly practiced way of premalignancy determination in such cases.[4,5] However, this methodology carries a significant risk of false negativity. Several recent research works have concluded that immunohistochemical markers such as Ki-67 and p53 are more sensitive and specific indicators of malignancy conversion in OL. Even these individual immunomarkers can be utilized as independent predictors for the dysplasia.[6,7]

Ki-67 is a nuclear protein that is expressed during the active phases of cell cycle.[8] Its expression proportionately increases with the severity of dysplasia in OL. Therefore, it acts as a useful tool for detection of initial conversion into dysplasia. On the other hand, an early discovery of incipient cancer is regarded as the cornerstone of modern cancer prevention programs. Unveiling of the maiden biological derangement

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during its early malignant transformation phase holds the uttermost importance.[9] However, the earlier studies emphasizing on the diagnostic/prognostic competency as well as on scoring/labeling indices (LIs) of Ki-67 were mostly attributed to the comparative evaluation through various grades of dysplasia up to squamous cell carcinoma (SCC).[6-11] In this context, the present study aimed at evaluating the Ki-67 expression in the OL cases, which specifically did not feature any evidence of dysplasia on routine histopathology. Furthermore, an attempt was made to correlate various clinicopathological factors with the LI of Ki-67 in these cases.

Subjects and Methods

Study design

The present study was an institution-oriented prospective research work. It was accomplished during January 2014 to December 2014 in the respective Departments of Pathology and Oral Pathology in the North Bengal Medical College and Hospital and North Bengal Dental College and Hospital, Darjeeling, India. Prior approval from the Institutional Ethics Committee and informed consent from each participating patient were obtained regarding this study.

Patient selection

Patients who satisfied the World Health Organization-defined criteria for the OL,[2] and also did not manifest any evidence of dysplasia on simultaneous histopathology, were included in this study. Patients with previously treated, recurrent, or refractory OL, those being earlier diagnosed with oral epithelial dysplasia, SCC, or papilloma, and those suffering from grievous general health or any such medical condition were excluded from this research tabulation.

Demographic evaluation

A prestructured questionnaire was used. Relevant clinical information was accumulated regarding their age, sex, addiction habits etc. During presentation, the patients who used to smoke regularly since at least past 12 months were recognized as smokers,[12] and those habituated to drink at a minimal frequency of thrice weekly were identified as alcoholic.[13] Mucosal location of each OL was noted. Clinically, these patches were classified into thin homogeneous and nonhomogeneous subtypes. Abiding by the OL staging system amended by van der Waal, the lesions were categorized according to their size, into three groups from L1 to L3.[14]

Histopathological examination

Biopsy was performed as an outdoor-based procedure in the Department of Oral Pathology. Lesions below 2 cm in diameter were completely excised; however, larger lesions were sampled by punch biopsy. The tissue was routinely processed and stained with hematoxylin and eosin at the Department of Pathology. Under the nondysplastic category, the histopathological diagnoses included: Epithelial hyperplasia, hyperkeratosis, and lichenoid keratosis.

Ki-67 staining

For the Ki-67 immunohistochemical staining, routine formalin-fixed paraffin-embedded tissue was utilized. Antigen retrieval was performed by heating the sections at 98°C in citrate buffer of pH 6.0. 3,3 α-diaminobenzidine tetrahydrochloride was used as the chromogen. Normal human tonsil tissue was processed as positive control, and a negative control was set up by skipping the step of primary antibody. An extra attention was paid to prevent desiccation of the sections at any stage of the procedure. Hence, the steps beyond antigen retrieval were performed in a humid chamber.

Ki-67 scoring

Distinct brown nuclear staining within the squamous cells, irrespective of its color intensity, was considered as positive. The Ki-67 LI was calculated semi-quantitatively. Portion of the section containing the highest number of reactive cells was subjected for the calculation. Percentage of a total number of Ki-67 positive cells to a total number of cells was primarily deducted. The ultimate Ki-67 LI was then simplified into four scores according to that percent reactivity as: 0 = 0%–5%; 2 = 6%–25%; 4 = 26%–60%; 6 = 61%–99%.[6,9]

Statistical analysis

In the purpose of statistical analyses, SPSS version 16.0 (SPSS Inc. in Chicago: United States of America) for Windows was utilized. Fisher's exact test and Chi-square test were used for comparing the data. *P* value was appreciated as statistically significant only when it was under 0.05.

Results

Clinicopathological evaluation

In conformity to the inclusion criteria, a total of 97 OL patients were investigated within the stipulated study period. Table 1 highlights the principle clinical and pathological characteristics of these patients. Owing to the convenience with statistical analysis, the age group and OL sites were subcategorized into >50 or ≤50 years, and buccal mucosa, tongue, or other sites, respectively. Alcohol and tobacco were the most frequently consumed addictive substances. Among these, smokeless tobacco (45 cases, 46.4%) in the form of betel quid or locally available preparations, namely, "gutkha," "khaini," and "paan masala," were most popular [Table 2]. Any other substance abuse was extremely scarce in frequency and was so left out of the research tabulation. In addition to the histopathological diagnoses, the extent of subepithelial lymphocytic infiltration was also considered for statistical comparison. It was dichotomized as sparse and dense [Figures 1 and 2].

Immunohistochemical evaluation

Score 0 (57 cases, 58.8%) was the most frequently detected Ki-67 LI. However, interestingly, 25.8% and 15.4% patients had the LI of 2 and 4, respectively, i.e., an increased proliferative activity than the normal mucosa. None of the

Table 1: Clinicopathological features of all leukoplakia cases (*n*=97)

Features	Categorical values (%)
Age in years	
Range	27-72
Mean±SD	45.8±10.1
Gender	
Males	43 (44.3)
Females	54 (55.7)
Male:female ratio	1:1.26
Location of the leukoplakic patch	
Buccal mucosa	52 (53.6)
Tongue	31 (32)
Lips	8 (8.2)
Gingiva	4 (4.1)
Floor of mouth	2 (2.1)
Size of the leukoplakic patch in centimeters	
Range	0.4-6.2
Mean±SD	2.4±1.4
Histopathological diagnoses	
Hyperkeratosis	61 (62.9)
Epithelial hyperplasia	27 (27.8)
Lichenoid keratosis	9 (9.3)
Subepithelial lymphocytic infiltration	
Sparse	75 (77.3)
Dense	22 (22.7)
Ki-67 labeling index	
Score 0	57 (58.8)
Score 2	25 (25.8)
Score 4	15 (15.4)

SD: Standard deviation

Table 2: Distribution of the patients according to their addictive behavior

Type of addictive measures	Number of patients (%)
Alcohol	10 (10.3)
Tobacco smoking	9 (9.3)
Smokeless tobacco	11 (11.3)
Alcohol + tobacco smoking	8 (8.2)
Alcohol + smokeless tobacco	15 (15.4)
Tobacco smoking + smokeless tobacco	12 (12.4)
Alcohol + tobacco smoking + smokeless tobacco	7 (7.2)
Total	72 (74.2)

lesions expressed a score as high as 6. Contextual images are displayed in Figure 3. For analytical ease, the LI scores were divided into two groups as 0/2 or 4. Thenceforth, the LIs were compared with various addictive behaviors, clinical features, and histopathological findings from the patients and their patches. Purportedly, a statistically significant correlation was established between higher LI with the age beyond fifth decade ($P = 0.0101$), nonhomogeneous leukoplakia ($P = 0.0016$), and tobacco habits either in the form of smoking ($P = 0.0028$) or chewing ($P = 0.0094$). However, their gender distribution, alcoholic status, and lesion site, size or histology did not pose any significant relevance with the LI [Table 3].

Discussion

Ki-67 is a perichromosomally located, cell cycle-related antigen. Its expression is strictly restricted to dividing cells. Therefore, it is regarded as one of the most potent proliferation marker in the field of molecular pathology.[6] In normal oral mucosa, Ki-67 positive cells are detected dispersedly within the parabasal layer, i.e., the two cell strata above basal layer. However, its expression beyond that within the suprabasal layer indicates epithelial dysplasia.[8]

The proliferative activity of the epithelial cells in nondysplastic OL has been assessed by several researchers, mostly as a part of discriminative analyses between normal epithelium, hyperkeratotic OL, intraepithelial neoplasia, and SCC. However, there is notable disparity among the observations. In this respect, Humayun and Prasad[6] examined four cases of OL without any dysplasia. The same Ki-67 LI scoring system, as in currently discussed study, was implicated by them. They found two cases with score 0, and one case each with scores 2 and 6. Sinanoglu *et al*.[11] isolated an average LI of 36% within 19 cases of hyperkeratotic OL, which was almost at par with the cases of oral intraepithelial neoplasia 1 and 2 they examined. On the contrary, Dwivedi *et al*.[8] noticed very little disagreement between the mean LIs of normal oral epithelium (13.65%) and nondysplastic OL (12.78%). Same was the experience of Kumar *et al*.[15] too. They recorded the mean LIs in normal and leukoplakic oral epithelia without dysplasia as 6.38% and 9.80%, respectively. In the present study only, the nondysplastic patches were evaluated in detail. Here, 58.8% cases accounted for the LI of ≤5%, i.e., score 0 and 25.8% cases accounted for 6%–25%, i.e., score 2. Rest of the lesions belonged to score 4, and none were scored with 6. Under this beacon, the discrepant and relatively higher mean LIs within OLs lacking any dysplasia from earlier literature, are possibly the misconsequences of smaller sample sizes.

In a review of 14 prospective studies encompassing whole 992 OL patients, the cumulative mean MTR was derived as 12.1%. It was again directly related to the grades of oral dysplasia.[16] Bouquot *et al*.[17] reported that severe dysplasia has an overall MTR of around 7%–50%; and Speight[18]

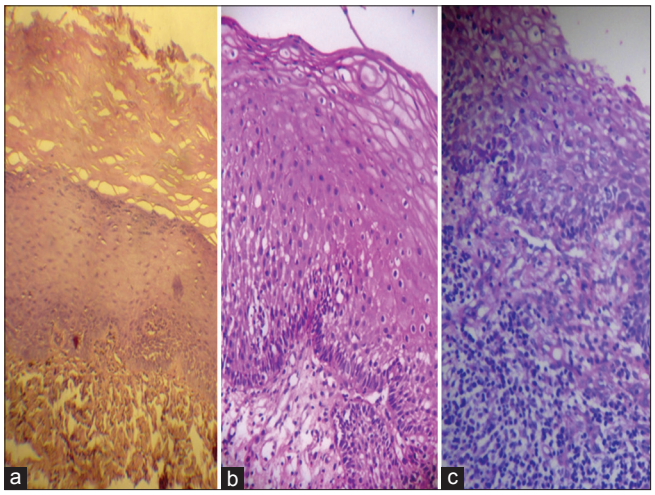

Figure 1: (a) Hyperkeratosis (H and E, ×40); (b) epithelial hyperplasia (H and E, ×100); (c) lichenoid keratosis (H and E, ×100)

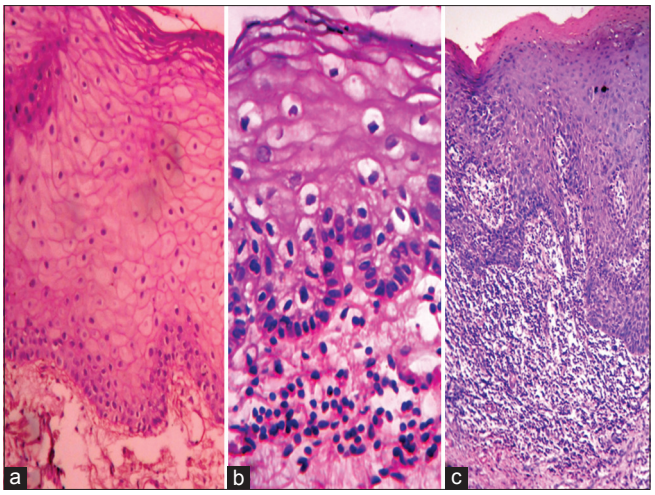

Figure 2: (a) None and (b) mild subepithelial lymphocytic infiltration: Categorized as "sparse" (H and E, ×400); "dense" (c) subepithelial lymphocytic infiltration (H and E, ×100)

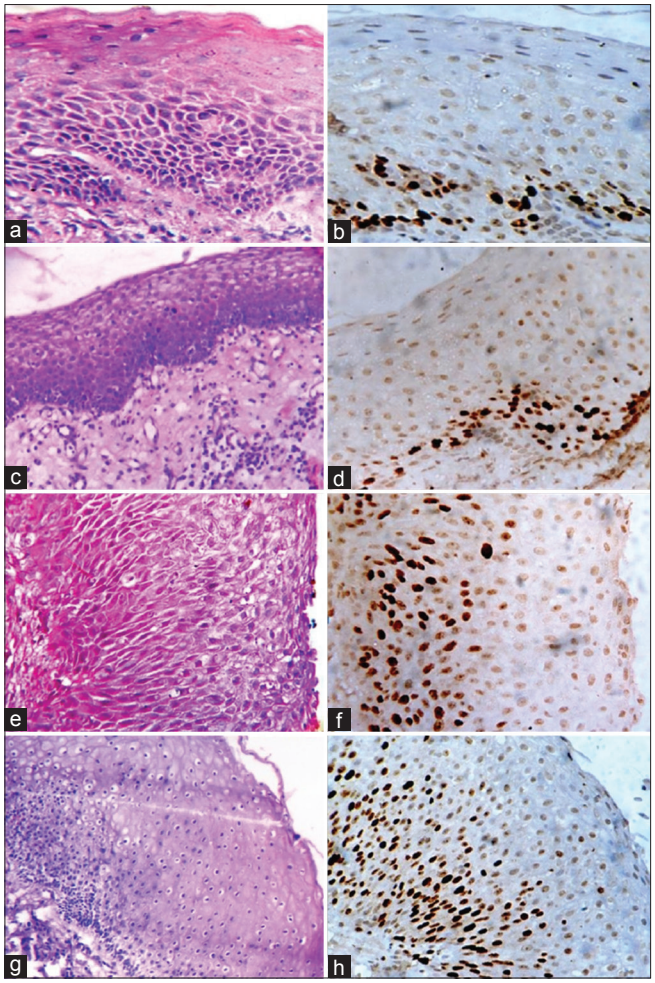

Figure 3: Representative histopathological and immunohistochemical images of various Ki-67 labeling index scores: (a and b) score 0; (c and d) score 2; (e and f) score 4; (g and h) score 4 with nearly 50% stained cells (a, e, and g: H and E, ×400; c: H and E, ×100; b, d, f, and h: Ki-67 IHC, ×400)

reported that moderate and mild dysplasia harbor the MTR of approximately 3%–15% and <5%, respectively. Molecular expression of p53, Ki-67, CK13, p16INK4a, and some other markers has been proposed as important predictive factors for oral dysplasia. Even if there is a lack of histological evidence for dysplasia, these molecules can be used as independent predictors for the dysplasia. Ki-67 is the most widely studied immunomarker in this context.[7,10,19] The previous literature on Ki-67 expression demonstrated the range of positivity for oral dysplastic epithelia from 13.5% to 51.2% and for oral SCC from 25% to 74.6%.[6-11,15] On the other end, many earlier studies and also this presently discussed study of ours have revealed that the lesions lacking any histopathological evidence of dysplasia may immunohistochemically express high and abnormal proliferative activity.[9,11] These OLs carry a significant potential to develop into SCC and should be managed in the same way as the corresponding grades of dysplasia. Such scenario of premalignancy in histopathologically nondysplastic epithelium may undermine any yet to be unfolded mechanism of carcinogenesis.[16]

Among the clinical factors: Increasing age, feminine gender, glossal location, large patch size, and nonhomogeneous phenotype indicate poor prognosis of OL, in terms of recurrence or malignancy transformation. More than 90% of OLs progressing to dysplasia or carcinoma have been seen to arise in the tongue and floor of the mouth. Exposure of these areas to a higher amount of carcinogens suspended in saliva could explain its pathogenesis.[20,21] Quite identically a large size of the patch, i.e., L3 >4 cm has been identified as another important determinant of malignant transformation.[22] However, in the discussed study, there was no significant correlation between OL site/size or its gender distribution with higher Ki-67 scores. Malignant transformation of an OL usually supervenes over 40 years

Table 3: Clinicopathological correlation of Ki-67 expression in all leukoplakia cases (*n*=97)

Categories	Ki-67 labeling index		*P*
	Score 0/2 (%)	Score 4 (%)	
Age in years			
>50	19 (19.6)	9 (9.3)	0.0101*,#
≤50	63 (64.9)	6 (6.2)	
Gender			
Males	35 (36.1)	8 (8.2)	0.5738#
Females	47 (48.5)	7 (7.2)	
Location of the leukoplakia			
Buccal mucosa	44 (45.4)	8 (8.2)	0.7629δ
Tongue	27 (27.8)	4 (4.1)	
Other sites	11 (11.3)	3 (3.1)	
Type of the leukoplakia			
Thin homogeneous	67 (69.1)	6 (6.2)	0.0016*,#
Nonhomogeneous	15 (15.4)	9 (9.3)	
Size of the leukoplakia			
L1	49 (50.5)	6 (6.2)	0.0818δ
L2	17 (17.5)	2 (2.1)	
L3	16 (16.5)	7 (7.2)	
Addictive measure			
Alcohol			
Consumers	33 (34.0)	7 (7.2)	0.7768#
Nonconsumers	49 (50.5)	8 (8.2)	
Smoking			
Consumers	25 (25.8)	11 (11.3)	0.0028*,#
Nonconsumers	57 (58.8)	4 (4.1)	
Smokeless tobacco			
Consumers	33 (34.0)	12 (12.4)	0.0094*,#
Nonconsumers	49 (50.5)	3 (3.1)	
Histopathological diagnosis			
Hyperkeratosis	52 (53.6)	9 (9.3)	0.2736δ
Epithelial hyperplasia	21 (21.6)	6 (6.2)	
Lichenoid keratosis	9 (9.3)	0 (0.0)	
Subepithelial lymphocytic infiltration			
Sparse	65 (67.0)	10 (10.3)	0.3195#
Dense	17 (17.5)	5 (5.2)	

*Statistically significant *P* values, #Fisher's exact test, δChi-square test

of age. Less than 1% males experience such precancerous transformation before the fourth decade, whereas alarming 8% men are affected beyond 70 years.[21,23] Similarly, in the current study, a significant correlation was procured for a high Ki-67 score, with age beyond the fifth decade and nonhomogeneous leukoplakia.

Tobacco addiction, either in the form of smoking or chewing, substantially augments the oral carcinogenesis. Many chromosomal deletions, translocations, and structural abnormalities caused by tobacco play the pathogenic role.[24] Although there is no clear evidence that alcohol consumption leads to the development of OL or its malignant transition, its synergistic effect together with tobacco in oral oncogenesis is beyond doubt.[25] This present study substantiated a significant relationship between tobacco addiction and a high Ki-67 score of 4. However, alcoholism did not yield any statistical significance in this respect.

Histopathologically, the 97 cases from the current study were diagnosed as hyperkeratosis, epithelial hyperplasia, and lichenoid keratosis. When the Ki-67 scoring patterns from

these three histological categories were compared together, the difference was found to be statistically insignificant. Previously, Kannan *et al*.[9] classified the nondysplastic OLs in their experience into proliferative/hyperplastic and nonproliferative/hyperkeratotic groups. They also failed to manipulate any significant difference in this context. Same was the observation of Dwivedi *et al*.[8] in 2013 as well.

Subepithelial lymphoplasmacytic infiltration has long been proposed as an indicator of dysplasia as well as malignancy transformation in OLs.[26] Yagyuu *et al*.[7] examined the OL patches from 94 Japanese patients and correlated the subepithelial lymphocytic infiltration with various grades of epithelial dysplasia as well as Ki-67 expression. They noticed that although there is an apparent tendency of higher subepithelial lymphocyte infiltration with increasing grades of dysplasia, such association did not manage any statistical significance. Similar consequences were encountered again in this current study. Here, dense subepithelial lymphocytic infiltration was noted in 33.3% (5 out of 15) lesions expressing Ki-67 LI score of 4, but also in 20.7% (17 out of 82) lesions with low Ki-67 LI score of 0/2. Such an apparent difference did not account for any statistical significance [Table 3].

The management protocol of OL differs between the clinicians. In the absence of dysplastic epithelial changes, clinicians mandate: Smoking cessation with a periodic reexamination and repeat biopsy if there is any change in the appearance of the lesion, or use of serial clinical photographs of the lesion to demonstrate any change between visits.[27] If an OL shows mild dysplasia, many physicians opt for the above-mentioned approach of watchful waiting. However, their recommended recall intervals vary significantly, ranging from 3 months to annual.[16] Moreover, it is difficult to predict which of these patients will return at the stipulated interval. Therefore, physicians in favor of the removal of the mildly dysplastic lesions are also there. If significant morbidity results owing to the lesion's location or size, it is acceptable for them to make a surveillance approach.[27] A complete excisional biopsy is the universally accepted choice of therapy for moderate to severe dysplasia. Surgical methods such as scalpel excision, cryosurgery, electrosurgery, or laser surgery – all carry similar effectiveness in curing these lesions. Large lesions may require grafting following surgery.[20] On the other hand the surgical removal of an OL by no means alters the natural history of the disease. As a result, several OLs, including many nondysplastic ones, often recur after complete excision. However, obviously, those lesions with dysplastic morphological changes carry the worst prognosis.[27] In this respect, the present study was aimed at evaluation of the Ki-67 expression in histologically nondysplastic OLs. Of which, a total of forty lesions were identified as having high Ki-67 scores amounting to dysplasia. These lesions actually have a much worse prognosis than other contemporary patches with a Ki-67 LI score of 0 and need a more aggressive treatment approach. Subsequently, this study also derived some clinicopathological factors having a significant association with high Ki-67 scores. Hence, by keeping these risk factors in mind while planning for a Ki-67 labeling of nondysplastic OLs will ease out the patient segregation.

Conclusions

The present study reinstalls the fact that Ki-67 is an important immunohistochemical tool to recognize different grades of epithelial dysplasia in OL, even if there is no definite histopathological evidence of that. However, its application on a routine basis for nondysplastic OL may prove economically cumbersome. As obtained from the current results, patients older than 50 years, nonhomogeneous OL, and tobacco abuse have a significant association with higher Ki-67 positivity. Hence, consideration of this fact while planning for a Ki-67 labeling in OLs without dysplasia would be most appropriate.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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