



## Head and Neck Cancer

# Importance of Ki-67 Labeling in Oral Leukoplakia with Features of Dysplasia and Carcinomatous Transformation: An Observational Study over 4 Years

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## Abstract

**Background** Early detection of dysplastic changes within oral potentially malignant disorders is the mainstay to prevent oral cancer. Ki-67 is one of the most useful antigens in this purpose.**Aims** The study aims were to recognize and mutually compare the proliferative status of idiopathic oral leukoplakia (OL) patches, which presented through different forms of dysplasia and carcinoma.**Settings and Design** In 4 years of observation, cumulatively 140 OL lesions were included for examination. The wholesome Ki-67 labeling scores in each of the subgroups were calculated.**Subjects and Methods** The World Health Organization recommended histopathological classification was used to categorize the dysplastic and malignant lesions. Paraffin-embedded tissue sections were processed for Ki-67 immunostaining. The labeling indices (LIs) were quantified semiquantitatively at the site of maximal reactive cells on tissue sections.**Statistical Analysis** The statistical comparison was performed by means of the SPSS software (Version 16.0 SPSS Inc.). A *p*-value < 0.05 was considered as the benchmark for statistical significance.**Results** A steady and significant increment in Ki-67 expression was discovered from dysplastic to malignant OL patches compared with normal mucosa. The labeling differences were significant between normal mucosa and mild dysplasia, as well as between mild, moderate, and severe dysplasia. However, the expression did not differ significantly with the severity of oral cancers.**Conclusions** Ki-67 is a useful molecular marker of carcinogenesis in OL. It also serves worthwhile in separating marginally dysplastic lesions, such as mild dysplasia or verrucous carcinoma from their benign epigones.

## Keywords

- ▶ immunohistochemistry
- ▶ Ki-67
- ▶ oral epithelial dysplasia
- ▶ oral leukoplakia
- ▶ squamous cell carcinoma

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## Introduction

The 13 different centers of population-based cancer registry in India calculated oral cancer as the third most prevalent malignancy after carcinoma breast and carcinoma cervix. Nearly 90 to 95% of oral cancers consist of squamous cell carcinoma (SCC). Its age-standardized incidence rate has been 12.6 per 100,000 population, which encompasses approximately 30% Indians suffering from cancer.<sup>1,2</sup> Approximately 80% of oral SCC develop from any preexisting potentially malignant disorder (PMD). Oral leukoplakia (OL) is the frequent most PMD encountered in India, with a prevalence of 0.2 to 5.2%, and up to 10% of malignant transformation rate.<sup>1</sup>

The physiological proliferative activity of oral epithelium is limited to its parabasal layer, the two-cell-thick-strip immediately overlying the basal layer. On dysplastic transformation abnormally, excessive cellular proliferation ensues and the physiological limitations are then breached. Multiple upregulated proliferation markers can thus be useful in determining the dysplastic transition in oral PMDs.<sup>3</sup> Ki-67 is the best marker to demonstrate the proliferative fraction of oral epithelium. Its nuclear expression is restricted to the G1-S-G2 phases of cell cycle and mitosis. Its short half-life makes it undetectable beyond the proliferative stage. Furthermore, Ki-67 holds the added advantage for the remaining uninfluenced from any extracellular biological alterations. Therefore, Ki-67 not only detects the hyperactive cells in oral epithelial dysplasia (OED), but also its quantitative positivity is comparable to the clinical course or prognostication of the disease.<sup>3,4</sup> Previously, several researchers evaluated the percentage reactivity of Ki-67 in different histological grades of OED,<sup>3-7</sup> whereas a handful number of studies incorporated the Ki-67 labeling index (LI) into a simple scoring system for the purpose of comparative ease.<sup>8,9</sup> In this latest approach, we focused on the corroboration of Ki-67 labeling scores with multitudes of OED and epithelial malignancy, which clinically presented as idiopathic OL.

## Subjects and Methods

It was a 4-year prospective study orchestrated from January 2013 to December 2016. At the Department of Oral Pathology, North Bengal Dental College and Hospital, idiopathic OL patches are routinely biopsied on an outdoor basis. Subsequent histopathological and immunohistochemical proceedings are performed at the Department of Pathology, North Bengal Medical College and Hospital. Here, during this study, the de novo cases of idiopathic OL that featured dysplastic or carcinomatous changes on histopathology were only analyzed. Persons with a prior history of similar or any other oral PMD, and also in whom therapy has already been commenced for the present patch, were left out. Twenty specimens of normal-appearing buccal mucosa were included from the archives as controls. In accordance with the World Health Organization (WHO) classification, the lesions were diagnosed as mild, moderate, and severe dysplasia, SCC, and verrucous carcinoma (VC). The lesions of SCC in situ were clustered together with severe dysplasia. The SCC lesions were further subclassified into well-differentiated

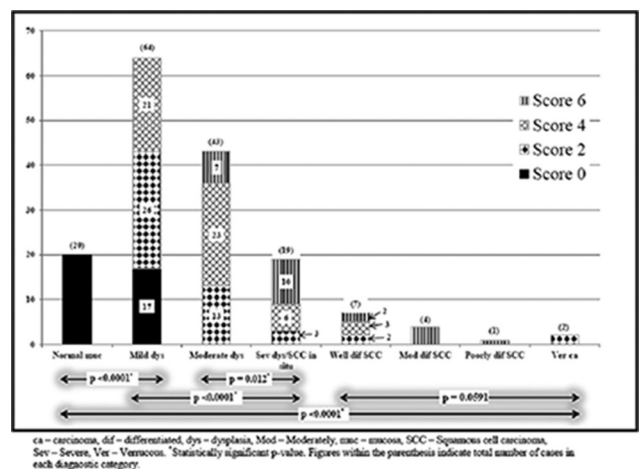
(WDSCC), moderately differentiated (MDSCC), and poorly differentiated (PDSCC) spectrums.<sup>10</sup>

Ki-67 immunohistochemical (IHC) staining was performed with formalin-fixed paraffin-embedded tissue-sections using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Positive and negative controls were devised from the sections of normal tonsil tissue and by omitting the step with primary antibody, respectively. Any recognizable nuclear browning regardless of its intensity was qualified for positivity. Ki-67-positive cells were enumerated at the segments fetching maximal reactive cells. Next, the percentages of overall Ki-67 positive cells in respect of the absolute amount of epithelial cells were determined semiquantitatively. Finally, the Ki67 LI was computed in four scoring categories as follows: 0 = 0–5%, 2 = 6–25%, 4 = 26–60%, and 6 = 61–99%.<sup>8,9</sup> For statistical comparison, chi-square test, as incorporated with SPSS software Version 16.0 (SPSS Inc., Chicago, Illinois, United States) for Windows, was utilized. Statistical significance was inflicted upon *p*-value regressing under 0.05.

## Results

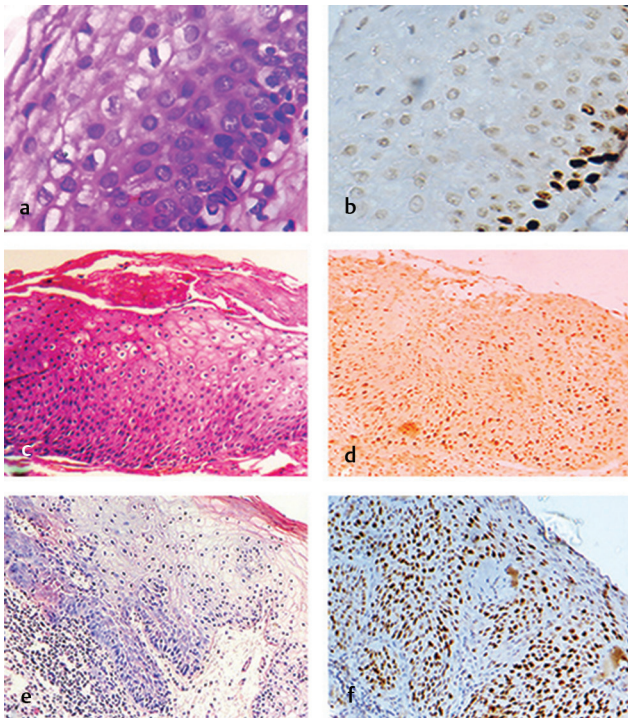
A total of 786 idiopathic OL patches were biopsied during the observed 4 years. Thereof, 126 patches were histopathologically diagnosed with dysplasia and 14 patches turned out as definite malignancies. Besides SCC (12 cases), 2 cases of VC were recognized (► Fig. 1). On Ki-67 IHC, all 20 normal mucosal samples were labeled as score 0, as was also evidenced with 17 out of 64 patches of mild dysplasia. The remaining mildly dysplastic lesions expressed LI scores of 2 (26 cases) and 4 (21 cases) (► Fig. 2). Henceforth, a significant labeling discrepancy ( $p < 0.0001$ ) was noted between normal and mildly dysplastic oral mucosae (► Fig. 1).

The lesions featuring moderate and severe dysplasia were immunohistochemically distributed into scores 2, 4, and 6 (► Figs. 3 and 4). Their labeling diversity fetched a low statistical significance ( $p = 0.012$ ) in this regard. However, the overall intensifying Ki-67 LI scores in respect of worsening histological grades of dysplasia garnered significant statistical association with  $p < 0.0001$  (► Fig. 1).

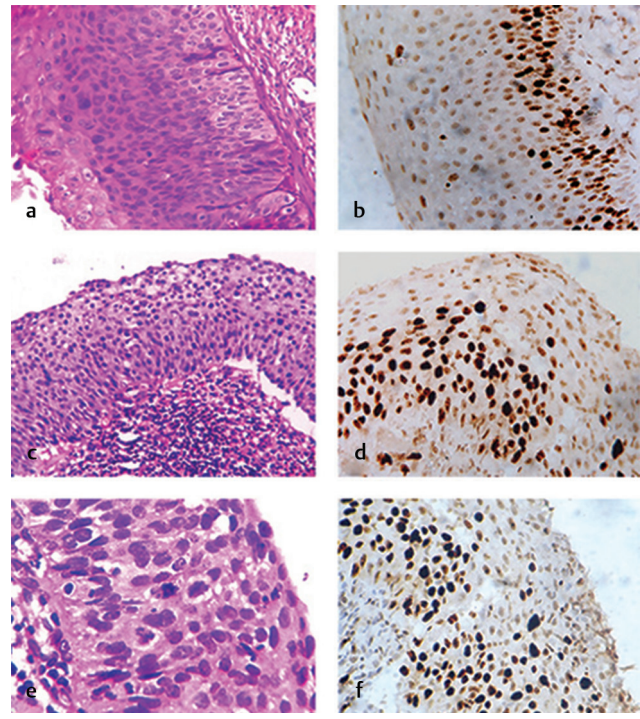


**Fig. 1** Comparison of Ki-67 labeling in normal oral mucosa, oral epithelial dysplasia, and carcinoma.

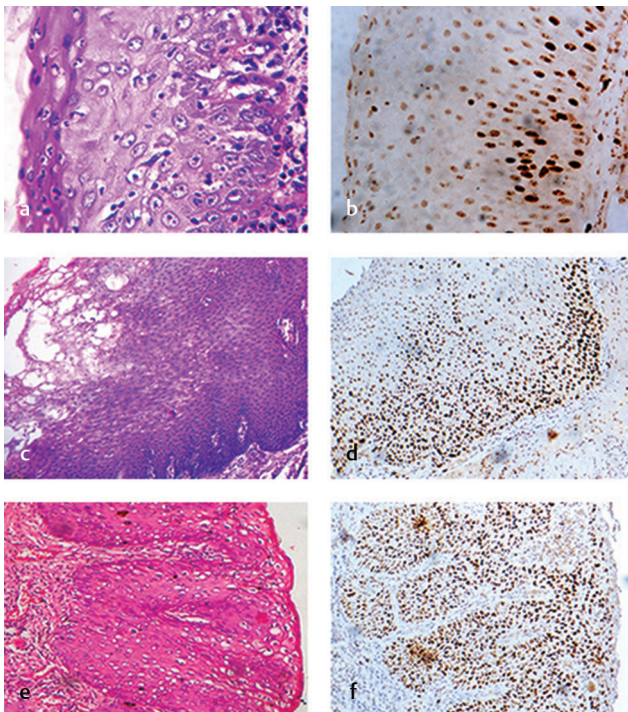




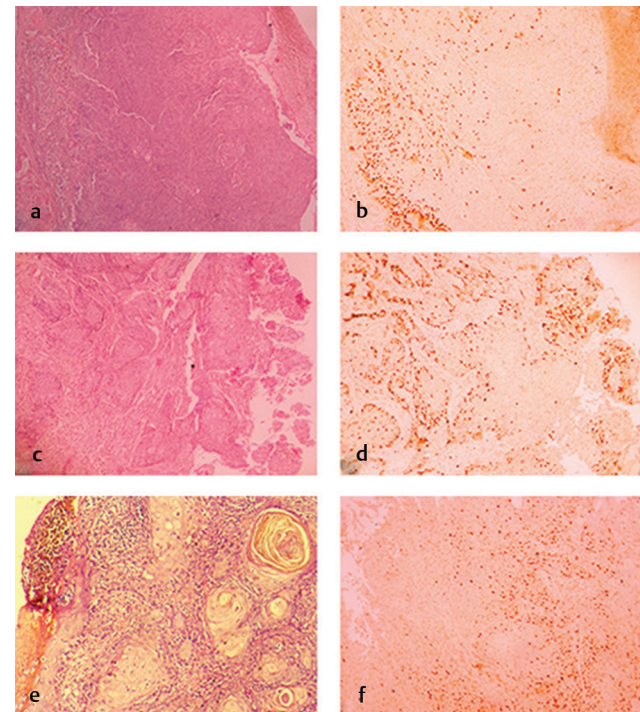
**Fig. 2** Representative histopathological and immunohistochemical (IHC) images of mild dysplasia: (a, b) score 0; (c, d) score 2; (e, f) score 4. (a) Hematoxylin and eosin (H&E), x400. (c, e) H&E, x100. (b) Ki-67 IHC, x400. (d, f) Ki-67 IHC, x100.



**Fig. 4** Representative histopathological and immunohistochemical (IHC) images of severe dysplasia/squamous cell carcinoma in situ: (a, b) score 2; (c, d) score 4; (e, f) score 6 (a, c) Hematoxylin and eosin (H&E), x100. (e) H&E, x400. (b, d, f) Ki-67 IHC, x100.

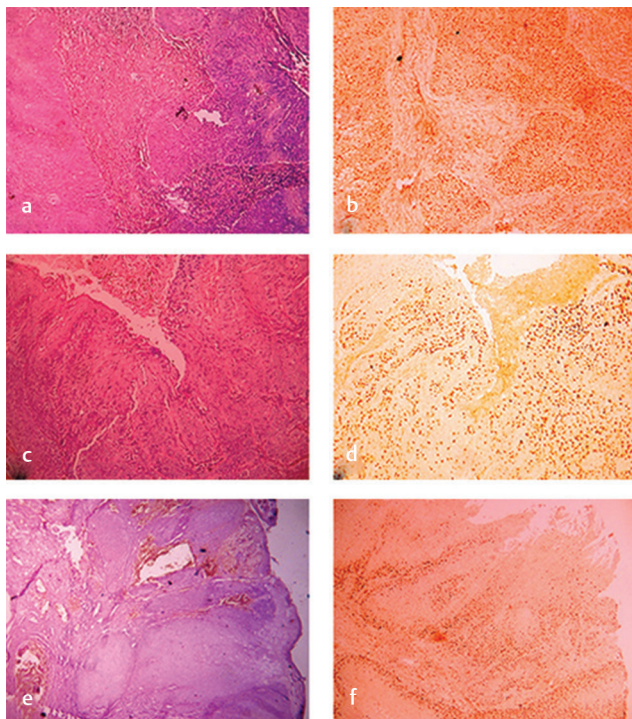


**Fig. 3** Representative histopathological and immunohistochemical (IHC) images of moderate dysplasia: (a, b) score 2; (c, d) score 4; (e, f) score 6. (a) Hematoxylin and eosin (H&E), x400. (c, e) H&E, x100. (b) Ki-67 IHC, x400. (d, f) Ki-67 IHC, x100.



**Fig. 5** Representative histopathological and immunohistochemical (IHC) images of well-differentiated squamous cell carcinoma: (a, b) score 2; (c, d) score 4; (e, f) score 6. (a, c, e) Hematoxylin and eosin (H&E), x100. (b, d, f) Ki-67 IHC, x100.





**Fig. 6** Representative histopathological and immunohistochemical (IHC) images of (a, b) moderately differentiated squamous cell carcinoma, (c, d) poorly differentiated squamous cell carcinoma, and (e, f) verrucous carcinoma. (a, c) Hematoxylin and eosin (H&E), x100. (e) H&E, x40. (b, d) Ki-67 IHC, x100. (f) Ki-67 IHC, x40.

The seven cases of WDSCCs expressed LI scores of 2 to 6 in near-equivalent distributions (►Fig. 5). Alongside, all cases of MDSCCs and PDSCCs were labeled as score 6. Both lesions of VC conveyed a mere score 2 on Ki-67 labeling (►Fig. 6). This difference among carcinomatous lesions did not generate any statistical significance ( $p = 0.0591$ ). Although on ultimate comparison, a statistically significant association of escalating Ki-67 LI scores was found between normal oral mucosae, through various grades of OED, up to multitudes of oral epithelial malignancies ( $p < 0.0001$ ) (►Fig. 1).

## Discussion

The normal biological proliferation within stratified squamous epithelium is maintained by the stem cells at the basal layer and their direct progenies at the parabasal layer. Its regulatory proteins are delivered through the underlying connective tissue, whereas its synchronized differentiation toward surface is nourished by the factors produced from keratinocytes. In PMDs, abnormal cell proliferation commonly prevails. The synchronicity in mucosal maturation is lost. Even the suprabasal squamous cells are then capable of proliferating independently from their regulatory molecules.<sup>4</sup> The causative genetic derangements lead to the expression of certain molecular markers that apparently correspond with different grades of OED. These markers are classified into: (1) genomic markers, for example, p53;

(2) proliferation markers, for example, Ki-67; and (3) differentiation markers, for example, CK13 and CK17.<sup>11</sup>

p53 and Ki-67 are the most widely implemented molecular antigens to discriminate OED from normal oral mucosa. The p53 is a tumor suppressor gene, and its resultant protein is normally expressed at the G1 phase. On its mutation, the protein resumes a longer half-life. Therefore, abnormal suprabasal expression of p53 is an important indicator of oral carcinogenesis.<sup>6,8,11</sup> However, sometimes, a defective catabolism or binding with other proteins can also prompt its intranuclear precipitation. Several researchers derived low positive predictive value of independent p53 expression in this context. On the contrary, Ki-67 is bereft of such limitations, which makes it an individualistic proliferation marker. It tallies with the existence as well as the virulence of dysplasia. Often, it denotes the aggressiveness of an already invasive SCC and also the proliferative activity of neoplastic cells at the edge of infiltrating fronds. Such a phenomenal upregulation of Ki-67 in dysplastic and carcinomatous epithelium makes it an unparalleled prognostic marker.<sup>9,12,13</sup> Otherwise, only CK13, with its loss of expression beyond severe dysplasia, is comparable to Ki-67 for independent prognostic significance.<sup>14</sup> Other anticipated antigens such as p16INK4a, CK17, bcl-2, proliferating cell nuclear antigen, cyclooxygenase-2, and cyclin D1 have mostly been considered as an adjunct to Ki-67 IHC.<sup>7,11-14</sup>

In all the 20 normal mucosal samples from this study, the Ki-67-positive cells were sprinkled within its basal and parabasal layers only. With the progression of dysplasia till SCC, these cells gradually grew more populous at the suprabasal epithelium. Such trend of Ki-67 reactivity in OED or oral SCC has been discussed previously by many researchers.<sup>7,12-14</sup> But unlike theirs, in this study, the OL patches that did not exhibit any dysplasia were excluded. Instead, we solely concentrated upon the dysplastic and malignant patches. Therein, a statistically significant difference of proliferative activity was procured between normal mucosa, OED, and SCC (►Fig. 1).

Earlier, many researchers did fail to find any significant difference in Ki-67 labeling between normal and mildly dysplastic oral lesions. Often, these both were incorporated together within the same low-risk category for statistical comparison. It might have been potential pitfalls of their limited sample size.<sup>3,4</sup> In the discussed study, the Ki-67 labeling pattern of mild dysplasia significantly differed from normal epithelium. Compared with all the normal tissues being scored as 0, 26 out of the 64 mild dysplasia lesions were labeled as score 2, and 21 lesions were labeled as score 4 (►Fig. 1). In this situation, the 17 samples with score 0 could possibly be representatives of reactive atypical epithelium.<sup>14</sup> Raju et al<sup>12</sup> in 2005 and, more recently, Sinanoglu et al<sup>7</sup> also computed a similar kind of statistical comparison as of ours.

Multiple antecedent studies suggested that the abnormal Ki-67 LI corresponds best with moderate dysplasia and the lesions beyond. A significant labeling difference between low-grade and high-grade dysplasia is almost always there. Though sometimes, the researchers could not segregate

moderate and severe dysplasia on the basis of Ki-67 labeling. They preferentially clubbed these two lesions for comparative ease.<sup>3,4,14,15</sup> Exceptional reports suggesting an indifferent proliferative activity at different stages of OED are also documented.<sup>5,12</sup> Ki-67 labeling according to the WHO-defined trimodal grades of OED<sup>10</sup> has only been reminisced by a handful number of studies ahead of our present deliberation. Similarly, Kumar et al examined 19, 7, and 3 samples of mild, moderate, and severe dysplasia, respectively. There was a significantly high proliferative activity in severe > moderate > mild dysplasia.<sup>6</sup> Sinanoglu et al recognized the three phases of dysplasia as oral intraepithelial neoplasia (OIN) subgroups 1 to 3. The Ki-67 expression within their OIN1 and OIN2 cases was nearly the same. Neither did the overall exaggerating LI from OIN1→OIN2→OIN3 gather any statistical significance. Still, there was a significantly high Ki-67 expression in OIN3 compared with OIN2.<sup>7</sup> Identically, the Ki-67 LI in this study steadily aggravated in a statistically significant way from mild through moderate up to severe dysplasia. The isolated variation of LI between moderate and severe dysplasia was also significant (►Fig. 1). Because of such heterogeneous observations in this particular context, additional research attributes are still required to reach a definite opinion.

Normally, the Ki-67 responsiveness enhances through the progressive grades of oral SCC. In WDSCC, the proliferative activity is localized at the periphery of the tumoral fronds. Centrally, the tumor nests mature into frequent squamous pearls and lack Ki-67 expression. Onto MDSCC, the Ki-67-reactive cells sporadically begin to populate more centrally as much at the edge of tumor nests. The relatively undifferentiated neoplastic cells in PDSCC express more diffuse staining pattern.<sup>3,16-18</sup> The presently described report was no different either. Herein, the seven WDSCC cases acquired LI scores of 2 (two cases), 4 (three cases), and 6 (two cases). However, all the lesions of MDSCC and PDSCC were labeled as score 6. Despite this apparently improving Ki-67 expression in respect of diminutive differentiation of oral SCC, the same did not manage any statistical significance. Similar kind of insignificant proliferative activity among oral cancers has already been reiterated by Raju et al.<sup>12</sup> Under similar situation, Birajdar et al<sup>4</sup> and Piffkó et al<sup>19</sup> achieved a strange response. The Ki-67 labeling in their examined MDSCC patches fell short of WDSCC, which then again augmented to a significant high in PDSCC cases. In fact, the histological grades of oral SCC bear a dismal correlation with its prognosis, and this nature of unworthy proliferative discrepancies within oral cancers could just be the reason behind.<sup>10</sup>

VC is a locally invasive low-grade subtype of SCC. It is characterized by an expansile bulbous downgrowth of mature squamous epithelium penetrating into the submucosa. The irregular stromal infiltration seen in classic SCC is absent. Metastasis also never happens with VC.<sup>20</sup> Its closest mimickers are verrucous hyperplasia (VH) and WDSCC. Ki-67 IHC can unanimously discriminate VC from VH. The reactive cells in VC are localized at the basal and the suprabasal regions in the lower half of the epithelium, whereas in VH, these positive cells are strictly confined within the

basal layer just as good as the normal mucosa.<sup>20,21</sup> Rather, conflicting opinions are there regarding the role of Ki-67 IHC in the separation of VC from WDSCC. Previously, Saito et al<sup>22</sup> and Adegboyega et al<sup>23</sup> considered the presence of positive cells throughout the entire epithelial thickness as the principal discriminating point in favor of WDSCC. Contradictorily, Zargaran et al<sup>21</sup> did not find this way-out any helpful. Whatsoever, such dilemma did not surface in this study. None of the examined two VC patches did come along with VH or WDSCC either histologically or immunohistochemically. In WDSCC, the Ki-67-positive cells were scattered along full thickness of the epithelium, and in VC, these cells were restricted within the suprabasal 4 to 6 layers only (►Figs. 5 and 6).

Conclusively, this latest study implements that a significantly escalating Ki-67 immunorexpression along with increasing grades of dysplasia and furthermore into the carcinoma actually makes it a predictive marker for cancerous transformation in OL. Its differential overexpression in various grades of dysplasia beholds the necessity of WHO-recommended tripartite classification for dysplasia. Ki-67 IHC is of particular importance in mild dysplasia and VC, where it clearly secludes the erroneously included reactive atypical epithelium. Reciprocally, an insignificant labeling difference within multimodal oral carcinomas argues the negligible correlation between its histological grades and outcome.

## Conclusion

Therewith Ki-67 is conclusively depicted as an important immunohistochemical marker for dysplastic and malignant transformation in OL, and also its credibility toward discrimination of mildly dysplastic patches and VCs from reactive epithelial atypia has been ratified.

### Funding

None.

### Conflicts of Interest

There are no conflicts of interest.

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