**Original Article**

**An Immunohistochemical Study Showing Ki-67 as an Analytical Marker in Oral Malignant and Premalignant Lesions**

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**Introduction:** Ki-67 is a nuclear protein. It is a proliferation marker that has an essential function in tumorigenesis due to its positive connection with tumor expansion. **Aim:** The aim of this study was to evaluate the articulation of Ki-67 as prognostic marker in various grades of oral epithelial dysplasia (OED) and in oral squamous cell carcinoma (OSCC). **Materials and Methods:** A total of 100 histologically affirmed samples of normal oral mucosa (NOM), OED, and OSCC were divided into three groups—Group I (10 samples of normal oral mucosa), Group II (45 samples of OED), Group III (45 samples of OSCC). Routine hematoxylin and eosin and immunohistochemical staining with Ki-67 monoclonal antibody were carried out in all the samples. **Results:** Within Group I, articulation of Ki-67 was constrained to the basal layers. In Group II, cells showing positive expression of Ki-67 were available in the basal, suprabasal, and spinous layers. Cells showing positive expression of Ki-67 among well-differentiated OSCC were presented mainly in the periphery of the tumor nests; in moderately differentiated OSCC, cells were located in both peripheral and part of a center of the tumor nests; and in most cases of poorly differentiated OSCC, cells were diffused. Statistically significant difference in positive expression of Ki-67 was appreciated between three groups. **Conclusion:** Ki-67 antigen may perhaps be used as a marker for the histological reviewing of OED and OSCC. With the increase in the severity of OED, cells showing positive expression of Ki-67 also increased.

**Keywords:** Ki-67 antigen, oral epithelial dysplasia, oral squamous cell carcinoma, tumor markers

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of tumor cell is considered as significant natural factor for tumor detection. Ki-67 has appeared to serve a significant part in tumorigenesis because of its positive relationship with tumor multiplication and invasion.\(^2\)\(^4\)

Ki-67 is a monoclonal marker used for proliferation. It is exclusively related to proliferation of cells and aggressiveness of malignant tumors.\(^5\) During cell cycle, the presence of Ki-67 is seen during prophase and metaphase but is absent during resting cells.\(^6\) The aforementioned features make Ki-67 as one of the best markers to calculate cell proliferation, and it can be used as a reagent that helps in determining a patient prognosis for several types of tumor.\(^7\)

This research intended to evaluate the positive appearance of Ki-67 as prognostic marker in diverse grades of OSCC and OED.

**MATERIALS AND METHODS**

A total of 100 paraffin-embedded blocks that were formalin fixed were included in the study sample as follows [Figure 1]:

- **Group I** (15 samples of normal oral mucosa [NOM])
- **Group II** (45 samples of OED)
- **Group III** (45 samples of OSCC)

All the blocks were collected from archival files of department of oral pathology and microbiology of Kalinga Institute of Dental Sciences, Kalinga Institute of Industrial Technology, Deemed to be University, Bhubaneswar, Odisha, India. OED was further subdivided into 15 mild OED, 15 moderate OED, and 15 severe OED. OSCC was subdivided into 15 well-differentiated OSCC, 15 moderately differentiated OSCC, and 15 poorly differentiated OSCC. Paraffin sections of formalin-fixed tissues were used for both histological and immunohistochemical evaluation.

Hematoxylin and eosin stained sections of 4 μ were used for routine histological examination.

**Immunohistochemistry**

IHC recognition of Ki-67 was done with Dako antibody detection system. For IHC staining, the segments were sliced at approximately 3-μ thickness using semiautomatic microtome. In case of IHC, segments were positioned on lysine-coated slides. For antigen recovery, the segments were dipped in a 1 mM citrate buffer (pH 6), and microwave was used with patterns of high, medium high, low, and very low, each going for 5 min and afterward cooled to room temperature.

The endogenous peroxidase action was blocked with 3% hydrogen peroxide for 10 min, after that washing in 0.05 mM Tris-buffered saline (TBS) at pH 7.4 was done. The segments were incubated with ready to use primary mouse monoclonal antibodies against Ki-67 1 h at 37°C. Subsequent to washing in TBS, the segments were incubated by means of a secondary antibody conjugated amid peroxidase-labeled dextran polymers for half hour at room temperature. Subsequent to washing with TBS, they were treated with 0.5 mg/mL 3, 3′-diaminobenzidine solution containing 0.001% hydrogen peroxide to envisage reaction products, and for 3 min further stained with Mayer’s hematoxylin.

**Statistical Analysis**

**Labelling Index**

Labeling index was calculated in cases of OSCC as the number of positive cells per 100 cells. In cases of OED, nuclear expression of Ki-67 was confirmed by basal, parabasal, and suprabasal layers [Figure 2].

Figure 2: Mean labeling index for Ki-67 protein in normal oral mucosa, oral epithelial dysplasia, and oral squamous cell carcinoma
**Results**

Clinicopathologically, age range of 100 participants was between 20 and 75 years (average 40 years), the sex ratio was 1:1.

In Group I, positive Ki-67 cells were constrained in basal layer only. In Group II, positive Ki-67 cells were present in the basal, suprabasal, and spinous layers. Positive Ki-67 cells in well-differentiated OSCC were presented chiefly in the periphery of the tumor nests; in moderately differentiated OSCC, Ki-67 positive cells were located in both peripheral and part of a middle of the tumor nests; and in most cases of poorly differentiated OSCC, positive Ki-67 cells were diffused. The Ki-67 positive cells in various groups were scrutinized for statistical significance with one-way analysis of variance (ANOVA) test. A P value of <0.05 indicated significant difference.

**Discussion**

Oral mucosa comprises stratified squamous epithelium. Presence of stratifications in the epithelium is due to differentiation and proliferation of cells.[8] Differentiation begins when recently divided cells segregate from underlying extracellular matrix.[9] With time when differentiating cells mature, they move toward the epithelial surface due to force generated in the underlying proliferation compartment.[8] Cell proliferation, imperative natural procedure, is a noteworthy addition to histological tumor classification and has prospective importance such as a marker of treatment success and setback. Numerous investigations have revealed that abnormal cell increase seems to be a precursor and can act as an indicator for tumorigenesis.[10] Different IHC markers are used to identify cell proliferation, of which Ki-67 is used as an increasingly dependable marker of proliferation in our investigation.

In cases of NOM, the mean labeling index of Ki-67 protein was 12.56; for OED, it was 28.4; and for OSCC, it was 40.18 [Table 1]. There was a statistical noteworthy distinction in between three groups. Further labeling index of mild OED was found to be 15.42; for moderate OED, it was 29.77; for severe OED, it was 58.5; for well-differentiated OSCC, it was 35.23; for moderately differentiated OSCC, it was 48.22; and for poorly differentiated OSCC, it was 59.13 [Table 2]. No statistical noteworthy distinction was present amid NOE and mild OED, this shows that the pace of carcinomatous change is more in entities that display moderate to severe dysplasia.[11] There was a statistical noteworthy distinction in the expression of Ki-67 among well-differentiated OSCC, moderately differentiated OSCC, poorly differentiated OSCC, and NOM, this shows that the appearance of Ki-67 amplified progressively with the increase of differentiation of OSCC. Macluskey et al.[12] showed statistical noteworthy relation with mean Ki-67 in healthy tissue, dysplasia, and in carcinomatous tissue, thus suggestive of increase in epithelial propagation throughout the changeover from dysplasia to cancer [Table 3].

In Group I, expression of Ki-67 was limited to basal layers. Comparable results were reported by Takeda et al.,[13] Dwivedi et al.,[14] and Birajdar et al.[15]

### Table 1: Mean labeling index for Ki-67 in normal oral mucosa, oral epithelial dysplasia, and oral squamous cell carcinoma

| Group | Ki-67 labeling index (mean ± SD) | P value |
|-------|----------------------------------|---------|
| Group I | 12.56 ± 6.9 | 0.00 |
| Group II | 28.24 ± 13.2 | |
| Group III | 40.18 ± 15.9 | |

### Table 2: Mean labeling index for Ki-67 protein in normal oral mucosa; mild, moderate, and severe oral epithelial dysplasia; and well, moderate, and poor oral squamous cell carcinoma

| Group | Grade | Ki-67 (mean ± SD) | P value |
|-------|-------|------------------|---------|
| Group I | Normal | 12.56 ± 6.9 | 0.00 |
| Group II | Mild | 15.42 ± 10.13 | 0.01 |
| | Moderate | 29.77 ± 14.02 | |
| | Severe | 58.5 ± 11.00 | |
| Group III | Well | 35.23 ± 14.27 | 0.013 |
| | Moderate | 48.22 ± 14.00 | |
| | Poor | 59.13 ± 16.11 | |

### Table 3: Comparison of labeling index for Ki-67 protein in different subgroups

| Sample 1 | Sample 2 (control) | P value | Significance |
|----------|---------------------|---------|--------------|
| Mild dysplasia | Normal oral mucosa | 0.71 | Nonsignificant |
| Moderate dysplasia | Normal oral mucosa | 0.01 | Significant |
| Severe dysplasia | Normal oral mucosa | 0.013 | Significant |
| Well OSCC | Normal oral mucosa | 0.00 | Significant |
| Moderate OSCC | Normal oral mucosa | 0.00 | Significant |
| Poor OSCC | Normal oral mucosa | 0.00 | Significant |
In Group II, Ki-67 positive cells were present in the basal, suprabasal, and spinous layers. There was an increase in the expression of Ki-67 with the severity of dysplasia. Comparable results have been reported by Gonzalez-Moles et al.\[16\] and Kumar et al.\[17\]

In the mild OED, the maximum expression of Ki-67 was observed in the basal and parabasal layers of the epithelium. A statistical noteworthy distinction was observed between the moderate OED and the NOM, and in the severe OED, the nuclear Ki-67 positivity was noted in the basal, parabasal, and most of the spinous layers of the epithelium; it can be concluded that the speed of malignant transformation is higher in lesions that have moderate to severe dysplasia. In future, the Ki-67 protein may serve as prognostic tools in finding malignant changes in OED.

This increased proliferation in parabasal layers of premalignant oral epithelium is likely related with the loss of heterozygosity in 3p, 9p, and 17p, which carries on as a marker of precancerous fields and builds the plausibility of developing multiple tumors.\[18\]

The pace of malignant transformation may rely on the level of dysplasia, and lesions that have high grade of dysplasia may be 4.5-times bound to experience malignant transformation when contrasted with mildly dysplastic lesions.\[19\]

Ki-67-positive cells in well-differentiated OSCC were displayed fundamentally in the periphery of the tumor nests; in moderately differentiated OSCC, Ki-67-positive cells were situated in both peripheral and part of middle of the tumor nests; and most of the time in poorly differentiated OSCC, Ki-67 positive cells were diffused. We saw that the cell expansion in OSCC expanded with expanding histological evaluations of OSCC. Comparable outcomes were accounted for by Takkem et al.\[11\] and Tumuluri et al.\[20\]

We saw that the cell proliferation in OSCC expanded with increasing histological grades of OSCC. Comparable outcomes were accounted by Takkem et al.\[11\] and Tumuluri et al.\[20\]

The high expression of the Ki-67 protein in OSCC tissues may play an important role in the development of OSCC.\[21\]

As indicated by our examination and past investigations performed by Raju et al.,\[22\] Dragomir et al.,\[23\] and Ahmad et al.,\[24\] Ki-67 ends up being helpful in surveying tumor aggressiveness. Increased cellular proliferation is associated with more advanced lesions, and the distribution of proliferating cells in tissues may disclose about the regulatory mechanisms that become dysfunctional through carcinogenesis.\[25\]

Owing to the expression of Ki-67 protein in all proliferating cells and the prognostic significance of the Ki-67 marker in many cancers, Ki-67 protein is a potential beneficial target in cancer, and approaches that inactivate Ki-67 protein region, promising anti-proliferative approach, can have potential applicability in cancer treatment.\[26\]

**CONCLUSION**

Ki-67 antigen may possibly be used as a marker for the histological reviewing of OED and OSCC. Expression of Ki-67 expands with the severity of OED.

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**Conflicts of interest**
There are no conflicts of interest.

**REFERENCES**

1. Jing Y, Zhou Q, Zhu H, Zhang Y, Song Y, Zhang X, et al. Ki-67 is an independent prognostic marker for the recurrence and relapse of oral squamous cell carcinoma. Oncol Lett 2019;17:974-80.
2. Yadav P, Malik R, Balani S, Nigam RK, Jain P, Tandon P. Expression of p-16, Ki-67 and p-53 markers in dysplastic and malignant lesions of the oral cavity and oropharynx. J Oral Maxillofac Pathol 2019;23:224-30.
3. Muller S. Oral epithelial dysplasia, atypical verrucous lesions and oral potentially malignant disorders: focus on histopathology. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:591-602.
4. Antonarakis ES, Keizman D, Zhang Z, Gurel B, Lotan TL, Hicks JL, et al. An immunohistochemical signature comprising PTEN, MYC, and Ki-67 predicts progression in prostate cancer patients receiving adjuvant docetaxel after prostatectomy. Cancer 2012;118:6063-71.
5. Dadfarnia T, Mohammed BS, Eltorky MA. Significance of Ki-67 and p53 immunoexpression in the differential diagnosis of oral necrotizing sialometaplasia and squamous cell carcinoma. Ann Diagn Pathol 2012;16:171-6.
6. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311-22.
7. Whitfield ML, George LK, Grant GD, Perou CM. Common markers of proliferation. Nat Rev Cancer 2006;6:99-106.
8. Jones PH. Epithelial stem cells. Bioessays 1997;19:683-90.
9. Blumenberg M, Tomic-Canic M, Jiang CK, Yang DR, Magnaldo T, Bernerd F, et al. Regulation of keratin gene expression by hormones, vitamins and growth factors. Pharmacol Skin 1993;5:75-82.

10. Bacci CE, Gown AM. Detection of cell proliferation in tissue sections. Braz J Med Biol Res 1993;26:677-87.

11. Takkem A, Barakat C, Zakaraia S, Zaid K, Najmeh J, Ayoub M, et al. Ki-67 prognostic value in different histological grades of oral epithelial dysplasia and oral squamous cell carcinoma. Asian Pac J Cancer Prev 2018;19:3279-86.

12. Macluskey M, Ogden GR, Green M, Chisholm DM, Schor SL, Schor AM. The association between epithelial proliferation and disease progression in the oral mucosa. Oral Oncol 1999;35:409-14.

13. Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JJ, Semb J. Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. J Oral Pathol Med 2006;35:369-75.

14. Dwivedi N, Chandra S, Kashyap B, Raj V, Agarwal A. Suprabasal expression of Ki-67 as a marker for the severity of oral epithelial dysplasia and oral squamous cell carcinoma. Contemp Clin Dent 2013;4:7-12.

15. Birajdar SS, Radhika M, Paremala K, Sudhakara M, Soumya M, Gadiyan M. Expression of Ki-67 in normal oral epithelium, leukoplakic oral epithelium and oral squamous cell carcinoma. J Oral Maxillofac Pathol 2014;18:169-76.

16. Gonzalez-Moles MA, Ruiz-Avila I, Rodriguez-Archilla A, Martinez-Lara I. Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. Head Neck 2000;22:658-61.

17. Kumar KV, Chaithanya K, Punde P, Thorat A, Jangam AG, Deepthi S. Comparative evaluation of immunohistochemical expression of Ki-67 in oral lichen planus, oral leukoplakia and normal mucosa cases. J Int Oral Health 2015;7:82-7.

18. Tabor MP, Braakhuis BJ, van der Wal JE, van Diest PJ, Leemans CR, Brakenhoff RH, et al. Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oro pharynx. J Pathol 2003;199:354-60.

19. Liu W, Wang Y-F, Zhou H-W, Shi P, Zhou ZT, Tang GY. Malignant transformation of oral leukoplakia: a retrospective cohort study of 218 Chinese patients. BMC Cancer 2010;10:685.

20. Tumuluri V, Thomas G, Fraser I. Analysis of the Ki-67 antigen at the invasive tumour front of human oral squamous cell carcinoma. J Oral Pathol Med 2002;31:598-604.

21. He W, Xiao Y, Chen W. Expression of Ki-67 and P53 protein in oral squamous cell carcinoma and its clinical significance. Shanghai J Stomatol 2015;24:228-31.

22. Raju B, Mehrotra R, Ojordsbakken G, Al-Sharabi AK, Vassstrand EN, Ibrahim SO. Expression of p53, cyclin D1 and Ki-67 in pre-malignant and malignant oral lesions: association with clinicopathological parameters. Anticancer Res 2005;25:4699-706.

23. Dragomir LP, Simionescu C, Mărgăritescu C, Stepan A, Dragomir IM, Popescu MR. P53, p16 and Ki67 immunoeexpression in oral squamous carcinomas. Rom J Morphol Embryol 2012;53:89-93.

24. Ahmad B, Asif M, Ali A, Jamal S, Zaib Khan MZ, Khadim MT. Expression of Ki-67 and β-catenin in pseudoepitheliomatous hyperplasia and squamous cell carcinoma in oral mucosal biopsies: an immunohistochemical study. Asian Pac J Cancer Prev 2019;20:157-61.

25. Endl E, Gerdes J. The Ki-67 protein: fascinating forms and an unknown function. Exp Cell Res 2000;257:231-7.

26. Kausch I, Lingnau A, Endl E, Sellmann K, Deinert I, Ratliff TL, et al. Antisense treatment against Ki-67 mRNA inhibits proliferation and tumor growth in vitro and in vivo. Int J Cancer 2003;105:710-6.