

Cellular Proliferation in Oral Mucosal Atypia

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Abstract— The study was an attempt to study the cellular proliferation, by a means of Ki-67 labeling index, in oral mucosal intraepithelial lesions and oral squamous cell carcinoma to evaluate its potential association with their histologic grades. The cellular proliferation was also evaluated on sections taken from hyperplastic and normal mucosal epithelium in order to determine whether it can be used as a diagnostic aid in evaluating oral mucosal atypia. Fifty five patients with oral squamous cell carcinoma (n=19), intraepithelial lesions (n=16), hyperplastic epithelium (n=13) and normal mucosal epithelium (n=7) were enrolled in the study. The Ki-67 labeling index was determined by immunohistochemistry on paraffin sections using avidin–biotin technique and antigen retrieval was done by a pressure pot. Overall, high Ki-67 labeling index was observed in 52.6% of squamous cell carcinoma cases, 62.5% of intraepithelial lesions, 23.1% of hyperplastic epithelium, and nearly none in benign epithelium (apart from the basal layer). The high labeling index was significantly high in both carcinoma and intraepithelial lesions compared with that of hyperplastic epithelium. In addition, the high index was associated with graded both carcinoma and intraepithelial lesions. This study confirms and extends previous findings that the Ki-67 labeling index can be used as an indicator to predict oral mucosal atypia as a pre-malignant or malignant lesion. Its immunohistochemistry emerges as a clinically useful supplement for histopathological assessment of severity of oral squamous cell carcinoma and intraepithelial lesions.

Index Terms— Cellular proliferation, Ki-67 labeling index, oral epithelial atypia, oral squamous cell carcinoma.

1 INTRODUCTION

ORAL squamous cell carcinoma (SCC) is relatively uncommon but especially aggressive cancer. It is associated with a high rate of local recurrence and poor survival.¹ Like other cancers, oral SCC represents an accumulation of defects in genes that encode the key proteins associated with proliferation and growth. This appears through a series of precancerous stages, manifested morphologically as epithelial dysplasia or intraepithelial lesions (IEL) in the sequence of dysplasia-carcinoma.²⁻⁵ The increased proliferative activity observed in oral SCC and IEL, compared with non-neoplastic hyperplasia, illustrates a continuum between benign and malignant squamous epithelia.⁶⁻¹¹ The human Ki-67 protein, a nuclear antigen strictly associated with cellular proliferation is expressed in all phases of the cell cycle excepting G0.^{2,12}

In oral mucosal lesions, the expression of Ki-67 has been reported to increase according to the proliferative activity and degree of epithelial dysplasia, suggesting that it is a good marker of cellular proliferation in premalignant and malignant lesions and is informed as a marker of the presence and severity of oral IEL.^{2,9,11,12} The Ki-67 labeling index (LI), i.e. the percentage of tissue cells stained for Ki-67, is a widely used marker of cell proliferation and can be used as an indicator to predict pre-malignant or malignant lesions.^{9,10,11,13}

The present study is an attempt to elucidate the overexpression of cell proliferation protein (Ki-67) in a series of graded oral IEL and untreated oral SCC at invasive front, to evalu-

ate the potential association between Ki-67 LI and the histologic grade of both IEL and SCC. The overexpression of this marker in hyperplastic epithelium and normal mucosal epithelium from the same patients was also evaluated in order to determine whether it can be used as a diagnostic aid in evaluating oral mucosal atypia.

2. METHODS

2.1 Histopathology

The study was conducted in Duhok Central Laboratory, Duhok, Iraq. A total of 55 archival biopsy specimens were taken from histopathologically confirmed oral squamous cell carcinoma (n=19), intraepithelial lesions (n=16), hyperplastic epithelium (n=13) and normal mucosal epithelium (n=7). The blocks were retrieved from histopathology laboratories in Erbil and Duhok cities/Iraq between January and December 2010. For SCC, blocks showing invasive front were selected. Two serial (4- μ m thick) sections were cut from each case, one for hematoxylin and eosin while the other was subjected to immunostaining. All histology slides were reviewed again to confirm the diagnoses. Squamous cell carcinoma cases were graded as well differentiated (n=7), moderately differentiated (n=5) and poorly differentiated (n=7). No undifferentiated carcinoma case was present. Intraepithelial lesions were graded, according to the WHO classification, as low grade intraepithelial lesions (LGIL) (n=6) and high grade intraepithelial lesions (HGIL) (n=10).^{14, 15}

2.2 Immunohistochemistry

Unstained tissue sections were mounted on coated slides with polylysine and incubated overnight at 37°C then heated at 60°C before staining. Sections were deparaffinized in xylene, rehydrated in graded alcohol, and then rinsed with phosphate buffered saline (PBS, Dako Denmark A/S). Endog-

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TABLE 1
 PATTERN OF KI-67 LABELING INDEX ACCORDING TO THE HISTOLOGY

Histology (n)	High Ki-67 (%)	Low Ki-67 (%)
Normal epithelium (7)	0/7 (00)	7/7 (100)
Hyperplastic epithelium (13)	3/13 (23.1)	10/13 (76.9)
Intraepithelial lesion (Total= 16)*	10/16 (62.5)	6 (37.5)
LGIL (6)	2/6 (33.3)	4/6 (67.7)
HGIL (10) *	8/10 (80) *	2/10 (20)
Squamous cell carcinoma (Total= 19)*	10/19 (52.6) *	9/19 (47.4)
Well differentiated SCC (7)	1/7 (14.3)	6/7 (85.7)
Moderately differentiated SCC(5)	3/5 (60)	2/5 (40)
Poorly differentiated SCC (7)	6/7 (85.7) *	1/7 (14.3)

*P-value ≤ 0.05

enous peroxidase was quenched by 3% hydrogen peroxide in methanol. Epitopes were retrieved by heating sections in a pressure pot in sodium citrate buffer (pH 6.0) up to 3 minutes after boiling started. Slides were washed with PBS and incubated overnight at 4°C with primary antibodies for Ki-67 using a mouse, anti-human, monoclonal antibody (clone MIB-1 ready to use, Dako, Denmark A/S). Slides were incubated for 10 minutes with the biotinylated goat antipolyvalent antibody (Dako, Denmark A/S) at room temperature, and an Envision Dual link system-HRP (ready to use, Dako, Denmark A/S) was used as a secondary antibody. Incubation with 3,3-diaminobenzidine tetra hydrochloride was performed for 5 minutes at room temperature as a substrate chromogen solution to produce brown colored stained nuclei. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. Appropriate positive control section from a lymph node with Burkitt's lymphoma was processed in parallel. Negative control in which the primary specific antibody was substituted by a buffer solution was used.

A semiquantitative study was carried out and LI was calculated using the following formula: LI= number of positive cells/total number of cells × 100 as described by Dudderidge et al.¹⁶ We adopted a 2-scale Ki-67 LI: low (<50%) and high (≥50) considering one cut-off point depending on the median value (50%).

2.3 Statistical Analyses

All statistical tests were performed using SPSS version 16 for windows (SPSS Inc, Chicago, IL, USA). The chi-squared test was used to compare the categorical variables. In all cases, p-value ≤ 0.05 was considered as significant.

3. RESULTS

The mean age of patients was 59.8 years (range: 35-78) with a male: female ratio of 1.7:1 (22 males, 13 females). Patterns of Ki-67 immunostaining, according to histology, are summarized in Table 1. Overall, the mean proliferative index, determined by Ki-67 LI, was low (<50%) if not absent in all cases of normal epithelium. This index was also low in 76.9% of hyperplastic epithelium (Figure 1) and 67.7% of LGIL cases. In contrast, Ki-67 LI was high (≥50%) in 80% of HGIL (Figure 2). A significantly higher Ki-67 LI was observed in HGIL compared with benign hyperplastic epithelial proliferation (p< 0.05). The index was also significantly associated with the grade of intraepithelial lesions (p< 0.05), highest among HGIL. Ki-67 LI was high in 52.6% of SCC (Figure 3-5). It was significantly higher than that of hyperplastic epithelium (p< 0.05). Most well differentiated SCC revealed a low labeling index while the high index was observed mainly among high-grade carcinomas; the difference was statistically significant (p< 0.05). The staining pattern also differed; it was stronger and more diffuse among high grades IEL and SCC than their counterpart low grade lesions. On the other hand, no significant difference was noted between SCC and intraepithelial lesions staining patterns for Ki-67 (p> 0.05).

LGIL: Low grade intraepithelial lesion, HGIL: High grade intraepithelial lesion, SCC: Squamous cell carcinoma.

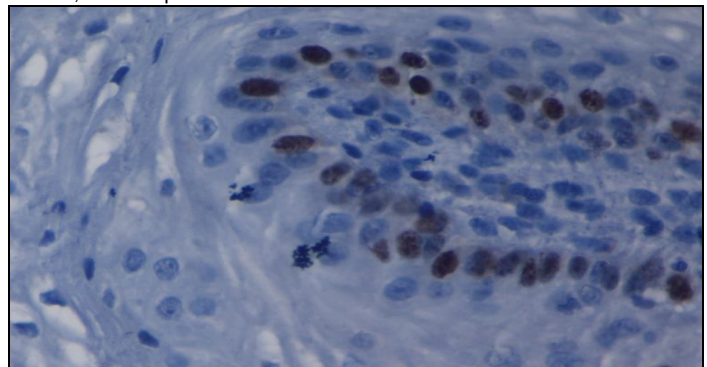


Fig 1. Ki-67 expression in hyperplastic oral epithelial proliferation (x 400).

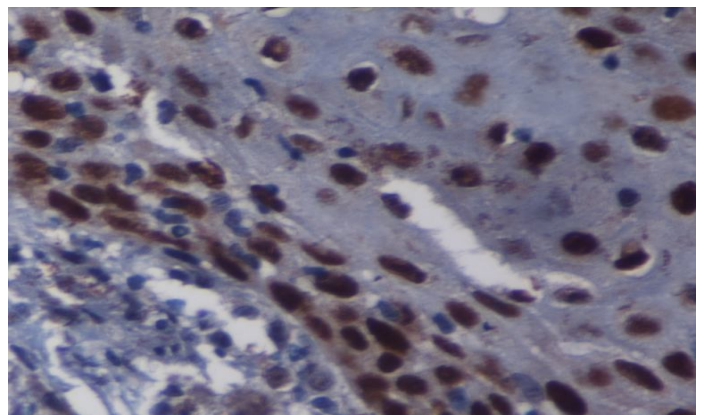


Figure 2. Ki-67 expression in high grade oral intraepithelial lesion (x 400).

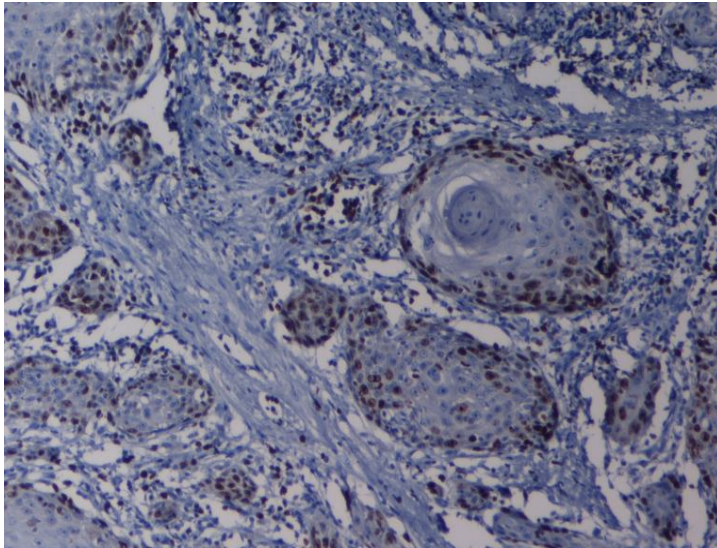


Fig 3. Ki-67 expression in the infiltrative margin of well differentiated oral squamous cell carcinoma (x 400).

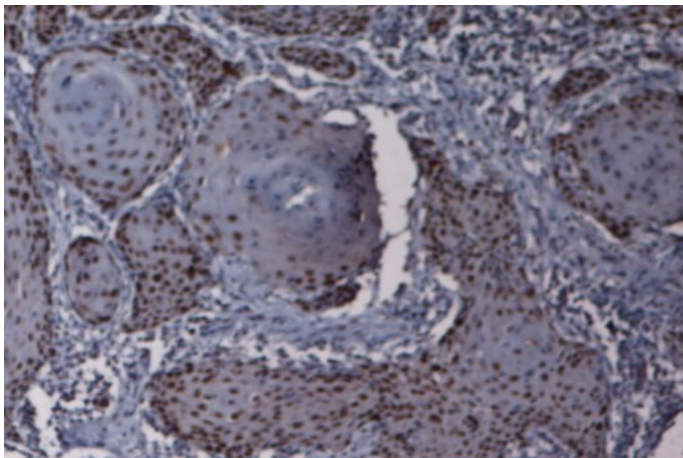


Fig 4. Ki-67 expression in the infiltrative margin of moderately differentiated oral squamous cell carcinoma (x 100).

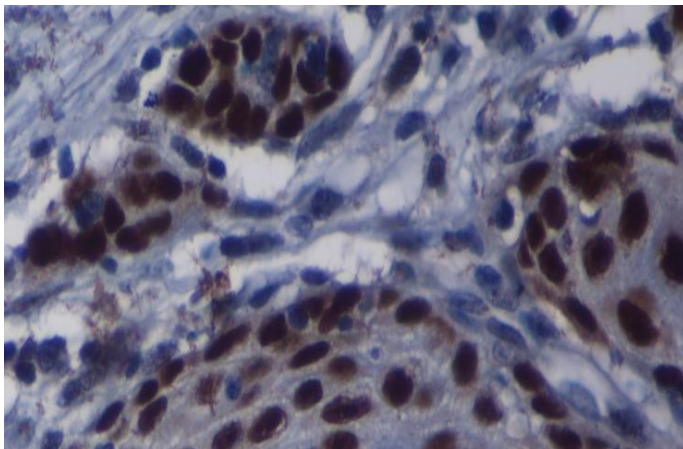


Fig 5. Ki-67 expression in the infiltrative margin of poorly differentiated oral squamous cell carcinoma (x 400).

67 LI was high in 62.5% of IEL and 52.6% of SCC. The marker was significantly high in both IEL and SCC when compared with hyperplastic epithelium suggesting that hyperproliferation is thought to be an early event in oral carcinogenesis,^{5,9,10,11,12,17,18} In accordance with our results, a similar panel was composed in many studies to separate SCC and IEL from hyperplastic epithelium of the oral cavity and larynx; they noticed that Ki-67 overexpression can be considered as reliable indicator for SCC development.^{6,9,10,17-21} The invasive front of SCC was selected for protein evaluation, because this is considered to be more relevant for the understanding of the cell-cycle kinetics of cells involved in invasion into the adjacent tissues.⁸ In our series, we also observed an increased Ki-67 overexpression, as a function of grade of histopathologic abnormalities in oral IEL and SCC. The highest levels of Ki-67 LI belonged to high grades IEL (80%) and poorly differentiated SCC (85.7%). The staining pattern was also different. In low grade IEL, the distribution of Ki-67 stained nuclei was patchy, just in contrast to high grade lesions in which the staining distribution was compact and diffuse. Likewise, in well differentiated SCC the number and distribution of the stained nuclei were obviously less marked than in poorly differentiated SCC. The increased cellular proliferation associated with advancing lesions and the distribution of proliferating cells in tissue may tell more about the regulatory mechanism that become dysfunctional during the multistep process of carcinogenesis. This finding was also observed by other authors who found that the more differentiated the epithelium is the smaller positivity they found in contrast to poorly differentiated epithelia in which nearly all strata were positive for these markers. They also noted that this expression increased in a marked way, as there was advancing in the progression of oral IEL, with significant differences in the cellular proliferation between hyperplastic epithelium and graded IEL. Larger differences had been noticed in *in situ* and microinvasive carcinomas, suggesting that Ki-67 LI is an excellent marker for the presence and severity of IEL and SCC.^{7,8,9,11,13,20,22}

Although these results need to be confirmed by other similar studies, Ki-67 LI showed no significant difference between the IEL and SCC groups. These findings suggest that cells in IEL as those in SCC have more or less a similar increased number of cells licensed to proliferate and that cellular proliferation begins early in oral carcinogenesis.

The low Ki-67 expression in non-neoplastic oral epithelial proliferation (23% of cells) in this study, suggests that these tissue compartments have a low and controlled proliferation rate. Low Ki-67 LI in non-neoplastic tissues have also been observed by other studies done in oral mucosa,¹⁰ larynx,²⁰ and prostate tissues.²³ In our series, there was nearly none or very low Ki-67 in normal epithelium. Absence of Ki-67 in some cases of normal epithelium suggests, according to some authors, that the proliferating cells could be stem cells that may pass through a prolonged cell cycle. I.e. these cells are in a temporary G0 state.⁴

4. DISCUSSION

In the present study, we evaluated cellular proliferation, calculated by Ki-67 LI in oral mucosal atypia from 35 patients with intraepithelial lesions and squamous cell carcinoma. Ki-

5. CONCLUSIONS

This study confirmed and extended the previous findings that the Ki-67 labeling index can be used as an indicator to predict oral mucosal atypia as a pre-malignant or malignant

lesion. Its immunohistochemistry emerges as a clinically useful supplement for histopathological assessment of severity of oral squamous cell carcinoma and intraepithelial lesions.

6. ACKNOWLEDGEMENTS

This work was supported by Duhok University, Faculty of Medicine and Directorate of health. The authors would like to thank all staff of histopathology department, Duhok Central Laboratory.

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