ACTUAL PROLIFERATING INDEX AS AN INDICATOR OF PROGNOSIS IN ORAL MALIGNANCY AND PREMALIGNANCY

Tekade S,* Wanjari S,** Gadbail A.R.***

The purpose of this study was to use actual proliferation index toexamine the possible association between epithelial proliferation and disease progression in the oral mucosa. Ki-67 labeling index (LI) and argyrophilic nucleolar organizer regions (AgNORs) count per nucleus were used to draw actual proliferation index (API). The immunohistochemistry (IHC) was carried out for ki-67 by using MIB-1 clone in 30 leukoplakia, 30 oral squamous cell carcinoma (OSCC), and 10 normal oral mucosa (NOM). The AgNORs count per nucleus, ki-67 LI and API was significantly higher in OSCC followed by leukoplakia and NOM. Leukoplakia with dysplasia showed significantly higher API as compared to leukoplakia without dysphasia. The API is useful as a prognostic factor and also helps to determine treatment modality for patients with premalignant and malignant lesions.

Key words: Oral squamous cell carcinoma, leukoplakia, Ki-67 labelling index, AgNORs count, Actual proliferating index.

Squamous cell carcinoma is the most common malignant neoplasm of the oral cavity, accounting for more than 90% of all oral malignancies. It is usually preceded by premalignant lesions. The identification of these premalignant lesions through clinical, morphological and molecular means helps in early detection and treatment of OSCC. Therefore, markers of cellular proliferation may help to establish more objective diagnostic criteria. Immunohistochemical staining using MIB -1 antibody against Ki-67 antigen have emerged as a marker of choice for cell proliferation. Ki-67 expression assesses the state of cell proliferation i.e. growth fraction. [1] Proliferation rate represents measurements in which a cell cycle phase transit time is calculated to allow estimation of a doubling time.[2] This can be evaluated by argyrophilic nucleolar organizer regions (AgNORs) parameter.

In this study, we tried to find possible association between actual proliferation index (API) and disease progression in the oral mucosa using Ki-67 LI and AgNORs count to determine true proliferative index.

*Assistant Professor, **Professor & HOD, Department of Oral and Maxillofacial Pathology & Microbiology, Modern Dental College & Research Centre, Indore, Madhya Pradesh, India.

***Assistant Professor, Department of Oral and Maxillofacial Pathology & Microbiology, Sharad Pawar Dental College & Hospital, Sawangi (M), Wardha, Maharashtra, India.

Material and Methods

The present study was carried out in the Department of Oral & Maxillofacial Pathology, Modern Dental College & Research Centre, Indore, Madhya Pradesh, India. The study included histopathologically diagnosed 30 OSCC patients, 30 oral leukoplakia and 10 normal oral mucosa. The study was performed on tissues fixed in 10% neutral buffered formalin, paraffin embedded tissue.

Leukoplakia samples were divided into two groups – with and without dysplasia. In OSCC, Clinical TNM staging and Histological malignancy grading (WHO & Bryne's) was carried out.

Procedure

AgNORs staining was carried out according to "International Committee on AgNOR Quantitation" (Derenzini et al., 2004)[3]. Slides were hydrated and heated in microwave oven at 750 watt for 10 min in 10mM sodium citrate buffer (pH 6.0). These sections were then washed with double distilled water three times for 5 minutes each and incubated in dark for 20 to 25 minutes in working solution at room temperature. The slides were washed in running double distilled water for 10 to 15 minutes. Counting was done at a 1000× magnification by the criteria of Giri et al., 1989[4]

Immunohistochemical method for detection Ki-67 antigen

For immunohistochemistry, prediluted Ki-67 antibody (clone MIB-1; Product code: N1633, Dako, Denmark) and Peroxidase Detection System (XViz Polymer-HRP Detection System, Product Code: QD550-YCX,Biogenex) was employed. 3, 3' Diaminobenzidine (DAB) was used to visualize the antigen-antibody reaction and sections were counterstained in Mayer's hematoxylin. Sections were examined by conventional light microscopy.

The Ki-67 labelling index (LI) was calculated from the ratio of the number of tumor cells stained by Ki-67 to the total number of tumor cells counted per section.

$$\text{Ki-67 LI} = \frac{\text{Number of Ki-67 positive cells} \times 100}{\text{Total number of tumour cells observed}}$$

Actual proliferative activity was calculated by using the following equation.

'API = Ki-67 LI × AgNOR count.'

Statistical Analysis was done by using one-way analysis of variance (ANOVA), Mann-Whitney U test and Kruskal-Wallis test. The level of statistical significance is at $p<0.05$.

Results & Observations

The Ki-67 immunostaining was observed as brown colored nucleus in all the groups (Figure 1). AgNORs appears as dark brown or black intranuclear bodies against yellowish background in all the groups (Figure 2). The mean AgNOR count per nucleus, Ki-67 LI and Actual PI was significantly higher in OSCC than leukoplakia (with and without dysplasia) ($p<0.05$) and higher in leukoplakia than NOM ($p<0.05$). Leukoplakia with dysplasia showed significantly higher Ki-67 LI and Actual PI as compared to leukoplakia without dysplasia. The means increased from well- to moderately- to poorly-differentiated SCC. There was significant correlation of Bryne's histological malignancy grading with AgNOR count

Table 1

Groups	n	Subgroups	n	Ki-67 LI Mean(±SD)	AgNORs Count / Nucleus Mean(±SD)	Actual PI Mean(±SD)
NOM	10	-	-	3.77(±2.58)	2.01(±0.70)	7.41(±5.08)
Leukoplkia	20	-	-	20.50(±13.16)	2.55(±0.59)	52.51(±34.24)
OSCC	20	With Dysplasia	09	27.30(±16.51)	2.73(±0.49)	71.91(±39.42)
		Without Dysplasia	11	14.90 (±5.86)	2.40(±0.64)	36.60(±19.18)
		-	-	53.20(±10.26)	3.67(±0.94)	180.90(±87.76)
		WDSCC	13	3.49 (±0.85)	40.2(±13.1)	140.49 (±55.4)
		MDSCC	06	3.91 (±1.15)	62.53(±12.31)	243.99(±92.51)
		PDSCC	01	4.49 (±0.0)	73.0 (±0.0)	327.77 (±0.0)

NOM: Normal oral mucosa; OSCC: Oral squamous cell carcinoma; SD: Standard deviation, WDSCC: Well differentiated squamous cell carcinoma; MDSCC: Moderately differentiated squamous cell carcinoma; PDSCC: Poorly differentiated squamous cell carcinoma.

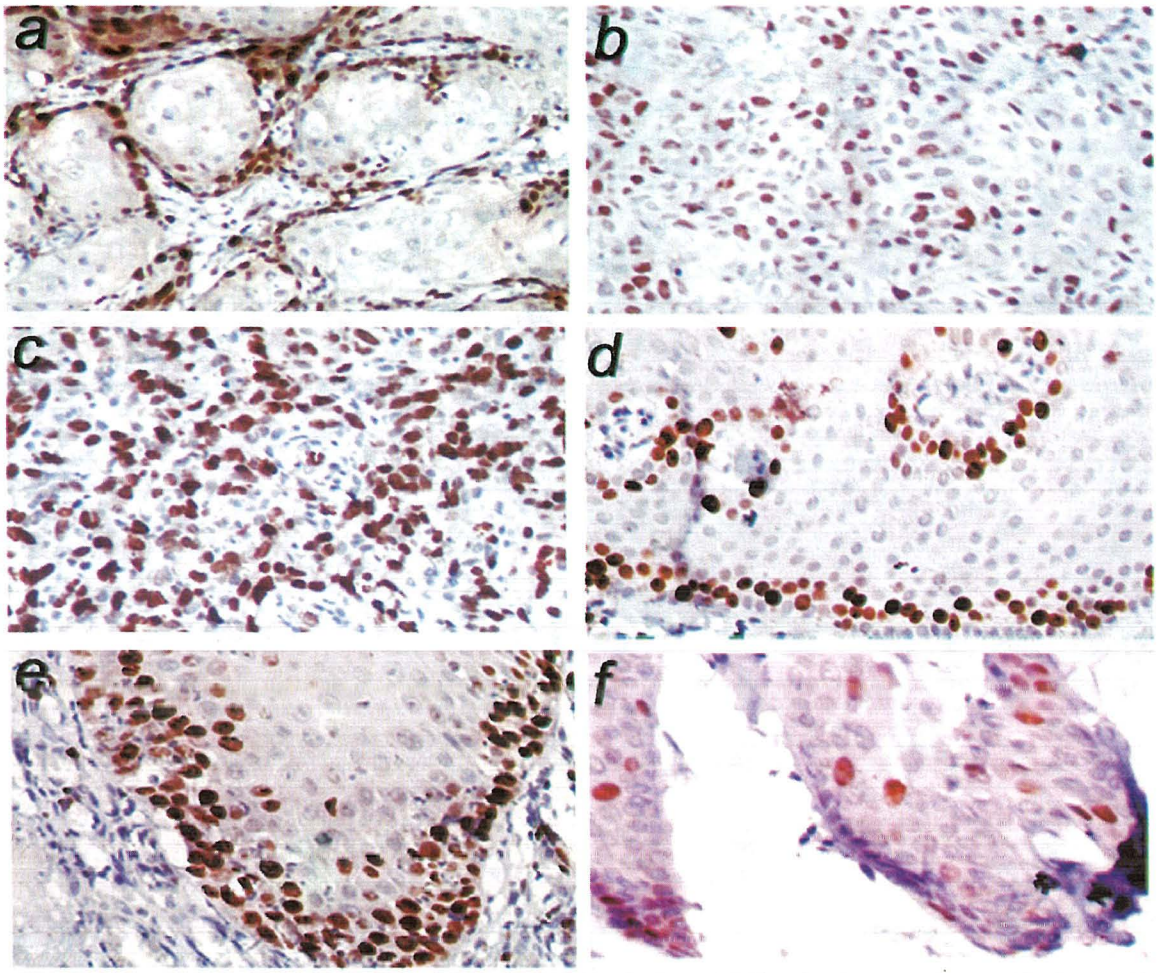


Fig 1. Photomicrograph of immunostaining for Ki-67. (a) In Well differentiated squamous cell carcinoma, the Ki-67 positive cells are noted little to nil in central areas of cellular maturation and keratinization to high reactivity in the more actively proliferating peripheral area. (b) The Ki-67 positive cells are noted in Moderately differentiated squamous cell carcinoma (c) The Ki-67 positive cells are noted highest number in Poorly differentiated squamous cell carcinoma. (d) In Leukoplakia without dysplasia, the Ki-67 positive cells are found in basal cell layer and suprabasal cell layer. (e) In Leukoplakia with dysplasia, the Ki-67 positive cells are found in basal cell layer, predominantly in suprabasal cell layers and few in prickle cell layer. (f) In NOM, the Ki-67 positive cells are predominantly noted in the basal cells and few in suprabasal cells of epithelium. (Original magnification x400).

and Ki-67 LI. There was significant positive correlation between AgNOR count and Ki-67 LI amongst all the groups ($p<0.05$). [Table 1]

Discussion

The ki-67 positive cells increased in number from Nom to Leukoplakia without dysplasia to with dysplasia and maximum in OSCC and these results are in accordance with previous studies[5,6]

In this study, significant increase in mean Ki-67 LI was noted from well differentiated to moderately differentiated SCCs. In Bryne's histological

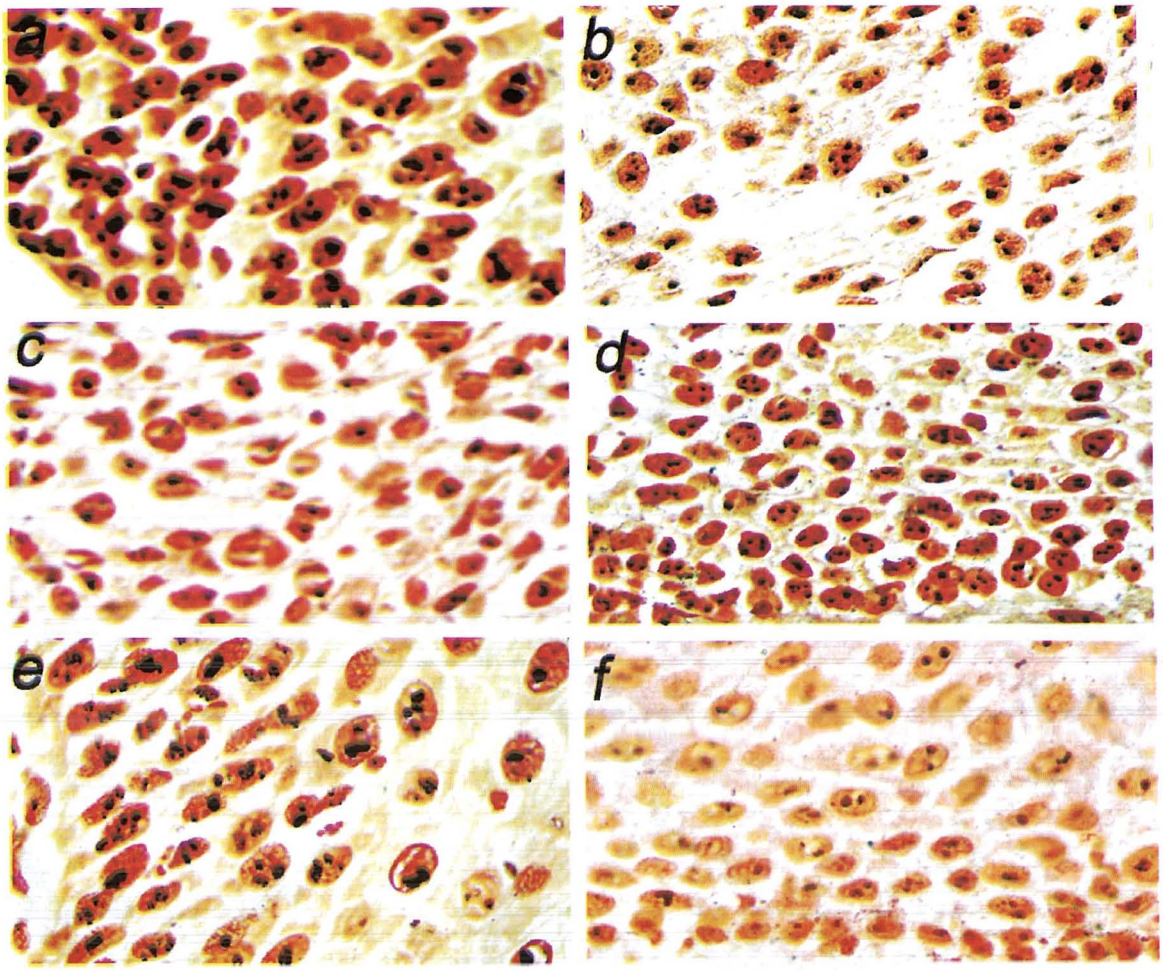


Figure 2. Photomicrograph of AgNORs staining. (a) Well differentiated squamous cell carcinoma, (b) Moderately differentiated squamous cell carcinoma, (c) Poorly differentiated squamous cell carcinoma, (d) Leukoplakia without dysplasia (e) Leukoplakia with dysplasia, (f) NOM. (original magnification x1000).

malignancy grading system, Ki-67 LI was minimal in good prognostic group and higher in poor prognostic group with intermediate value in moderate group.

It is clear from various studies[9] and our study that high Ki-67 LI were observed in OSCC and it correlates with disease progression. Our results showed no significant correlation with TNM staging, but there was significant correlation of Ki-67 LI with WHO and Bryne's histological malignancy grading.

In this study, significant increase in mean AgNOR counts was observed from NOM to non-dysplastic leukoplakia, to dysplastic leukoplakia to OSCC. Previous several studies reports[10] are in agreement with these findings.

There was no correlation between AgNOR count and TNM staging and histological grade. Some studies have got similar and contradicting results because of inadequate distribution of various categories.[10,11]

AgNOR count was highest in poor prognostic group, followed by moderate and good prognostic group according to Bryne's histological grading.

Our results re-affirm the finding of previous studies[12] i.e. increase in AgNOR count in dysplastic leukoplakia as compared to non-dysplastic leukoplakia and NOM. Thus, the AgNORs may be quantitative, discriminative and easy to monitor routinely in detecting incipient cellular alterations.

Progressive increase in API from NOM followed by non-dysplastic leukoplakia and dysplastic leukoplakia to OSCC was observed. Significant correlation of API was noted with histological malignancy grading system. However, we could not draw statistically significant correlation of API with clinical staging. Our study also demonstrated significant positive correlation between AgNOR count and Ki-67 LI in all the groups. This suggest the increased proliferative state and turnover rate of epithelial cells was found to be correlated with different biological behavior of epithelium in NOM, leukoplakia, leukoplakia with dysplasia and OSCC.

Considering the malignant potential of dysplastic leukoplakia, it is always better not to miss a dysplastic case. Assessment of cell proliferation can make a false-negative diagnosis of dysplastic premalignant lesions if assessed solely on the basis of routine H & E staining. Hence, it is suggested that tissues of leukoplakia or suspected epithelial dysplasia should be additionally stained for AgNORs and Ki-67 because using cell proliferation as diagnostic criteria has stronger biological basis. Multiple larger studies will standardize the use of actual proliferative activity to distinguish between non-dysplastic leukoplakia and dysplastic leukoplakia. It is postulated that pharmacological modulators of cell proliferation and differentiation are the future of cancer-preventive drugs. Thus, use of Ki-67 and AgNORs could be of great value in monitoring the effects of these agents during the course of future chemoprevention trials.

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