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Original Research

Comparative Evaluation of Imunohistochemical Expression of Ki-67 in Oral Lichen Planus, Oral Leukoplakia and Normal Mucosa Cases

K Vinay Kumar[1], K H Chaithanya[2], Prashant Punde[3], Ashutosh Thorat[4], Aditya Gopinath Jangam[5], S Deepthi[6]

Contributors:

[1]Assistant Professor, Department of Oral Pathology, Sridevi Institute of Medical Sciences and Research Hospital, Tumkur, Karnataka, India; [2]Professor & Head, Department of Periodontics, RKDF Dental College and Hospital, Bhopal, Madhya Pradesh, India; [3]Assistant Professor, Department of Oral and Maxillofacial Surgery, School of Dental Science Krishna Institute of Medical Sciences, Karad, Maharashtra, India; [4]Resident, Department of Oral and Maxillofacial Surgery, Swargiya Dadasaheb Kalmegh Smruti Dental College and Hospital, Nagpur, Maharashtra, India; [5]Resident, Department of Oral and Maxillofacial Surgery, MA Rangoonwala Dental College, Azam Campus, Pune, Maharashtra, India; [6]Department of Oral Medicine and Radiology, Ashok Multispeciality Dental Clinic, Manvi, Raichur, Karnataka, India.

Correspondence:

Dr. Kumar KV. Department of Oral Pathology, Sridevi Institute of Medical Sciences and Research Hospital, Tumkur - 572 106, Karnataka, India. Email: dr.vinaykumar.k@gmail.com

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Abstract:

Background: Cell proliferation is a biological process of vital importance to all living organisms and is fundamental to both embryonic and post embryonic existence. As epithelial dysplasia is characterized by the number of cell and tissue alterations at molecular and genetic level, there is an alteration of the cellular maturation in the epithelium leading to increase in the proliferative activity of the suprabasal layer. Ki-67 was used as a proliferation marker. Ki-67 is often used to estimate the growth fraction of tumors and other tissues. The present study was conducted to compare immunohistochemical expression of Ki-67 of Oral Lichen planus (OLP) and Leukoplakia patients with normal mucosa.

Materials and Methods: A study was conducted on sections obtained from the paraffin embedded archival blocks of patients diagnosed histologically as oral leukoplakia, lichen planus, and normal mucosa. A total number of 60 archival blocks were studied. The blocks were further divided in three groups as Group-I normal buccal mucosal, Group-II patients histologically diagnosed as epithelial dysplasia, Group-III patients diagnosed clinically and histologically as OLP. The sections were subjected to Ki-67 staining.

Results: The Ki-67 expression was found in all the cases of normal oral epithelium studied and was found to be restricted to the basal layer of the epithelium. There were no significant differences between groups by age, sex, and site. The Ki-67 expression was mainly seen in the parabasal layer where proliferating cells were restricted rather than in the basal layer. Ki-67 positive cells in leukoplakia were located in the periphery of the tumor nests as compared to the center, where frequent mitoses were observed. In Lichen planus cases, the Ki-67 expression was less pronounced than compared to leukoplakia but the expression was more than the normal mucosa.

Conclusion: The Ki-67 expression was detected in all the cases of normal oral mucosa and was found to be restricted in the basal layer of the epithelium. Generally, it has been observed that the mean ki-67 LI increases between high and low risk epithelial dysplasia and different grades of oral squamous cell carcinomas.

Key Words: Cell proliferation, Ki-67, oral leukoplakia, oral squamous cell carcinoma

Introduction

Cell is a basic, living, structural and functional unit of the body. Cell proliferation is defined as "increase in cell numbers resulting from completing the cell cycle." Cell proliferation is a biological process of vital importance to all living organisms and is fundamental to both embryonic and post embryonic existence.[1] The control on this important biological process is thought to be impeded in cancer,[2] and many studies have reported that abnormal cell proliferation appears to be a precursor as well as a predictor of tumorogenesis.[3] The risk factor for oral cancers is closely related to lifestyle, such as tobacco use, alcohol use, poor oral hygiene, and betel quid chewing.[4] In the oral cavity, oral squamous cell carcinoma (OSCC) is thought to develop by a multi-step carcinogenesis.[5] OSCC frequently coexists with or is surrounded by epithelial leukoplakia or dysplasia.[6] Clinicopathologically malignant transformation of oral precancerous lesion is observed up to 17.5% of the cases.[6] In societies where the incidence of oral cancer is high, clinically recognizable pre - malignant lesions are particularly common. Even small dysplastic lesions can be followed by multiple carcinomas.[7] Oral leukoplakia is the best-known early lesion which exhibits the histopathologic features of epithelial dysplasia.[8] The term "epithelial dysplasia" is assigned to histopathological changes associated with an increased risk of malignant development (i.e., SCC). The individual cellular changes are referred to as atypia.[7]

Oral epithelial dysplasia is not associated with any specific clinical appearance. Thus, white, red, or mixed white and red lesions in the oral mucosa are most frequently revealing epithelial dysplasia. The frequency of epithelial dysplasia in leukoplakia varies between >1% and <30%. The lowest frequency reported originated from a population-based

study in India in which all clinically diagnosed leukoplakias were biopsied, emphasizing again the dependency on case selection and, possibly, geographical variations. The presence of epithelial dysplasia is generally accepted as one of the most important predictors of malignant development in pre-malignant lesions. Oral lesions with epithelial dysplasia more often develop into carcinoma than those without dysplasia. Furthermore, features of epithelial dysplasia are commonly seen in epithelium adjacent to oral carcinomas. However, all epithelial dysplasias will not necessarily develop into cancer, and some may even regress.[9] Theories of carcinogenesis suggest that premalignant change may occur in any area of mucous membrane exposed to a carcinogen; hence patients with oral cancer have the risk of developing a second or multiple primary carcinomas within the upper aerodigestive tract- field cancerization proposed by Slaughter in 1953.[10,11]

Immunostaining with antibodies to Ki-67 antigen is well-established as a quick and efficient method for evaluating growth fractions of various tumor types because of its distinctive reaction patterns that exclusively involve proliferating cells.[12] The Ki-67 antibody was first isolated during attempts to raise monoclonal antibodies to antigens specific for Hodgkin and Reed-Steinberg cells.[13] Ki-67 stood out from other antibodies produced because it only reacted with cells which were proliferating, for example cortical thymocytes and cells in the crypts of the small intestine, whereas it would show no reaction with cells which were known to be in a resting or terminally differentiated state, such as liver cells and neurons.[12] The Ki-67 antigen was named after its place of characterization in Kiel, Germany, and because the clone producing the antibody was grown in the 67th well of tissue culture plate.[14] The Ki-67 antigen is a large basic protein found as peptides with molecular weights of 345 kD and 395 kD[15] which have been detected within the nucleus and its gene is located on chromosome 10q25-ter.[16] However, Ki-67 is not expressed in arresting cycling cell. Ki-67 antigen starts to be expressed in the S phase, and increases through S and G2 phases, reaching a plateau at mitosis. Epithelial dysplasia at molecular and genetic level is characterized by the number of cell and tissue alterations. There is an alteration of the cellular maturation in the epithelium leading to increase in the proliferative activity of the suprabasal layer, which helps to establish a more objective diagnosis.[17]

Hence, the present study has been undertaken to evaluate a potential association of abnormal cell proliferation of cells in the leukoplakic epithelium and oral lichen planus (OLP) by the proliferative marker Ki-67 and both are compared to histologically normal appearing mucosa.

Materials and Methods

A retrospective study was designed on sections obtained from the paraffin embedded archival blocks of patients diagnosed histologically as oral leukoplakia, lichen planus, and normal mucosa from the Department of Oral Pathology and Microbiology, Sri Hasanamba Dental College and Hospital Hassan and Sridevi Institute of Medical Sciences and Research Hospital, Tumkur. A total number of 60 archival blocks were studied. The case details of all the patients were retrieved. The cases were selected on the basis of the case details obtained from the patients. The blocks were further divided in three groups as follows: Group-I: Normal buccal mucosa (n = 20). Group-II: Patients diagnosed clinically as potentially malignant disorders and histologically diagnosed as oral leukoplakia (n = 20). Group-III: Patients diagnosed clinically and histologically as OLP (n = 20).

Procedure

Sections cut at 4 µ were floated on to Poly - L - Lysine coated slides and incubated overnight at 58°C (Figures 1 and 2). The sections were then deparaffinized in two changes of xylene for 15 min each. Dexylinization was done by immersing the slides in two changes of absolute alcohol for 1 min each. Sections were alcoholized by immersing the slides in 90% and 70% alcohol for 1 min each and then washed for 10 min and 5 min each in tap water and distilled water, respectively.

Antigen retrieval was done by placing the sections in citrate buffer and then kept in micro-oven for 10 min. Borosil jar was then cooled for 20 min in the sink with water. Sections were then rinsed with distilled water for 5 min and were then washed with two changes of tris buffer solution (TBS) for 5 min each. To block the endogenous peroxidase enzyme activity, the sections were treated with peroxidase block for 10-15 min

Figure 1: Immunohistochemical reagents and equipments.

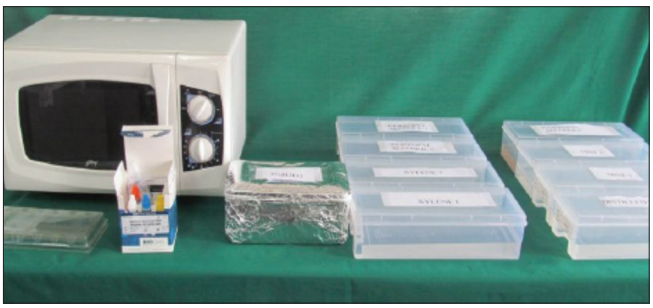


Figure 2: Other reagents and equipments.

and then again washed with 3 changes of TBS for 5 min each. Sections were then treated with power block for 15 min in order to block non-specific reaction with other antigens. Sections were then drained and covered with primary antibody against Ki-67 with dilution of 1:100 for 1 h to identify tumor markers by antigen - antibody reactions and again washed with TBS as described earlier. To enhance the reaction between primary and secondary antibodies, sections were then treated with super enhancer for 30 min. And again washed with TBS. Enzymes were labeled by treating the sections with super sensitive poly - HRP secondary antibody and washed with TBS. Chromogen was then added to the sections for 5 min to give color to the antigens and sections were again washed with TBS. Sections were then washed with tap water for 5 min and were counterstained with hemotoxylin for 1 min and washed in tap water, dried, cleared in xylene, and mounted with DPX. Diagnosed SCC cases were taken as positive control for Ki-67 expression and for negative control normal mucosa was taken.

Interpretation of staining

Presence of brown colored end product at the site of target antigen (nucleus) was considered as positive immunoreactivity. This was considered based on the findings observed in the positive control slide of SCC. SCC demonstrated nuclear staining with Ki-67 monoclonal antibody in the malignant cells. Leukoplakia, OLP, normal cell demonstrated nuclear staining with Ki-67 monoclonal antibody in the epithelial cells.

Selection of field for counting cells

The stained sections were scanned under low power to determine the areas that were most evenly stained. Such representative fields were selected carefully in each slide by scanning the slides from left to right of every slide to avoid recounting of same areas.

Statistical analysis

To find out the significant difference between the mean Ki-67 labeling indices descriptive statistical analysis has been carried out. Results on continuous measurements are presented as mean standard deviation (min-max). Significance is assessed at 5% level of significance. Analysis of Variance (ANOVA) has been used to find the significance of study parameters between three or more groups of patients, *post-hoc* Tukey test has been used to find the significance pair wise.

Results

The present study was carried out using histopathologically diagnosed cases of Leukoplakia, Lichen planus, and these were compared with normal oral mucosa (NOM). Immunohistochemical features obtained are described below: Group-I ($n = 20$): Normal buccal mucosal specimens. Group-II ($n = 20$) patients diagnosed clinically as potentially malignant lesion and histologically diagnosed as Leukoplakia. Group-III ($n = 20$) patients diagnosed clinically as malignant lesion and histologically diagnosed as lichen planus. Significance is assessed at 5% by using ANOVA to find the significance of study parameters between three or more groups of patients, *post-hoc* Tukey test has been used to find the significance pairwise.

Table 1 shows that the NOM (Group I), the number of Ki-67 positive cells value ranges from 19 to 25 with a mean and standard deviation of 22.05 ± 2.06 respectively. In histopathologically diagnosed as leukoplakia cases the number of Ki-67 positive cells value ranges from 61 to 105 with mean and standard deviation of 75.8 ± 11.32 and in lichen planus the range was 20-80 with mean and standard deviation of 44.15 ± 12.19. An ANOVA revealed statistically highly significant difference in expression of Ki-67 positive cells with a $P < 0.001$.

Table 2 shows the multiple comparisons done by using Tukeys *post hoc* showed statistical significant difference between leukoplakia and lichen planus, leukoplakia and normal mucosa, and lichen planus and normal mucosa with a $P < 0.001$.

Graph I shows mean number of Ki-67 expression of 400 cells, Graph II shows percentage of Ki-67 expression among leukoplakia, lichen planus compared with normal mucosa.

Discussion

Our aim was to study and interpret the relationship of Ki-67 labeling index (LI) with leukoplakia and lichen planus and these were compared with the NOM for the proliferative index. These histological examinations of proliferating cells and alteration of stem cells have mainly focused on the total number of positive cells within the epithelium as an index of malignancy rather than for architectural distribution within the altered epithelium. In our study, the Ki-67 expression was detected in all the cases of NOM and was found to be restricted in the basal layers of the epithelium. The number of Ki-67 positive cells in our study were significantly more in the high risk group when compared to the low risk group which is in accordance with the studies which have revealed that the number of Ki-67 positive cells increased according to the proliferative activity and degree of epithelial dysplasia thus implicating Ki-67 as a marker of the presence and severity of epithelial dysplasia in the oral mucosa.[18] The assessment of changes at the molecular level may become the primary means of diagnosis and may guide management since these changes mediate morphologic changes that occur after genetic changes, and knowledge of current morphologic changes is based on the subjective assessment of clinical and histopathologic changes.[19] Present study results are similar to a study conducted in 1996 for expression of p53 in oral leukoplakia and SCC of the oral mucosa. A significant correlation was revealed with of both p53 and Ki-67 with the histopathological stage of the tumor.[20]

An immunohistochemistry study done in 2001 by 10 specimens of OLP, oral leukoplakia, dysplasia, and normal mucosa were compared for Ki-67 expression and found that all the tissue analyzed showed some grade of proliferation in the basal layer.

Table 1: Comparison of Ki-67 in three groups studied.

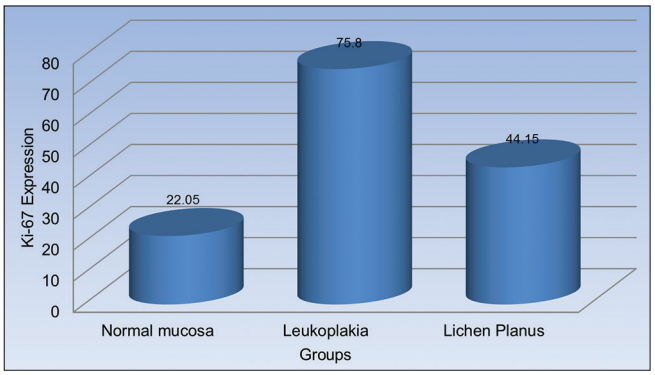
Ki-67	Min-Max	Mean±SD	ANOVA (F)	*P* value	Significance
Normal mucosa	19-25	22.05±2.06	155.735	<0.001	HS
Leukoplakia	61-105	75.80±11.32			
Lichen planus	20-80	44.15±12.19			

P<0.001, HS: Highly significant, SD: Standard deviation, ANOVA: Analysis of variance

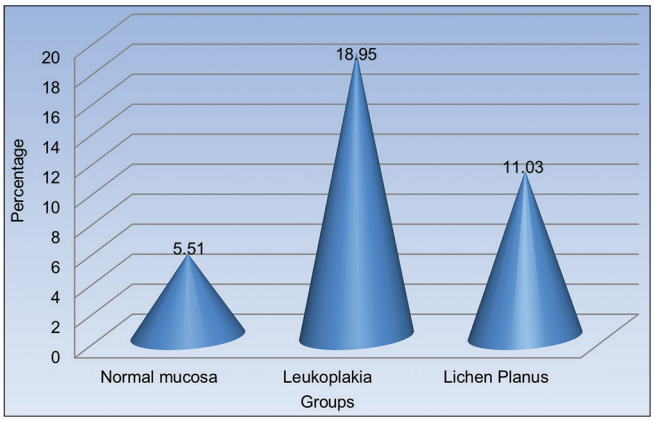
Table 2: Multiple comparisons using Tukeys *post hoc* test.

Groups	Comparison	Mean difference	Standard error	Significant	95% confidence interval	
					Lower bound	Upper bound
Normal mucosa	Leukoplakia	53.75000*	3.06156	0.0001	−61.1174	−46.3826
	Lichen planus	22.10000*	3.06156	0.0001	−29.4674	−14.7326
Leukoplakia	Normal mucosa	53.75000*	3.06156	0.0001	46.3826	61.1174
	Lichen planus	31.65000*	3.06156	0.0001	24.2826	39.0174
Lichen planus	Normal mucosa	22.10000*	3.06156	0.0001	14.7326	29.4674
	Leukoplakia	31.65000*	3.06156	0.0001	−39.0174	−24.2826

*The mean difference is significant at the 0.05 level



Graph 1: Mean Ki-67 expression in different groups.



Graph 2: Percentage of Ki-67 expression in different groups.

Results were statistically significant among mean of the positive nuclei per mm of length in normal mucosa and oral leukoplakia and between normal mucosa, OLP and oral leukoplakia with dysplasia, the present study results are also similar.[21] Previous studies have compared lichen planus but in our study we have tried to compare the expression of Ki-67 in leukoplakia and lichen planus with normal mucosa and expression was significantly higher in leukoplakia than in lichen planus. OLP is a chronic immunologic mucocutaneous disease. The possible malignant transformation of OLP is the subject of study of an ongoing and controversial discussion in the literature. The main criticism of studies on this subject relates to the lack of sufficient data to support the initial diagnosis of OLP in cases that finally developed into SCC.[22] The occurrence of SCC in most series ranges from 0.4% to 2.0% per 5 year observation period.[23] The most recent and extensive study reported a rate of 1.5% for patients observed during 7.5 year.[24] The presence of dysplasia in OLP increases the risk of malignant transformation, mandating management, and close follow-up.[25] Potentially malignant lesions are those occurring in a morphologic altered tissue. In order to analyze the malignant potential, it is important to consider that oncogene activation and inactivation of tumor suppressor genes occur precociously in carcinogenesis.[26]

Ki-67 is the protein that plays a pivotal role in maintaining cell proliferation. This protein is used as a prognostic marker in many tumors. The proliferative index determined by the number of cells stained by Ki-67/MIB-1 per number of tumoral cells counted has proven to be a prognostic factor in several neoplasms.[27] However, in potentially premalignant lesions its value is still being analyzed.[26] The increased proliferative activity of the epithelium in our patients in case group stressed by the results of Ki-67, which showed significantly higher mean values of both indices than control group. Over - expressions of Ki-67 in OLP patients have been reported in other studies and OLP has been postulated to have a secondary proliferative disorder probably due to repeated breakdown of the cycling cells leading to an increased state of proliferation.[28] In another study was found a strong correlation between Ki67 over - expression and cell proliferation (Ki-67).[29] This result is consistent with our result; however, this study evaluated various malignant and pre-malignant lesions other than OLP. In conclusion, since Ki-67 extensively accepted as important biomarkers in diagnosis, prognosis and treatment of malignant and premalignant lesions, high expression of these biomarkers are useful for the identification of OLP lesion with a more aggressive pattern and

with a major tendency to OSCC development. Lichen planus represents as a set of lesions including white involvements (striation, papule, plaque), erythema, erosions, and blisters mainly on the mucosa, gingival structures and tongue.[30] Although it is believed that a T-cell mediated autoimmune mechanism plays a major role in pathogenesis of lichen planus, the exact underlying etiology is still under debate.[31]

OLP is generally considered as a benign entity. However, there are case reports of SCC which are suspected to be developed from a previous mucosal lichen planus. The odds of such transformation are not determined.[26] In the present study, we examined the rate of Ki-67 expression in cases with leukoplakia and oral OLP and normal subjects were considered as controls. Ki-67 LI was significantly more prevalent in specimens with leukoplakia (100%) and lichen planus (86.7%) than in normal samples (20%) as evaluated by immunohistochemical evaluations. These findings are indicative of a potential tendency for malignancy in OLP. In previous reports, the rate for expression of Ki-67 was from 96% to 100%.[32] Present results are also similar to these reports. A study conducted in 2004 by tissue expression of proliferative antigens (PCNA and Ki-67) in oral lichen ruber was compared to clinical status in lesions of 30 patients. Results showed the reaction on Ki-67 tissue antigen ranged from low to moderately high intensity. Intensely high reaction was observed in lesions of lichen ruber erosivus. There was a positive correlation of the inflammation grade with degree of hyperkeratosis in lesions.[33]

In a study in 2009, the expression of Ki-67 protein in OLP was compared with NOM. Specimens of 44 lesions of OLP and 30 specimens of control group were evaluated. Result revealed that the mean expression of Ki-67 in patient with OLP was more than the control group.[34] In line with the present findings, the rate of Ki-67 positivity was significantly higher in patients with leukoplakia and lichen planus than in normal counterparts in another series.[35] Lichen planus might be considered a premalignant disease based on these findings.

Conclusion

The Ki-67 expression was detected in all the cases of NOM and was found to be restricted in the basal layer of the epithelium. Generally, it has been observed that the mean Ki-67 LI increases between high and low risk epithelial dysplasia and different grades of oral SCCs.

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