Research Article

JRAD Journal of Research and Advancement in Dentistry

J Res Adv Dent 2016;5:1:417-422.

Analysis of Ki-67 Expression In Leukoplakia

Jazib Nazeer[1*] Tanoj Kumar[2] Avanindra Kumar[3] Soumen Mandal[4] Asad Iqubal[5] Parmeshwar Kumar Ranjan[6]

[1]Senior Lecturer, Department of Oral Pathology, Patna Dental Collage & Hospital, Patna, Bihar, India.
[2]Professor, Department of Oral Pathology, Patna Dental Collage & Hospital, Patna, Bihar, India.
[3]Reader, Department of Oral Pathology, Patna Dental Collage & Hospital, Patna, Bihar, India.
[4]Private Consultant, Department of Oral Surgery, Kolkata, West Bengal, India.
[5]Senior Lecturer, Department of Oral Medicine and Radiology, Patna Dental Collage & Hospital, Patna, Bihar, India.
[6]Tutor, Department of Periodontics, Patna Dental Collage & Hospital, Patna, Bihar, India.

ABSTRACT

Background: Leukoplakia has been studied for many years and is commonly agreed upon as representing the most common pre-malignant oral mucosal lesion. Oral leukoplakia (OL) is the most common potentially malignant disorder of the oral cavity, which can precede oral squamous cell carcinoma. Ki-67 is a nuclear antigen which is present in the peri-chromosomal region during mitosis and seems to be a non-histone protein, the objective of this research was to detect the expression of Ki-67 in leukoplakia cases with dysplasia and without dysplasia and co relate its expression in relation to normal mucosa.

Materials and Method: Two sections were made from each paraffin block of respective group. The H&E staining was done for one of section. The second section was used for IHC demonstration of Ki-67expression.

Results: In this study Ki-67 positive nuclei were found only at basal and parabasal layer, In the present study 87.5% of the leukoplakia shows positivity for Ki-67 staining, no significant difference were observed in expression of Ki-67 between non dysplastic and dysplastic group (p=.0589)

Conclusion: it can also be documented that Ki-67 may also be considered as a tumor marker. Expression of Ki-67 protein may help in determining the prognosis as well as play a key role in early detection of squamous cell carcinoma, which may help not only in determining the prognosis but also plays a role in gene therapy in future.

Keywords: Leukoplakia, ki-67, Epithelial dysplasia.

INTRODUCTION

Oral leukoplakia (OL) is the most common potentially malignant disorder of the oral cavity, which can precede oral squamous cell carcinoma. Leukoplakia is a term describing "a white lesion/patch of the oral mucosa that cannot be characterized clinically or microscopically as any other defined oral disease entity". According to World Health Organization (WHO) in 2005, oral leukoplakia can be defined as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer".[1]

Leukoplakia has been studied for many years and is commonly agreed upon as representing the most common pre-malignant oral mucosal lesion.[2,3] About 70–90% of oral leukoplakia are found to be related to smoking tobacco and areca nut use, either alone or in combination. A direct relationship has been evaluated between the

Received: May. 18, 2015: **Accepted:** Aug. 21, 2015
***Correspondence Dr. Jazib Nazeer.**
Department of Oral Pathology, Patna Dental Collage & Hospital, Patna, Bihar, India.
Email: Not Disclosed

pISSN 2278-0076
eISSN 2321-9270

frequency, duration of cigarette, pipe, or cigar smoking and the prevalence of oral leukoplakia. The factors implicated in the pathogenesis of idiopathic leukoplakia are unknown. However it is possible that infection of the oral epithelium with human papilloma virus (HPV) and excessive consumption of alcoholic beverages may be associated with oral leukoplakia.

Ki-67 is a cell cycle associated human nuclear protein present in peri-chromosomal region.[4] Ki-67 is detected in the nucleus of proliferating cells in all active phases of the cell division cycle, but is absent in non-proliferating cells. The Ki-67 antibody reacts with a nuclear non-histone protein of 395 KD that is expressed in all active phases of the cell cycle, except the G0.[5]

Oral leukoplakia has greatest relevance in the study of the biology of carcinogenesis because the oral cavity is easily accessible for clinical examination and also because it has a multistage carcinogenesis pathway and of the concept of field cancerization.[6] Cancer is characterized by uncontrolled cell proliferation, markers of proliferation, such as Ki-67 and proliferating cell nuclear antigen, have been studied extensively in neoplastic lesions.

Ki-67 is a nuclear antigen which is present in the peri-chromosomal region during mitosis and seems to be a non-histone protein, representing a new class of cell cycle maintaining proteins.[7] Studies on proliferation markers in oral leukoplakia cases with and without involvement of epithelial dysplasia are few with the need of further investigation, hence the objective of this research was to detect the expression of Ki-67 in leukoplakia cases with dysplasia and without dysplasia and co relate its expression in relation to normal mucosa.

AIM AND OBJECTIVES

1. To assess role of Ki-67 in leukoplakia cases with dysplasia in oral tissue.

2. To assess role of Ki-67 in leukoplakia cases without dysplasia in oral tissue.

3. To co-relate the immune histochemical expression of Ki-67 between leukoplakia (with or without dysplasia) and normal oral mucosa.

MATERIALS & METHODS

The sample consisted of 50 tissue sections 20 each diagnosed case of leukoplakia with dysplasia and without dysplasia. The tissue sections were obtained from the archival of Department of Oral & Maxillofacial Pathology, D. J. College of Dental Sciences & Research, Modinagar. 10 tissue section of normal mucosa were selected as control group sourced from Department of Oral and Maxillofacial Surgery of the same institute. For the control group healthy volunteers without the habits of tobacco chewing were selected.

Fig 1: Chemicals Used For H & E Staining.

INCLUSION CRITERIA

1. Patients diagnosed clinically as leukoplakia and histopathologically as cases of epithelial dysplasia and without epithelial dysplasia with presence of evident clinical lesion.

2. Paraffin blocks of lesional tissue obtained from benign connective tissue lesion consisting of normal epithelium.

EXCLUSION CRITERIA

1. Any other systemic illness.

2. Metastatic lesions to the jaws.

METHODOLOGY

Two sections were made from each paraffin block of respective group. The H&E staining was done for one of section. The second section was used for IHC demonstration of Ki-67expression.

INTERPRETATION OF THE KI-67 STAINING

The slides stained for Ki-67 were observed under Motic microscope with a magnification of 10X

and 40X using image analysis software. The tissue samples were thoroughly examined and total number of 100 basal and suprabasal (always jointly) cells was counted. Then, among the 100 cells, the number of cells which had taken up the stain, were counted. A brown precipitate seen within the nucleus confirmed the presence of Ki-67 protein.

METHOD FOR COUNTING KI-67 POSITIVE CELLS

1. Each slide was placed on the optical photomicroscope and observed under the 10X and 40 X objectives.
2. Digital pictures were taken and stored as jpeg files.
3. Each file was opened and a grid was placed over the image using grid cell counter software.
4. To cover the immunohistochemical image completely, the grid was enlarged.
5. Positive cells were counted starting from left corner and then moving in upward direction.

The Ki-67 positive samples were then evaluated quantitatively on a 4 point scale based on the percentage of cells showing Ki-67 staining[7].

1. Point 0 if less than 05% of the cells show positive staining.
2. Point 2 if 06-25% cells show positive staining.
3. Point 4 if 26-60% cells show positive staining.
4. Point 6 if more than 60% of the cells show positive staining.

The data thus obtained was tabulated and statistically analyzed.

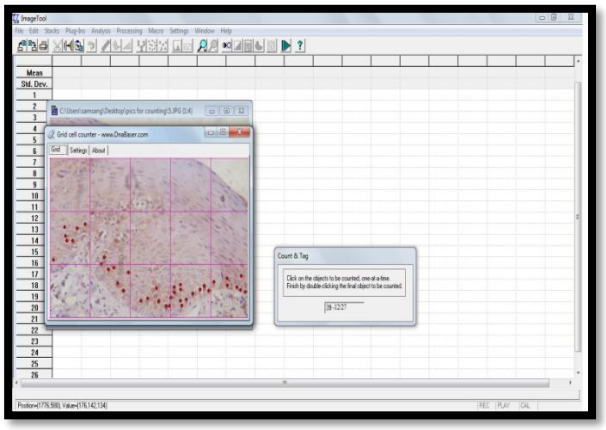

Fig 2: Image Analyzer Software For Counting Ki-67 Positive Cells.

STATISTICAL ANALYSIS

The statistical analysis used in the study was unpaired "t" test and one-way ANOVA test of significance and association between scores and different groups were done By „Fisher‟s Exact Test‟. In this test the p value of less than 0.05 was accepted as indicating statistical significance.

RESULTS

The present study was designed to determine the expression of Ki-67 protein in leukoplakia. The sample selected for this study consisted of 20 tissue sections each of leukoplakia with dysplasia and without dysplasia. Mean age among leukoplakia with epithelial dysplasia was 54.05 years, leukoplakia without epithelial dysplasia was 54.45 years and in normal group it was 59.3 years. The mean & standard deviation of Ki-67 expression with epithelial dysplasia group at basal layer was 6.65±4.82, and at suprabasal layer was 11.6±9.90, the mean & standard deviation of Ki-67 expression without epithelial dysplasia group at basal layer was 10.85±9.63, and at suprabasal layer was 6.2±7.42 and the mean & standard deviation of Ki-67 expression in control group at basal layer was 11±8.60, and at suprabasal layer was .3±.48.

Table 1 shows the comparison in basal counting between different pair of groups respectively at .05 level of significance by student "t" test, which shows no significant difference was present between all the groups in counting.

S. No	Pair of Groups	PROBABILITY OF Independent "t" Test for basal Counting's	p-value
1	Ki-67 Expression with Epithelial Dysplasia Group & Ki-67 Expression Without dysplasia group	.0921**	P>.05 (N.S.)
2	Ki-67 expression with Epithelial dysplasia group & normal group	.1631**	P>.05 (N.S.)
3	Ki-67 expression without dysplasia group & normal group	.9659**	P>.05 (N.S.)

**shows no significant difference at .05 level of significance. (p>.05)

Table 2 shows the comparison in suprabasal counting between different pair of groups respectively at .05 level of significance by student "t" test, which shows a significant difference was present between all the groups in counting.

S. No	Pair of groups	Probability of independent "t" test for supra basal counting's	P- VALUE
1.	Ki-67 expression with epithelial dysplasia group & ki-67 expression without dysplasia group	.0589**	P>.05
2.	Ki-67 expression with epithelial dysplasia group & normal group	.0000*	P<.05
3.	Ki-67 expression without dysplasia group & normal group	.0021*	P<.05

*shows a significant difference at .05 level of significance. i.e. (p<.05)

**shows no significant difference at .05 level of significance. i.e. (p>.05)

Since score distributions are asymmetrical, the rank correlation and the ANOVA test were used in analyses performed.

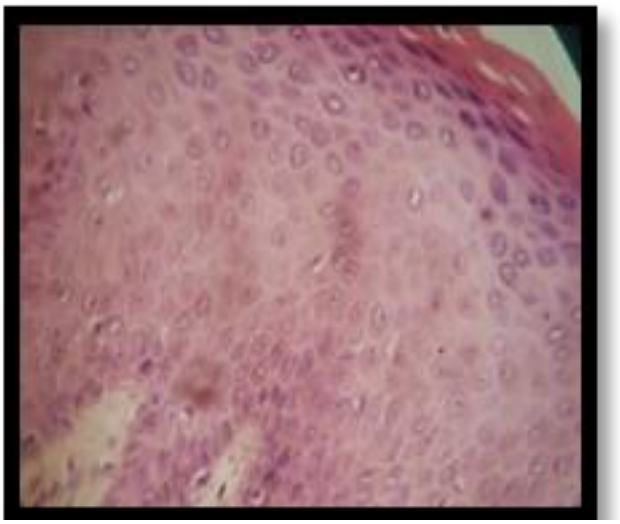

Fig 3: Leukoplakia (Dysplastic) (H & E 40x).

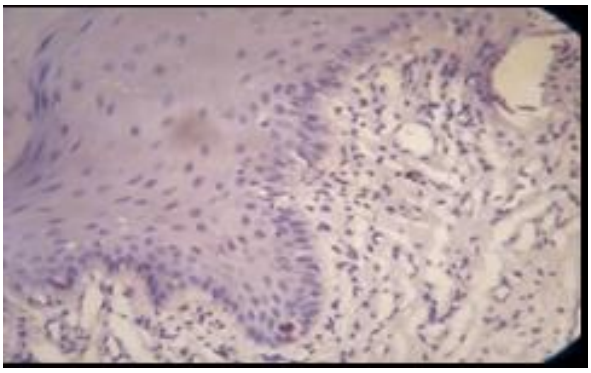

Fig 4: Leukoplakia (Dysplastic) (Ki-67-40x) 0 Score.

DISCUSSION

Oral leukoplakia (OL) is the most common potentially malignant disorder of the oral cavity, which can precede oral squamous cell carcinoma. Leukoplakia is a term describing "a white lesion/patch of the oral mucosa that cannot be characterized clinically or microscopically as any other defined oral disease entity".[1] Proliferation and differentiation are controlled by autocrine and paracrine factors generated by the keratinocytes, cytokines and growth factors originating in the underlying connective tissue and circulating systemic factors.[8, 9]

Proliferation is a property of stem cells seen in the basal layers of the stratified squamous epithelium and their immediate progeny, the transit-amplifying cells. Stem cells are slow cycling cells that are not distributed homogenously in the basal layer but are grouped in a pattern set by cell-to-cell communication among keratinocytes.[10, 11, 12] Ki-67 has been shown to be excellent for the estimation of the growth fraction in both normal and malignant human tissue. During inter phase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0), makes it an excellent marker for determining the so-called growth fraction of a given cell population.

In the present study, Ki-67 expression was positive in 35 (87.5%) cases and negative in 5 (12.5%) cases. Among control group 9 cases shows positive expression of Ki-67 and 1 case was negative. These results are in accordance with the

studies conducted by S.Humayun[15], Miguel A. Gonzalez-Moles[8], T. Takeda et al[10]. In the present study the expression of Ki-67 was brown granular nuclear staining, the distribution of staining was limited to basal and suprabasal layer in leukoplakia which is in accordance with the study done by Priya Kumar.

In our study Ki-67 positive nuclei were found only at basal and para basal layer which is in accordance with the study done by Takeda et al[10]. In the present study 87.5% of the leukoplakia shows positivity for Ki-67 staining which is likewise to the observation done by Humayun[15] who shows 75 % positive Ki-67 expression in leukoplakia cases and 100% in control group.

Our study is in accordance with the study and observation done by Kannan[7], he scored the presence of Ki-67 into 0, 2, 4, 6 and found that 14.28% cases were of 0 score, 28.57% cases of 2 and 57.14% were of 4 score, in our study it was 15% for 0 score, 55% cases were of 2 score and 30% cases were of score 4. Variation in techniques employed may account for these little discrepancies. In our study the comparison of basal cell counting in all the three groups were statistically insignificant this is because the basal layer is the only proliferative compartment for normal oral epithelium, whereas in rest of the epithelial layer, cellular maturation is seen without any proliferative activity.11 In our study no significant difference were observed in expression of Ki-67 between non dysplastic and dysplastic group (p=.0589) which is in accordance with the study done by Kannan et al[7].

Nasser et al[16] conducted a study and found that p53 and Ki-67 have low positive predictive value when expressed independently, but their dual expression could help discriminate between dysplastic and non-dysplastic leukoplakia's. In the present study the comparison between dysplastic and non-dysplastic group was not significant. This might be due to in present study we only uses the Ki-67 which is a proliferative marker but Nasser *et al* used the dual expression of Ki-67 and p53

In the present study the expression of Ki-67 is seen in the basal and suprabasal layer, maximum expression in suprabasal layer were present in leukoplakia with epithelial dysplasia and minimum in control group. This increased proliferation in parabasal layers of premalignant oral epithelium is likely related to loss of heterozygosity in 3p, 9p and 17p chromosome which behaves as a marker of precancerous fields and increases the risk of developing multiple tumors as stated by Smita S B et al[17].

SUMMARY AND CONCLUSION

Oral leukoplakia has greatest relevance in the study of the biology of carcinogenesis because the oral cavity is easily accessible for clinical examination and also because it has a multistage carcinogenesis pathway and of the concept of field concretization. The oral carcinogenesis pathway can be broadly classified histopathologically into normal, nondysplastic, dysplastic, carcinoma-in situ, and invasive carcinoma. Molecular biological studies on oral cancer have identified several genetic aberrations in the cells during various tumor stages.

As cancer is characterized by uncontrolled cell proliferation, markers of proliferation, such as Ki-67 and proliferating cell nuclear antigen, have been studied extensively in neoplastic lesions. The present study was designed to determine the expression of Ki-67 protein in leukoplakia the technique used was indirect immunoenzyme LSAB method in the present study; Ki-67 expression was positive in 87.5% cases and negative in 12.5% cases. Among control group 9 cases shows positive expression of Ki-67 and 1 case was negative.

Thus, it can also be documented that Ki-67 may also be considered as a tumor marker. Expression of Ki-67 protein may help in determining the prognosis as well as play a key role in early detection of squamous cell carcinoma, which may help not only in determining the prognosis but also plays a role in gene therapy in future. However, long term follow-up studies would further help to know the significance of Ki-67 in pathogenesis of leukoplakia and its malignant transformation.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

REFERENCE

1. Feller L, Lemmer J. Oral leukoplakia as it relates to HPV infection: a review. International Journal of Dentistry. 2012; 12(10): 1-7.

2. Sdubba JJ. Oral leukoplakia. Critical Reviews in Oral Biology & Medicine. 1995; 6(2): 147-160.

3. Caldeira PC, Aguiar MC, Mesquita RA, Carmo MA. Oral Leukoplakias With Different Degrees of Dysplasia: Comparative Study of HMLH1, p53, And Agnor. J Oral Pathol Med. 2011; 40(4): 305–311.

4. B. Neville, Damm D, Allen C, and Bouquot J, Oral and Maxillofacial Pathology. W.B. Saunders Company.2002; Ed 2nd: 337.

5. Kill IR. Localisation of the Ki-67 Antigen within the nucleolus evidence for a fibrillarin-deficient region Of the Dense fibrillar Component. Journal of Cell Science. 1996; 109(6): 1253-1263.

6. Andersen SN, Rognum TO, Clausen OP. Ki-67: A useful marker for the evaluation of dysplasia in ulcerative colitis. J Clin Pathol: Mol Pathol. 1998; 51(6): 327-332.

7. Kannan S, Jagadeesh GC, Raveendran K. Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: Correlation with expression of Ki-67. J Clinpathol Mol Pathol. 1996; 49(3): 170-175.

8. Gonzalez MA, Ruiz I, Rodriguez A, artinez I. Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. Head and Neck. 2000; 22(7): 658-661.

9. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer. 1983; 31 (1): 13–20.

10. Dierendonck J, Rob K, Cornelis J. Nuclear distribution of the Ki-67 Antigen during the Cell Cycle: Comparison with Growth Fraction in Human Breast Cancer Cells. Cancer Res. 1989; 49(11): 2999-3006.

11. Schonk DM, Van Drunen E, Van Dalen CH. Assignment of the gene(s) involved in the expression of the proliferation-related Ki-67 antigen to human chromosome 10. Hum Genet. 1989; 83 (3): 297-9.

12. Simony J, Jean-Louis, Radal M. In situ evaluation of growth fraction determined by monoclonal antibody Ki-67 and ploidy in surgically resected non-small cell lung cancers. Cancer Res. 1990; 50(14): 4382-4387.

13. Takeda T, Sugihara K. Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. J Oral Pathol Med. 2006; 35(6): 369–75.

14. Kumar P, Kane S, Rathod GP. Coexpression of p53 and Ki-67 and lack of c-erbB2 expression in oral leukoplakias in India. Braz Oral Res. 2012; 26(3): 228-34.

15. Humayun S, Prasad VR. Expression Of p53 Protein and Ki-67 Antigen in oral premalignant Lesions and OSCC: An IHC Study. National Journal of Maxillofacial Surgery. 2011; 2(1): 38-46.

16. Salati NA. Diagnostic potential of epithelial biomarkers in oral diseases-immunohistochemical basis. IOSR Journal of Dental and Medical Sciences. 2013; 5(2): 37-40.

17. Shrishail S, Radhika MB. Expression of Ki-67 in normal oral epithelium, leukoplakic oral epithelium and oral squamous cell carcinoma. Journal of Oral and Maxillofacial Pathology. 2014; 18(2): 169-176.