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MALIGNANT POTENTIAL OF DYSPLASTIC LEUKOPLAKIA AS EVALUATED BY KI-67 EXPRESSION: AN IMMUNOHISTOCHEMICAL STUDY WITH REVIEW OF LITERATURE

Oral Pathology

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ABSTRACT

Aim: This study aimed to evaluate the Ki-67 expression in leukoplakia (LPK) according to grades of epithelial dysplasia. **Materials and Methods:** Twenty samples of leukoplakia were selected from the archives of the institution and further divided into three groups: Group 1 (LPK with mild epithelial dysplasia),Group 2 (LPK with moderate epithelial dysplasia) and Group 3 (LPK with severe epithelial dysplasia). The slides stained with Ki-67 antigen were examined under light microscope at different magnifications. **Results:** A statistically significant ($p<0.05$) difference was noted when intensity of Ki-67 staining were compared between LPK with mild dysplasia (L1) and LPK with moderate dysplasia(L2) (p=0.049), L1 and leukoplakia with severe dysplasia(L3) (p=0.007) also between L2 and L3 (p=0.034). A statistically significant difference was noted when distribution of Ki-67 staining was compared between L1 and L2 (p=0.001) as well as between L1 and L3 (p=0.001), when L2 and L3 were compared, the difference was not statistically significant (p=0.74). A statistically significant difference was noted, when percentage of positive cells were compared between L1 and L2 (p=0.003) as well as between L1 and L3 (p=0.003), when L2 and L3 were compared, the difference was not statistically significant (p=0.070). A statistically significant difference was noted, when Ki-67 Labelling Index (LI) were compared between L1 and L2 (p=0.001) as well as between L1 and L3 (p=0.004), when L2 and L3 were compared, the difference was not statistically significant (p=0.079). **Conclusion:** The expression of Ki-67 antigen in LPK correlates well with the disease progression, it is a useful biomarker of malignant transformation in oral leukoplakia.

KEYWORDS

Ki-67, Leukoplakia, Epithelial Dysplasia

INTRODUCTION

It is a well accepted concept that most of the oral squamous cell carcinomas (OSCC) develop step wise, it means at first there is development of one of the potentially malignant disorders (PMD) like leukoplakia which later on with increase in the grades of epithelial dysplasia from mild to severe at last progresses to oral squamous cell carcinoma.[1] In India, oral squamous cell carcinoma represents about 30% malignancies, and five year survival rate after adequate treatment is only 50%.[2,3] It is the oral Leukoplakia which is the most common potentially malignant disorder which progresses to oral cancer, having malignant transformation rate of about 0.13 % to 10 % in India.[4,2] The malignant transformation rate of oral leukoplakia further increases when associated with epithelial dysplasia and it is about 36%.[5] Although Histopathological examination is routinely practiced to rule out the grade of epithelial dysplasia but changes occur first at molecular level which cannot be appreciated under the microscope.[6] Lot of phenotypic changes takes place when normal oral epithelial cells are exposed to carcinogenic agents (eg. Chemicals, radiation). These changes occurs in growth properties, morphology and antigens etc.[7] To detect these specific antigens by antigen antibody interaction, Immunohistochemistry (IHC) is used on formalin fixed and paraffin embedded tissues. A proliferation marker, Ki-67 antigen is absolutely associated with cell proliferation to measure the growth potential of cells in tissues.[8]

Following the above information, survival rates of OSCC can be increased if it is detected at an early stage by differentiating PMD into high risk and low risk categories.[9] The present study was formulated to analyse the Ki-67 expression in leukoplakia (LPK) and its correlation with grades of epithelial dysplasia.

MATERIALS AND METHODS

Twenty samples of optimally formalin fixed and paraffin embedded tissue of leukoplakia were selected from the archives of the institution. Diagnosis of Hyperkeratosis was confirmed by light microscopic examination of H & E stained slides. After confirmation of diagnosis all these samples were grouped into three categories depending upon the grade of epithelial dysplasia. LPK with mild epithelial dysplasia(n=7), LPK with moderate epithelial dysplasia(n=8) and LPK with severe epithelial dysplasia(n=5).

Reagents used-

Primary antibody - Ready to use mouse monoclonal antibody Ki-67 antigen with product code RTU-Ki-67-MMI was used.

Polymer detection kit – The Novolink ™ min. polymer detection kit was used. In this kit all the solutions were ready to use except for the diaminobenzidine (DAB), whose working solution was prepared every time using DAB chromogen and substrate buffer.

Preparation of Solutions-

Antigen retrieval solution: To prepare 0.001M of Ehylene diamine tetra acetic acid (EDTA) buffer solution, 0.37gm of EDTA salt is added to 1000ml of distilled water and pH of 8.0 is achieved by adding sodium hydroxide solution drop by drop.

Phosphate buffer saline (PBS) (pH 7.4): 0.45 gm of sodium dihydrogen phosphate dihydrate, 1.69 gm of disodium hydrogen phosphate dihydrate and 8gm of sodium chloride is added to 1000ml of distilled water.

DAB: 5µl of DAB chromogen with 100 µl of DAB substrate buffer (polymer) in a test tube. This solution was prepared few minutes before its use every time.

Immunohistochemical procedure: After standardizing the procedure, the following immunohistochemical method was adapted.

1. Three micron sections each of 20 samples of leukoplakia were prepared on semi automatic rotary microtome(LEICA RM2245)

and mounted on pre-coated slides.
2. After drying at room temperature, sections were incubated at 60^{0}C for 1 hour
3. The sections were deparaffinised as routine
4. The sections were cleared in xylene and rehydrated through descending grades of alcohol
5. Antigen retrieval procedure was carried out using conventional pressure cooker, for this, equal number of slides were kept in two coplin jars filled with antigen retrieval solution which were placed in pressure cooker 1/4th filled with pre-heated tap water. The pressure cooker was then sealed and heated at 100^{0}C for one hour. Constant temperature was maintained by using temperature adjustable hot plate.
6. For every cycle of antigen retrieval, one section of oral squamous cell carcinoma was used as a positive control and treated similarly as the test sample.
7. After one hour, the cooker was depressurized and cooled under running tap water. The lid was removed and coplin jars taken out from the pressure cooker, the sections were allowed to cool at room temperature in the coplin jar.
8. The cooled sections were washed with PBS for 5 min. the excess was wiped using absorbent wipes. The slides transferred to humidifying chamber for immunohistochemical staining.
9. The sections were treated with peroxidase block for 5 min. followed by PBS wash twice for 5 min. each, the excess was wiped with absorbent wipes.
10. The sections were treated with protein block for 5 min. followed by PBS wash twice for 5 min. each.
11. The sections were incubated for 60 min. at room temperature with primary antibody followed by PBS wash twice for 5 min. A negative control was performed by omitting the step of primary antibody during the staining.
12. The Sections were subjected to post primary for 30 min. and PBS wash twice for 5 min. each.
13. The sections were subjected to Polymer Horse radish peroxidise reagent for 30 min. and PBS wash twice for 5 min. each.
14. The sections were subjected to DAB for 5 min. and washed with distilled water for 5 min.
15. The slides were counterstained with Hematoxylin, washed with water, dehydrated through alcohol and cleared in xylene and mounted with Dibutylpthalate polyesterene xylene (DPX)

During the whole immunohistochemical staining procedure, all the tissue sections were not allowed to dry.

Evaluation of tissue sections-

The tissue sections were observed under light microscope at different magnifications. As Ki-67 is a nuclear marker, brown staining of the nucleus of the epithelial cells was considered to be positive.

Evaluation criteria for Ki-67 antigen –

The evaluation of nuclear staining was done according to the scoring criteria as described by Humayun S et al.[10]

Statistical Analysis-

Statistical analysis of the observed values for Ki-67 expression in leukoplakia were done by Mann Whitney U test. Inter-observer reliability was also tested by Cohen's Kappa test.(Table 1)

RESULTS

The slides were evaluated by the three observers who were blinded for the study and observations were statistically analysed.(Table 2),Fig.1 is showing the Ki-67 expression in well differentiated squamous cell carcinoma which was used as a positive control, while Fig.2, Fig.3 and Fig.4 showing the Ki-67 expression in leukoplakia accorging to the different grades of epithelial dysplasia.

In the present study, a statistically significant ($p<0.05$) difference was noted when intensity of Ki-67 staining were compared between LPK with mild dysplasia(L1) and LPK with moderate dysplasia(L2) (p=0.049), L1 and leukoplakia with severe dysplasia(L3) (p=0.007) also between L2 and L3 (p=0.034).

A statistically significant difference was noted when distribution of Ki-67 staining was compared between L1 and L2 (p=0.001) as well as between L1 and L3 (p=0.001), when L2 and L3 were compared, the difference was not statistically significant (p=0.74)

A statistically significant difference was noted, when percentage of positive cells were compared between L1 and L2 (p=0.003) as well as between L1 and L3 (p=0.003), when L2 and L3 were compared, the difference was not statistically significant (p=0.070)

A statistically significant difference was noted, when Ki-67 LI were compared between L1 and L2 (p=0.001) as well as between L1 and L3 (p=0.004), when L2 and L3 were compared, the difference was not statistically significant (p=0.079)

Table-1: Table showing Inter-observer reliability

IHC Marker	Variable	Cohen's Kappa testvalues
Ki 67	Intensity of staining	0.83
	Distribution o f staining	0.85
	Percentage of positive cells	0.75

Table-2: Immunohistochemical expression of Ki-67 in leukoplakia and its correlation with grades of epithelial dysplasia.

Diagnosis	Intensity of staining				Distribution of staining			Percentage of positive cells				Labelling index (LI)	
	S1	S2	S3	S4	D1	D2	D3	N1	N2	N3	N4	Mean	SD
L1(n=7)	0	5	1	1	7	0	0	5	2	0	0	4.43	3.409
L2(n=8)	0	1	4	3	1	6	1	0	4	3	1	39.00	21.314
L3(n=5)	0	0	0	5	0	2	3	0	0	3	2	63.80	14.043
Correlation	Mann Whitney U-test		p value		Mann Whitney U-test		p value	Mann Whitney U-Test		p value		Mann Whitney U-test	p value
L1VsL2	12.000		0.049		3.500		0.001	4.000		0.003		0.000	0.001
L1VsL3	2.500		0.007		0.000		0.001	0.000		0.003		0.000	0.004
L2VsL3	7.500		0.034		9.500		0.74	8.500		0.070		8.000	0.079

Abbreviations:- L1 = Leukoplakia with mild dysplasia, L2 = Leukoplakia with moderate dysplasia, L3 = Leukoplakia with severe dysplasia; S1 = Negative, S2 = Mild, S3 = Moderate, S4 = Intense; D1 = Basal layer, D2 = Both basal and suprabasal layer, D3 = All layers of the epithelium; N1 = 0 to 5 %, N2 = 6 to 25 %, N3 = 26 to 60 %, N4 = 61 to 99 %; LI = Labelling Index; SD = Standard Deviation; Vs = versus.

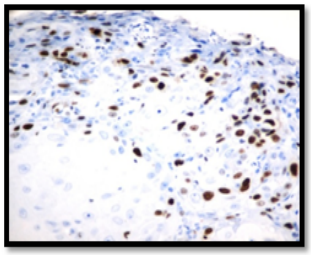

Fig.1: Oral squamous cell carcinoma, Positive control for Ki-67 antigen (40X)

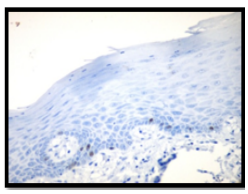

Fig.2: Ki-67 expression in leukoplakia with mild dysplasia (40X)

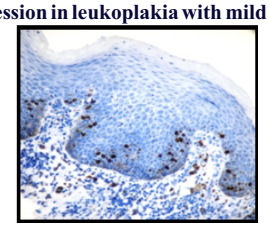

Fig.3: Ki-67 expression in leukoplakia with moderate dysplasia (40X)

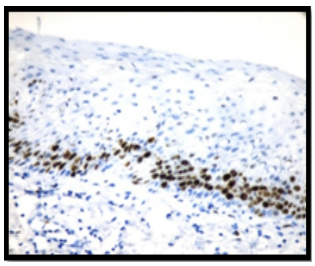

Fig.4: Ki-67 expression in leukoplakia with severe dysplasia (40X)

DISCUSSION

Oral mucosa is made up of stratified squamous epithelium; the stratification is the result of cell proliferation and sequential differentiation.[11] Proliferation is a property of stem cells and their immediate progeny, the transit-amplifying cells of the basal layer of the epithelium. Differentiation starts when recently divided cells detach from the underlying extracellular matrix.[12] As the differentiating cells mature, they are pushed towards the epithelial surface by the pressure generated in the underlying proliferation compartment.[11] Any sign of proliferative cellular activity beyond the basal layer should be considered as a alarming sign.[13]

Oral leukoplakia was first time defined by a World Health Organisation (WHO) in the year 1978 as a white patch or plaque which can not otherwise be characterized clinically or pathologically as any other disease. The etiology for the development of leukoplakia is multifactorial and it mainly includes use of tobacco especially smoking habit, alcohol, and possible role of infection from Human Papilloma Virus.[4]

The term'Dysplasia'was introduced by Reagon in 1958.[14] Dysplastic features are characterized by cellular atypia and loss of normal stratification and maturation.[15] Epithelial dysplasia is considered as a histologic marker of premalignancy and it is predictive of an increased rate of development of squmaous cell carcinoma.[16] According to WHO 1978 classification oral epithelial dysplaia is classified as mild, moderate and severe.[15]

In this study, a statistically significant difference was noted for the expression of Ki-67, in leukoplakia according to the grades of epithelial dysplasia.

According to Kannan S et al.(1996)[17] who found that the expression of Ki-67 antigen increases in OSCC as compared to normal or dysplastic mucosa, in their research they had not found much difference in Ki-67 expression in leukoplakia without epithelial dysplasia and leukoplakia with epithelial dysplasia. The probable reason for this could be, in their research they included only the few samples of frozen sections of leukoplakia with only low grade epithelial dysplasia, while in case of this research we tried to evaluate the Ki-67 expression in leukoplakia according to all the three grades of epithelial dysplasia.

Results of the present study were in accordance with Gonzalez-Moles MA et al. (2000)[13], who observed a statistically significant difference in the expression of Ki-67 according to oral epithelial dysplasia, but in their research they studied the non-neoplastic oral mucosa adjacent to oral squamous cell carcinoma.

The results of the present study were in accordance with Kurokawa H et al.[16] and highlighting the over expression of Ki-67 antigen in LPK with increasing grades of epithelial dysplasia.

Francesca A et al. (2008)[18] observed that the Ki-67 expression is directly associated with the increasing grades of epithelial dysplasia, in their study moderate and severe dysplastic epithelium irrespective of leukoplakia was considered in to one group, but this study includes LPK.

Study of Humayun S et al. (2011)[19] revealed, that expression of ki-67 increases as normal oral mucosa becomes dysplastic and undergoes malignant transformation. But, in their study leukoplakia group was not classified according to grades of epithelial dysplasia, which is unique for this study.

Ranganathan K et al (2011)[20] also observed altered expression of Ki-67 antigen in Oral submucous fibrosis and OSCC as compared to normal mucosa but leukoplakia group was not considered in their research, while LPK is considered in this study.

Dwivedi N et al. (2013)[21] also points towards valuable use of Ki-67 antigen in the early detection of oral premalignant and malignant lesions. Results of this study also pointing towards the same.

Birajdar S S et al.(2014)[22] also suggests that, the expression of Ki-67 antigen is a reliable proliferative marker which can be used for the diagnosis of oral epithelial dysplasia which have tendency to undergo malignant transformation, by conducting their study on normal oral epithelium, potentially malignant disorders including dysplastic epithelium and OSCC. While in this study, we are concentrating only on LPK.

According to the study by Beevi B H et al (2019)[23], the Ki-67 antigen expression is significantly higher in cases of LPK as compared to Oral lichen planus and normal mucosa, while in this study we further evaluated the Ki-67 expression in LPK according to the degree of dysplasia.

According to present study, the increased proliferating cell population in both basal and suprabasal layers of the epithelium suggest that proliferating cells might increase not only in a superficial direction but also downward to the basal layer of the epithelium in epithelial dysplasia.

Although efforts have been made in the present study to reduce the possible errors, certain limitations should be taken into consideration such as, less sample size of only 20 samples also Immunohistochemical staining and cell counting method was manual.

SUMMARY AND CONCLUSION-

Identification of high risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys in reducing the mortality, morbidity and cost of treatment associated with oral squamous cell carcinoma. The significant correlation between progression of oral epithelium from mild dysplasia to severe dysplasia and altered expression of Ki-67 antigen suggest that, it is a useful biomarker of malignant transformation in oral leukoplakia and may serve as intermediate point for cancer prevention programmes. The expression of Ki-67 antigen is probably reliable to grade the potentially malignant disorders into high risk and low risk categories.

Ethical issues –

Not applicable

Conflicts of interest –

There is no conflicts of interest of any author regarding the preparation of the manuscript and publication of paper.

REFERENCES

1. Sadiq H, Gupta P, Singh N, Thakar SS, Prabhakar I, Thakral J. Various Grading Systems of the Oral Epithelial Dysplasia: A Review. Int J Adv Health Sci 2015;1(11):20-26.
2. Mondal K, Mandal R, Sarkar B C. A study of Ki-67 expression and its clinicopathological determinants in nondysplastic oral leukoplakia. Contemp Clin Dent 2016;7:493-9.
3. Sajith Babu Thavarool , Geetha Muttath, Sangeetha Nayanar, Karthickeyan Duraisamy, Prasanth Bhat, Kalpita Shringarpure,Priyakanta Nayak, Jaya Prasad Tripathy, Alfonso Thaddeus, Sairu Philip and Satheesan B. Improved survival among oral cancer patients: findings from a retrospective study at a tertiary care cancer centre in rural Kerala, India. World Journal of Surgical Oncology (2019) 17:15
4. Ioanina Parlatescua; Carmen Gheorghea;Elena Coculescub; Serban Tovarua. Oral Leukoplakia – An Update. A Journal of Clinical Medicine, Volume 9 No.1 2014;88-93.
5. Sol Silverman Jr, Meir Gorsky, And Francina Lozada. Oral Leukoplakia and Malignant Transformation. Reprinted from Cancer, Vol. 53, No. 3,February 1, 1984
6. Mehrotra and Gupta:Exciting new advances in oral cancer diagnosis: avenues to early detection.Head & Neck Oncology 2011.3:33
7. Kumar, Cotran and Robbins. Basic pathology. 5th ed. Prism books Ltd, India.1992:pg184-93.
8. Schlüter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad HD, et al. The cell proliferation- associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J Cell Biol. 1993;123:513–22.
9. Messadi DV. Diagnostic aids for detection of oral precancerous conditions. International Journal of Oral Science 2013;5:59-65.
10. S. Humayun and V. Ram Prasad. Natl J Maxillofac Surg. 2011 Jan-Jun; 2(1): 38–46.
11. Jones PH. Epithelial stem cells. Bioessays 1997;19:683-90
12. Blumenberg M, Tomic-Canic M, Jiang CK, Yang DR, Magnaldo T, Bernerd F, et al. Regulation of keratin gene expression by harmones,vitamins and growth factors. Pharmacol Skin 1993;5:75-82
13. 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.
14. Sharma P, Singh D, Dixit J, Singh M K, Kumar N. Histological grading systems of epithelial dysplasia & squamous cell carcinoma. Journal of Oral Medicine, Oral Surgery, Oral Pathology and Oral Radiology;2015;1(1):7-20

15. Ranganathan K, Kavitha L. Oral epithelial dysplasia: Classifcations and clinical relevance in risk assessment of oral potentially malignant disorders. J Oral Maxillofac Pathol 2019;23:19-27.
16. Kurokawa H, Matsumoto S, Murata T, Yamashita Y, Tomoyose T, Zhang M, Fukuyama H, Takahashi T. Immunohistochemical study of syndecan -1 down regulation and the expression of p53 protein or Ki-67 antigen in oral leukoplakia with or without epithelial dysplasia. J Oral Pathol Med (2003) 32: 513-21
17. S Kannan, G Jagadeesh Chandran, K Raveendran Pillai, Babu Mathew, K Sujathan, K R Nalinakumary, M Krishnan Nair. Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: correlation with expression of Ki67. J Clin Pathol:Mol Pathol.1996;49:M170-M175
18. Francesca Angiero, Angiola Berenzi, Anna Benetti, Elisa Rossi, Rachele Del Sordo, Angelo Sidoni, Michele Stefani and Enrico Dessy. Expression of P16, P53 and Ki-67 Proteins in the Progression of Epithelial Dysplasia of the Oral Cavity. Anticancer Research *28*: 2535-2540 (2008)
19. Humayun S and Prasad R V. Expression of p53 protein and ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas: An immunohistochemical study. Natl J Maxillofac Surg. 2011 Jan-Jun; 2(1): 38–46.
20. Ranganathan K and Kavitha R. Proliferation and apoptosis markers in oral submucous fibrosis. Oral Maxillofac Pathol. 2011 May-Aug; 15(2): 148–153.
21. Nidhi Dwivedi, Shaleen Chandra, Bina Kashyap, Vineet Raj, and Akhil Agarwal. Suprabasal expression of Ki-67 as a marker for the severity of oral epithelial dysplasia and oral squamous cell carcinoma. Contemp Clin Dent. 2013 Jan-Mar; 4(1): 7–12.
22. Birajdar S S, Radhika M B, Paremala K, Sudhakara M, Soumya M, Gadivan 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.
23. Beevi B H, Nayak SR, Peter C D, Haridas A K, Jacob L, Aboobakker A. Analysis of Ki-67 expression in oral premalignant lesions and normal oral mucosa: An immunohistochemical study. J Pharm Bioall Sci 2019;11:S232-5.