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Evaluation of Ki-67 Expression in Oral Leukoplakia and Oral Squamous Cell Carcinoma: An Immunohistochemical Study

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ABSTRACT

Background: Oral cancer is a significant disease worldwide with up to 400000 new cases every year and almost 130,000 deaths annually. Oral leukoplakia is one of the most common precancerous lesions. Molecular pathogenesis is indeed our savior as it can be used for early diagnosis and prognosis. Cell proliferation is a fundamental process involved throughout life. It is hypothesized that in cancer, cell proliferation continues uncontrollably and may be the cause of tumorogenesis.

Aim: In the present study, Ki-67 (proliferative marker) expression was compared between lesions of OL with those of Oral squamous cell carcinoma (OSCC) and with that of normal Oral Mucosa. Grades of epithelial dysplasia and OSCC were also evaluated.

Material & Method: This retrospective study was done in Government Dental College & Hospital, Mumbai. The study comprised of 90 samples in total. Out of these, 48 were Oral Leukoplakia (OL) samples, 33 were Oral Squamous Cell Carcinoma (OSCC). 9 normal mucosal samples were taken. Tumor cells were considered positive for Ki-67 antigen if there was intranuclear light brown granular staining.

Results: The comparisons showed overexpression with increasing grades of Oral Epithelial Dysplasia (OED) and with those of OSCC. These results were statistically significant (p value<0.05).

Conclusion: We conclude that Ki-67 is a reliable marker to predict future outcome of OED and OSCC. It can be used routinely to monitor its progression and treatment modalities.

Keywords: Ki-67, epithelial dysplasia, carcinoma, leukoplakia.

INTRODUCTION

Head and neck cancers pose a serious threat to the society. The problem is further amplified by the fact that number of new cases goes up every year [1,2]. Oral cancer is a significant disease worldwide with up to 400000 new cases every year and almost 130,000 deaths annually (Ferlay et al., 2010). Incidence rates are much higher in developing regions like Southeast Asia, where they account for up to 50 % of all malignant tumors (Argawal et al., 2012; Siegel et al., 2012; Ghani et al., 2013; Krishna Rao et al., 2013)[3]. Malignancies of head and neck are notoriously known for their varied biologic

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behavior[4]. Tobacco, alcohol, environmental carcinogens are the risk factors associated to oral cancers [5,6,7,8]. While most oral pathologists are not prepared to become full time anti-tobacco crusaders, they can be extremely effective agents for change as they have the unique opportunity to examine their patients in the symptomless phase. It is well known now that if oral cancers are diagnosed at earlier stages, it can be easy to treat. However, ignorance of its seriousness leads many patients to delay seeking medical advice and the disease progresses rapidly. Oral leukoplakia (OL) is one of the most common precancerous lesions [9,10]. Leukoplakia is defined as "A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer"[11]. Diagnosis, treatment and prognosis of Oral cancer revolves round the newer techniques developed. The science of molecular biology has lead the investigators to think on the lines of molecular pathogenesis of cancer along with the introduction of tumor markers. Tumor markers are substances that are produced by cancer or by other cells of the body in response to cancer or certain benign (noncancerous) conditions. Most tumor markers are made by normal cells as well as by cancer cells; however, they are produced at much higher levels in cancerous conditions. These substances can be found in the blood, urine, stool, tumor tissue, or other tissues or bodily fluids of some patients with cancer [12]. Cell proliferation is a fundamental process involved throughout life [13]. It is hypothesized that in cancer, cell proliferation continues uncontrollably and may be the cause of tumorogenesis [14]. Proliferative marker Ki-67 protein is present in all active cell phases (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)). This makes it an excellent marker for growth fraction of given cell population [15]. In the present study, Ki-67 expression was compared between lesions of OL with those of Oral squamous cell carcinoma (OSCC) and with that of normal Oral Mucosa (OM). The Ki-67 expression was quantified and was observed in histological grades of OSCC. Immunohistochemical expression of Ki-67 was studied using a biotin streptavidin method on routinely processed paraffin sections.

MATERIALS & METHODS

This retrospective study was done in Government Dental College & Hospital, Mumbai. The study comprised of 90 samples in total. Out of these, 48 were Oral Leukoplakia (OL) samples, 33 were Oral Squamous Cell Carcinoma (OSCC). 9 normal mucosal samples were taken. The normal samples were obtained from patients undergoing third molar extractions, they were informed and their approval was sought. The study had the proper approvals of the authorities. The age of patients ranged from 25 to 65 years. All the patients of whose blocks were selected had history of consuming tobacco in either smoked or smokeless forms. Formalin fixed paraffin embedded blocks (FFPE) were sectioned at 4 micron thickness. 2 sets of slides were made, 1 for Hematoxylin & Eosin (H&E) and the other for Ki-67. Gelatin chrome coated slides were used for proper adherence of tissue sections. Deparaffinisation and rehydration were routinely carried out. The slides were stained with H&E. After this slides were stained for immunohistochemical evaluation. Staining protocol included incubation in 0.6% methanol Hydrogen peroxide for 15-20 minutes to block endogenous peroxidase. This was followed by rinsing with distilled water and tris buffer wash (pH 7.2-7.6). The slides were inserted in a petridish containing citrate buffer and placed in a microwave oven at 700 watts for 10 minutes. The petridish was allowed to cool to room temperature for 20 minutes and then the slides were taken out and washed with tris buffer. After washing excess buffer, sections were covered with optimally diluted primary antibody and incubated at room temperature for 1 hour. After washing in wash buffer, excess was removed and this was followed by treatment with biotinylated rabbit anti mouse IgG and incubated for 30 minutes. Again the slides were washed with buffer. Enough drops of streptavidin peroxidase were applied to cover the slides and were incubated for 45 minutes. Enough of freshly prepared substrate chromogen solution was applied to cover the sections and incubated at room temperature for 5-6 minutes. Finally, the slides were counterstained with Harri's hematoxylin. The sections were dehydrated, cleared and mounted in DPX (Distrene Debutyl-Pthalate Xylene).

Interpretation:

Histopathological diagnosis was confirmed in H&E stained sections. This was followed by analyzing immunohistochemically stained sections for Ki-67 at low power (10X). The selected fields were used for the quantitative assessment of Ki-67 positive cells at high power. Tumor cells were considered positive for Ki-67 antigen if there was intranuclear light brown granular staining. Expression of Ki-67 less than 10% were considered negative.

Table 1: Profile of sex distribution of the samples

Sex	Leukoplakia		Squamous cell carcinoma	
	No. of patients	Percentage (%)	No. of patients	Percentage (%)
Male	45	93.75	30	90.90
Female	3	6.25	3	9.10
n	48	100	33	100

Table 2: Distribution of cases according to Subtypes of OL and OSCC.

Group	Lesions	Histopathological grading	No. of samples
I	Oral Leukoplakia	Mild epithelial dysplasia	20
II		Moderate epithelial dysplasia	15
III		Severe epithelial dysplasia	13
IV	Oral Squamous cell carcinoma	Well differentiated squamous cell carcinoma	20
V		Moderately & Poorly differentiated squamous cell carcinoma	13
VI		Normal mucosa	9

Table 3: Comparison of Grades of OED with normal mucosa.

Control group n=9 **[Group VI]**	Mild epithelial dysplasia n=20 **[Group I]**	Moderate epithelial dysplasia n=15 **[Group II]**	Severe epithelial dysplasia n= 13 **[Group III]**	F Value	*p* value
3.47±23.36	35.7±7.23	39.35±5.68	42.36±4.57	**28.382**	**0.001**

Table 4: Comparison of Grades of OSCC with normal mucosa

Control group n=9 **[Group VI]**	Well Differentiated n=20 **[Group IV]**	Moderately & Poorly Differentiated n=13 **[Group V]**	F Value	*p* value
3.47±23.36	69.59±23.56	89.35±19.35	**41.904**	**0.001**

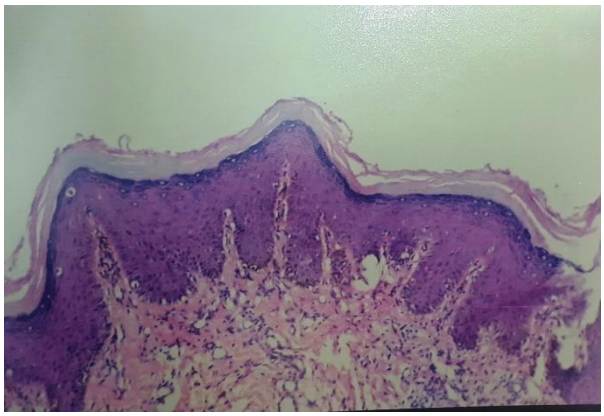

Fig 1: Mild epithelial dysplasia (H&E 200X).

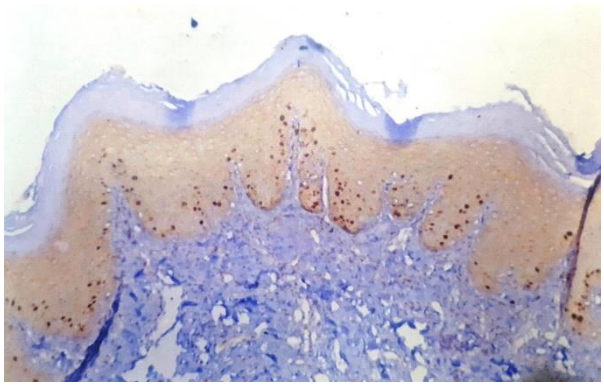

Fig 2: Mild epithelial dysplasia (Ki-67 expression 200X)

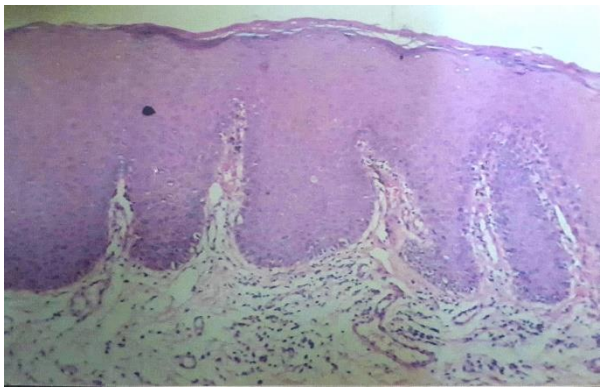

Fig 3: Moderate epithelial dysplasia (H&E 200X).

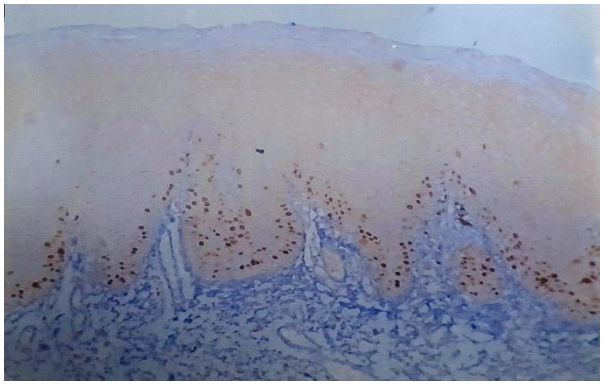

Fig 4: Moderate epithelial dysplasia (Ki-67 expression 200X).

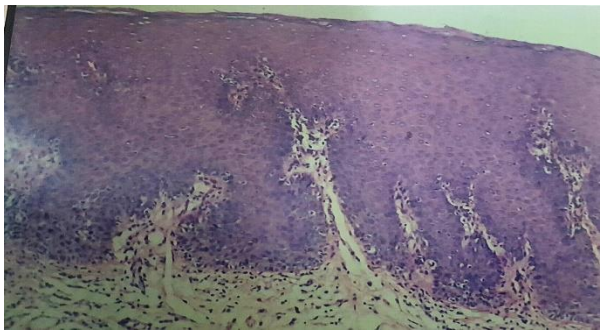

Fig 5: Severe epithelial dysplasia (H&E 200X).

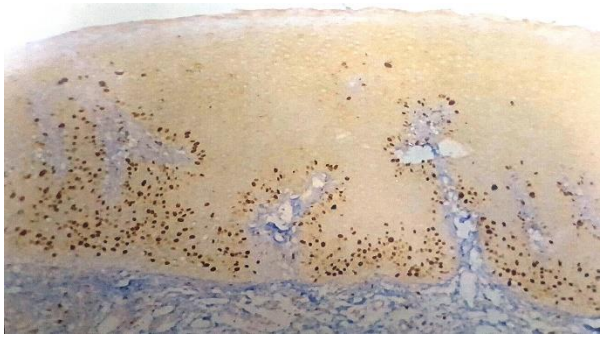

Fig 6: Severe epithelial dysplasia (Ki-67 expression 200X).

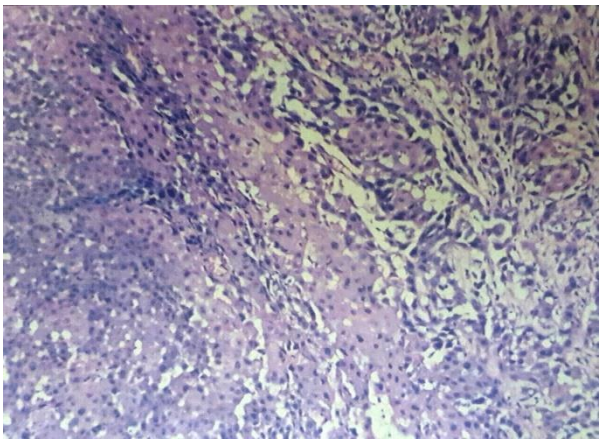

Fig 7: Poorly Differentiated squamous cell carcinoma (H&E 400X).

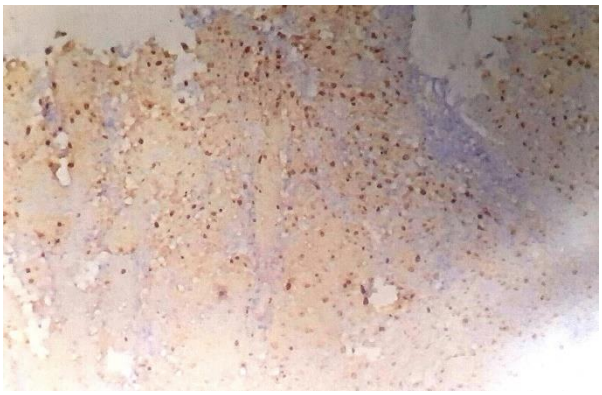

Fig 8: Poorly Differentiated squamous cell carcinoma (Ki-67 expression 400X)

RESULTS

This retrospective study carried out in Government Dental College & Hospital, Mumbai was aimed at correlating the ki-67 expression with grades of OSCC and OL (epithelial dysplasia). In a total of 48 OL, 45 were males and only 3 were females and out of 33 OSCC, 30 were males and 3 were females [Table1]. Out of 48 OL samples, 20 were mild dysplasia (Group I), 15 moderate dysplasia(Group II) and 13 severe dysplasia (Group III). Similarly, out of 33 OSCC samples, 20 were well differentiated (Group IV), 9 were moderately differentiated and 4 were poorly differentiated. Since poorly differentiated were very few they were clubbed with moderately differentiated and comprised of Group V. 9 samples from normal patients formed Group VI [Table 2]. The evaluation of the slides was done by scanning the immunohistochemically stained slides and 1000 tumor cells were observed and counted in five different histological fields using

a magnification of 40X. An eyepiece grid was used to prevent the overlapping of fields. Comparisons were made between Group I, II and III each with Group VI. Similarly, Group IV and V were compared with Group VI. Group I, II, III were compared with Group IV and V. The indices were calculated as the percentage of positively stained cells among the total number of cells. The results were tabulated and subjected to statistical analysis including Student's t-test, Chi-square test and Mann-Whitney U-test. The comparisons showed overexpression with increasing grades of Oral Epithelial Dysplasia (OED) and with those of OSCC. Comparisons of Ki-67 positive cells of Group I [Fig 1 & Fig 2], II [Fig 3 & Fig 4] and III [Fig 5 & Fig 6] showed increase in the mean value with increasing grades [Table 3]. These results were statistically significant (p value<0.05). Similarly, comparisons amongst Group IV and Group V [Fig 7 & Fig 8] too showed increase in the mean value of Ki-67 positive cells [Table 4]. Again the results were statistically significant (p value<0.05). For convenience and statistical purpose Group I, II and III were commonly grouped under Group X (denoting OED in total) whereas Group IV and V were commonly grouped under Group Y (denoting OSCC cases in total). Finally a comparison was done between Group X and Group Y which showed increased value in Group Y in comparison to Group X. These results were significant (p value<0.05).

DISCUSSION

The ability to predict reliably cancer outcome could substantially affect the course and biologic behavior of the lesion in order to achieve the best results in terms of loco-regional control, overall survival and quality of life. It is a known fact that OLs showing histologically moderate to severe degree of epithelial dysplasia are considered to have greater disposition for malignant transformation [16,17,18]. Routinely dysplasia is diagnosed by H&E staining of paraffin embedded tissue sections. However, its limitations in the assessment of epithelial changes of the oral mucosa have been recognized and represent an important constraint on the behavior of suspicious lesions. With the recent advancements in the field of molecular biology several markers of cellular proliferation such as PCNA (Proliferating cell nuclear antigen) and Ki-67 have been investigated in many diseased states [19]. Epithelial dysplasia is characterized by the number of cellular and tissue alterations that reveal an alteration of the cell maturation in the epithelium and an increase of the proliferative suprabasal activity. Thus changes in this proliferative capacity of Potentially Malignant Disorders (PMDs) may reveal the early preneoplastic changes and yield information about the risks of malignant transformation. Cell proliferation markers can be categorized as follows; (A) Growth fraction markers, (B) Markers of specific cell cycle phases and (C) Cell cycle time markers [20]. Ki-67 is the marker for growth fraction. The intensity of staining and the number of positive cells following Ki-67 demonstration tends to be lower than PCNA, reason being that Ki-67 is rapidly degraded following the M phase of mitosis whilst PCNA accumulate in the cells. The monoclonal antibody detects a nuclear antigen that is present in proliferating cells. Ki-67 is present in all phases of growth cycle except G0 phase. This may be because Ki-67 reacts with a nuclear non-histone protein which is present in all active phases of growth cycle and thus is considered as one of the most reliable marker for cellular proliferation [21]. This antigen is present in the nucleoli in G1followed by nucleoplasmic distribution in later cycle with intensity increasing in S and G2 and being maximum during mitosis [22].

The credit of introducing Ki-67 as a potential marker for proliferating cells goes to Johannes Gerdes et al in 1983. The word Ki is derived from the University of Kiel, where Johannes Gerdes in the laboratory of Herald Stein generated antibody. It was the 67th well of a 96-well microtitre plate and hence it was designated as Ki-67. After cloning and sequencing the gene, new antibodies were generated and they were named after the division at (M)olecular (I)mmunology (B)orstel [23]. Ki-67 gene is located on human chromosome 10 (10q25) [24].

We Examined 48 cases of OL and 33 cases of OSCC and 9 cases of normal oral mucosa. Out of these, 90 % (n=81) were smokers. Affected males were 93.75% in OL and 90.90% in OSCC.

In the present study, monoclonal antibody Mib-1 was used on paraffin embedded tissue sections to quantify the Ki-67 expression in the epithelium of

OL and OSCC. Comparisons were made among grades of OED and grades of OSCC. OED and OSCC were also compared amongst each other. It was noticed that in the normal mucosal epithelium, the Ki-67 postivity was minimal and was restricted to basal and very little to the parabasal layer. As the grades on OED increased i.e. from mild to moderate to severe, the positivity was more pronounced in the basal and parabasal cell layer. In cases of severe and some moderate OED, the positivity even extended in the spinous cell layer. In the OSCC cases, the peripeheral cells of the tumor islands showed more positivity compared to the inner cells. These results were very similar to other studies [14,25,26]. Comparisons within OED showed increased positivity of Ki-67 cells with significant results. Also OSCC compared with OED too showed higher positivity. Motta Rda R concluded that Ki-67 overexpression is directly proportional to metastasis to head and neck lymph nodes [20]. Similar results were also seen in various other studies [27,28]. There have been contradictory results regarding prognosis of OSCC and Ki-67 overexpression. In a meta-analysis performed by Xie S et al, they reviewed past published papers and found conflicting results. To conclude, they believed that over-expression of Ki-67 in OSCC relates to poor prognosis although suitable treatment timely provided could improve the prognosis [29]. They also stressed on an important point, that the results mentioned in many papers are based on a small sample size. Hence, the results are disputing.

Gonzalez-Moles MA found that ki-67 was over expressed in well differentiated compared to poorly differentiated OSCC and hence were of the opinion that Ki-67 falls short of being a reliable prognostic marker [30]. Again as pointed out by Xie S et al, it may be due to the fact that the sample size may be less.

Ki-67 is a very important and reliable tool for diagnosis and prognosis and has various advantages like simplicity and interpretation of results. Pharmacological modulators of cell proliferation and differentiation have been postulated as the future of cancer preventive drugs. Some of these agents like dimethylfluoronithine and retinoids have already been used in clinical trials of OL. Thus the use of Ki-67 could be of great value in montoring the effects of these agents during the course of future chemoprevention trials.

CONCLUSION

We conclude that Ki-67 is a reliable marker to predict future outcome of OED and OSCC. It can be used routinely to monitor its progression and treatment modalities.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

REFERENCES

1. Devi S, Singh N. Dental care during and after radiotherapy in head and neck cancer. Natl J Maxillofac Surg. 2014 Jul-Dec;5(2):117-25.

2. Bhateja S, Arora G. ABO blood groups and oral premalignancies: A clinical study in selected Indian population. Indian J Cancer. 2014 July-September;51(3):219-221.

3. Al-Maweri SA, Tarakji B, Alsalhani AB, Al-Shamiri HM, Alaizari NA, Altamimi MA, Darwish S. Oral cancer awareness of the general public in saudi arabia. Asian Pac J Cancer Prev. 2015;16(8):3377-81.

4. Fan CY. Epigenetic alterations in head and neck cancer: prevalence, clinical significance, and implications. Curr Oncol Rep. 2004 Mar;6(2):152-61.

5. Petersen PE, Oral cancer prevention and control – The approach of the World ..., Oral Oncol (2008), doi:10.1016/j.oraloncology.2008.05.023

6. Shiu MN, Chen TH, Chang SH, Hahn LJ. Risk factors for leukoplakia and malignant transformation to oral carcinoma: a leukoplakia cohort in Taiwan. Br J Cancer. 2000 Jun;82(11):1871-4.

7. Thomas SJ, Harris R, Ness AR, Taulo J, Maclennan R, Howes N, Bain CJ. Betel quid not containing tobacco and oral leukoplakia: a report on a cross-sectional study in Papua New Guinea and a meta-analysis of current evidence. Int J Cancer. 2008 Oct 15;123(8):1871-6.

8. Lee CH, Ko YC, Huang HL, Chao YY, Tsai CC, Shieh TY, *et al*. The precancer risk of betel quid chewing, tobacco use and alcohol consumption in oral leukoplakia and oral submucous fibrosis in southern Taiwan. Br J Cancer 2003;88:366-72.

9. van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol. 2009;45:317–323.

10. Thomas G, Hashibe M, Jacob BJ, Ramadas K, Mathew B, Sankaranarayanan R, Zhang ZF. Risk factors for multiple oral premalignant lesions. Int J Cancer. 2003;107:285–291.

11. Yardimci G, Kutlubay Z, Engin B, Tuzun Y. Precancerous lesions of oral mucosa.World J Clin Cases. 2014 Dec 16;2(12):866-72.

12. National Cancer Institute. http://www.cancer.gov/cancertopics/diagnosis-staging/diagnosis/tumor-markers-fact-sheet (accessed 10 May 2015).

13. Verma R, Singh A, Jaiswal R, Chandra A, Verma R, Tak J. Association of Ki-67 antigen and p53 protein at invasive tumor front of oral squamous cell carcinoma. Indian J Pathol Microbiol. 2014 Oct-Dec;57(4):553-7.

14. Birajdar SS, Radhika M, 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 May;18(2):169-76.

15. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. Cell Physiol. 2000 Mar;182(3):311-22.

16. Khan Z, Khan S, Christianson L, Rehman S, Ekwunife O, Samkange-Zeeb F. Smokeless tobacco and oral potentially malignant disorders in South Asia: a protocol for a systematic review. Syst Rev. 2016 Aug 24;5(1):142.

17. Ramsridhar S, Narasimhan M. Immunohistochemical Evaluation of Mast Cells in Leukoplakia and Oral Squamous Cell Carcinoma. J Clin Diagn Res. 2016 Aug;10(8):ZC100-3. doi:

18. Maia HC, Pinto NA, Pereira Jdos S, de Medeiros AM, da Silveira ÉJ, Miguel MC. Potentially malignant oral lesions: clinicopathological correlations. Einstein (Sao Paulo). 2016 Jan-Mar;14(1):35-40.

19. 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 Oct;32(9):513-21.

20. Motta Rda R, Zettler CG, Cambruzzi E, Jotz GP, Berni RB. Ki-67 and p53 correlation prognostic value in squamous cell carcinomas of the oral cavity and tongue. Braz J Otorhinolaryngol. 2009 Jul-Aug;75(4):544-9.

21. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol. 1984 Oct;133(4):1710-5.

22. Maheshwari V, Sharma SC, Narula V, Verma S, Jain A, Alam K. Prognostic and Predictive Impact of Ki-67 in Premalignant and Malignant Squamous Cell Lesions of Oral cavity. International Journal of Head and Neck Surgery. 2013;4(2):61-5.

23. 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 Jan; 31: 13–20.

24. Fonatsch C, Duchrow M, Rieder H, Schlüter C, Gerdes J. Assignment of the human Ki-67 gene (MK167) to 10q25-qter. Genomics. 1991 Oct;11(2):476-7.

25. Sinanoglu A, Soluk-Tekkesin M, Olgac V. Cyclooxygenase-2 and Ki67 Expression in Oral Leukoplakia: a Clinicopathological Study. J Oral Maxillofac Res. 2015 Jun 30;6(2):e3.

26. Humayun S, Prasad VR. 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;2(1):38-46.

27. Iamaroon A, Khemaleelakul U, Pongsiriwet S, Pintong J. Co-expression of p53 and Ki67 and lack EBV expression in oral squamous cell carcinoma. J Oral Pathol Med. 2004;33(1):30-6.

28. Lavertu P, Aldestein DJ, Myles J, Secic M. P53 and Ki-67 as outcome predictors for advanced squamous cell cancers of the head and neck treated with chemoradiotherapy. Laryngoscope. 2001;111(11 Pt 1):1878-92.

29. Xie S, Liu Y, Qiao X, Hua RX, Wang K, Shan XF, Cai ZG. What is the Prognostic Significance of Ki-67 Positivity in Oral Squamous Cell Carcinoma? J Cancer. 2016 Apr 10;7(7):758-67.

30. Gonzalez-Moles MA, Ruiz-Avila I, Gil-Montoya JA, Esteban F, Bravo M. Analysis of Ki-67 expression in oral squamous cell carcinoma: why Ki-67 is not a prognostic indicator. Oral Oncol. 2010 Jul;46(7):525-30.