Evaluation of Ki-67 Expression in Oral Submucous Fibrosis and Its Correlation with Clinical and Histopathological Features

Asif Iqbal, Sandhya Tamgadge, Avinash Tamgadge, Treville Pereira, Sourab Kumar, Siddarth Acharya¹, Abhishek Jadhav

Department of Oral and Maxillofacial Pathology and Microbiology, D. Y. Patil Deemed to be University School of Dentistry, ¹Department of Public Health Dentistry, D. Y. Patil University School of Dentistry, Nerul, Navi Mumbai, Maharashtra, India

Abstract

Objectives: Oral submucous fibrosis (OSMF) is a potentially malignant disorder. Although it shows atrophic epithelium, it has a high proliferative capacity. Therefore, this study correlates the Ki-67. (The name "ki" is derived from the city of origin [Kiel, Germany] expression with functional grading and epithelial thickness in OSMF). **Methods:** The study group comprised of thirty patients of OSMF, divided randomly into Group A, Group B, Group C, and Group D as per mouth opening (functional staging). Five participants without OSMF formed the negative control group. The positive control group comprised of five patients of diagnosed cases of squamous cell carcinoma. All the sections of biopsy were subjected for hematoxylin and eosin and immunohistochemistry staining and observed for expression of Ki-67. Epithelial thickness was evaluated using image analysis software of Leica research microscope. Images were analyzed by three independent observers who were blindfolded. All the findings were tabulated and statistically analyzed. **Results:** In the present study, as the functional staging increased, the Ki-67 expression also increased. Ki-67 expression was highest in severe functional staging/severely decreased mouth opening (100.78) and is least in mild functional staging/mild decreased mouth opening (10.39). However, there was no significant correlation between epithelial thickness and functional staging/mouth opening ($P > 0.05$). **Conclusion:** A decrease in functional staging (mouth opening) showed a greater expression of Ki-67, and there was no significant correlation between functional staging and epithelial thickness.

Keywords: Correlation, epithelial thickness, functional grading, Ki-67, oral submucous fibrosis

INTRODUCTION

Oral lesions which are caused by various forms of tobacco habits include oral leukoplakia, oral submucous fibrosis (OSMF), and squamous cell carcinoma. OSMF is a potentially malignant disorder which has risen rapidly in India reaching the count more than two millions in the last decade. The reported rate of malignant transformation in OSMF ranges from 3% to 19%.[1] OSMF shows atrophic epithelium but has a high rate of proliferation which cannot be detected under hematoxylin and eosin (H and E) by assessing dysplasia. Assessment of malignant potential of OSMF might be improved by quantitative evaluation of more specific diagnostic biomarkers, which could help to grade its aggressiveness.[2]

Extensive data for molecular markers such as p53, proliferation-associated antigens, cytokeratin, B-cell lymphoma 2 (BCL2) group of protein exist for leukoplakia, but data

for OSMF are limited, especially in India. One of the important hallmarks of neoplastic transformation is the uncontrolled growth rate, commonly reflected as increased cell proliferation which can be significantly detected as high Ki-67 index.[3]

Thus, this study evaluated and correlated Ki-67 expression with that of clinical staging and thickness of epithelium in different grades of OSMF.[4]

Ki-67 has been shown to be excellent for the estimation of the growth fraction in both normal and malignant human tissues, and this antibody is now used as the standard for the assessment

Address for correspondence: Dr. Sandhya Tamgadge,
Department of Oral and Maxillofacial Pathology and Microbiology,
D. Y. Patil Deemed to be University School of Dentistry, Sector 7, Nerul,
Navi Mumbai - 400 706, Maharashtra, India.
E-mail: sandhya.tamgadge@gmail.com

Access this article online

Quick Response Code:

Website:
http://www.jmau.org/

DOI:
10.4103/JMAU.JMAU_28_19

of cell proliferation as it does not get affected by internal and external factors. Due to its nuclear expression during a defined period of the cell cycle, it is considered as the most suitable biological marker of mitotic activity.[5] Ki-67 marker is reliable and widely used. It recognizes a proliferation-related nuclear antigen present at all phases of cell cycle except G_0.[3] Furthermore, it has a much shorter half-life, thus producing less residual staining after cells have gone through proliferative stage.[6,7]

The thickness of the epithelium in OSMF also varies with different functional grading.[8]

Therefore, this study tried to establish a possible correlation between Ki-67 expression with the thickness of epithelium and functional grading as there are very few such studies reported in the literature.

METHODS

This prospective study involved the use of paraffin-embedded tissues of previously histopathologically diagnosed cases of OSMF and oral squamous cell carcinoma (OSCC) between 1992 and 2015, retrieved from the archives of Department of Oral and Maxillofacial Pathology, Dr. D. Y. Patil Dental College, Nerul, Navi Mumbai.

Institutional ethical approval was not required from the ethical board as patients' identity has not been disclosed in the manuscript. Written informed consent was taken from patients.

This study was conducted from June 2014 to December 2015 at the Department of Oral and Maxillofacial Pathology, D. Y. Patil University School of Dentistry, Nerul, Navi Mumbai, and involved thirty patients of OSMF were randomly selected as the study group.

The control group comprised of 5 squamous cell carcinoma (positive control) patients. Five participants without OSMF, undergoing minor surgical procedures such as impaction and periodontal surgery, formed the negative control group. Demographic details were recorded for all the patients. Functional staging (mouth opening) was recorded for each patient of the study group, using a Vernier caliper as per More *et al.* (2012)[9] by recording interincisal opening to categorize patients in four groups.
- Group A: Mouth opening >35 mm
- Group B: Mouth opening between 25 and 35 mm
- Group C: Mouth opening between 15 and 25 mm
- Group D: Mouth opening <15 mm.

All the 30 patients were subjected to incisional biopsy, and the specimens were routinely fixed in 10% neutral-buffered formalin (24–48 h). Clinical diagnosis was confirmed with H and E staining.

In this study, patients were categorized with mouth opening >35 mm as normal, mouth opening between 25 and 35 mm as mild functional staging, mouth opening between 15 and 25 mm as moderate functional staging, and mouth opening <15 mm as severe functional staging.[9]

Immunohistochemical staining technique was based on the labeled Streptavidin–Biotin, method.[10]

Interpretation of staining

All images were clicked under oil immersion ×10 and ×40, and epithelial thickness was measured using image analysis software version of Leica research microscope (Model No. DM1000 LED) Ernst-Leitz Microsystems (CMS GmbH Ernst Street, 17-37, 35578 Wetzlar, Germany). The parameter used in this study was the intensity of immunohistochemical staining based on the subjective evaluation of color exhibited (brown color) by antigen, antibody, and chromogen complex as negative (−, no color), and light brown-to-dark brown color is considered as positive staining. The distribution of staining was graded in all layers of the epithelium. Only nuclear staining of epithelial cells was observed, and the nuclei with clear brown color, regardless of staining intensity, were regarded as positive.

Five different fields of immunohistochemistry-stained slide were viewed by three different pathologists who were blindfolded, and the number of stained nuclei was counted manually; the mean was calculated and tabulated using different statistical tools.[2]

The epithelial thickness was also measured in H and E staining in three different areas, and the mean was calculated using image analysis software (Leica Application Suite, LES core version 3.8) and Leica research microscope at ×10 (Model No. DM1000 LED) which was correlated with Ki-67 expression in various grades of functional staging.

Findings were observed and tabulated and analyzed. Further analysis was done using Pearson's correlation. The level of significance was set at 5%. All *P* < 0.05 were treated as significant. IBM SPSS 20.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). software was used for analysis.

In the study term "functional staging and mouth opening" have been used. One should consider severe functional staging as severely decreased mouth opening, mild functional staging as a mild decrease in mouth opening and moderate functional staging is considered as in between mild and severe functional staging/mouth opening.

RESULTS

Descriptive statistics for number of Ki-67-stained nuclei according to various grades of functional staging in OSMF was done by three observers and analyzed using Pearson's correlation. There was an increase in Ki-67 expression with increasing functional grades of OSMF. In the present study, (*P* < 0.01) was < 0.001, hence the study was statistically significant [Table 1].

Descriptive statistics for number of stained nuclei in various grades of functional staging in OSMF was also analyzed by different observers [Table 2].

Epithelial thickness according to various grades of functional staging in oral submucous fibrosis

Epithelial atrophy is said to be one of the significant features in OSMF. However, the present study did not show a consistent decrease in epithelial thickness with increasing functional grades of the disease [Figures 1 and 2]. This result indicates that there was no significant difference in the epithelial thickness according to functional staging/mouth opening ($P > 0.05$). Thus, there was no statistical correlation between various grades of functional staging/mouth opening and epithelial thickness.

DISCUSSION

Abnormal cell proliferation is a predictor of tumorigenesis and malignant transformation of potentially malignant lesions.[11]

OSMF is predominantly a connective tissue disorder but transforms into epithelial malignancy. What makes it more sinister is the malignant transformation rate, which has been reported to be around 7.6% over a 17-year period.[12]

Although extensive data for molecular markers such as p53, proliferation-associated antigens, cytokeratin, and BCL2 group of protein exist for other premalignant lesions such as

leukoplakia, data for OSMF are limited, especially in India. The epithelial changes in OSMF have been studied from the past five decades, and several hypotheses have been suggested for the role for its malignant transformation. It is suggested that the expression of Ki-67 increases with proliferative activity which is also observed in atrophic epithelium of OSMF. The rate of proliferation and the rate of recurrences have been widely studied using Ki-67 antigen in various oral pathological conditions such as leukoplakia, OSMF, squamous cell carcinoma, and other pathological tumors such as breast carcinomas, colorectal carcinomas, osteosarcomas, astrocytomas of the brain, and neoplasms of the thyroid. Few studies have also been documented in the literature where the authors have used Ki-67 to study odontogenic cysts such as dentigerous cyst, radicular cyst, and odontogenic keratocyst and odontogenic tumors such as adenomatoid odontogenic tumors and ameloblastoma.[13-15]

The available literature on the expression of Ki-67 in OSMF is sparse. Thus, this study aims to evaluate the Ki-67 expression and to correlate it with thickness of epithelium and functional staging, which has not been correlated so far.[1,16-20]

In this study, epithelial changes have been emphasized more than that of connective tissue changes.

Even though epithelium in OSMF is atrophic, it shows marked cellular proliferation and leads to malignant transformation. Therefore, this study is prominently focused on epithelial changes to evaluate neoplastic alterations.

In a recent study, it was observed that as the nuclear proliferation increased, the functional stage also increased.

Epithelial thickness decreases with increasing functional stage of OSMF.[18] Thus, epithelial atrophy is said to be one of the key features in OSMF. Therefore, there is a need of an hour

Table 1: Descriptive statistics for number of Ki-67-stained nuclei according to various grades of functional staging and thickness of epithelium in oral submucous fibrosis using Pearson's correlation

	Functional stage	Stained nuclei	Thickness of epithelium
Functional stage			
Pearson's correlation	1	0.612**	0.086
Significant (two-tailed) *P*		0.000	0.653
n	30	30	30
Dysplasia			
Pearson's correlation	0.587**	0.546**	−0.177
Significant (two-tailed) *P*	0.001	0.002	0.349
n	30	30	30
Stained nuclei			
Pearson's correlation	0.612**	1	−0.077
Significant (two-tailed) *P*	0.000		0.686
n	30	30	30
Thickness of epithelium			
Pearson's correlation	0.086	−0.077	1
Significant (two-tailed) *P*	0.653	0.686	
n	30	30	30

Table 2: Correlation between Ki-67 staining and thickness of the epithelium

	n	Minimum	Maximum	Mean	SD
Stained nuclei	30	0.0	128.6	22.177	30.0672
Thickness of epithelium	30	36.5	248.6	144.283	53.4202

SD: Standard deviation

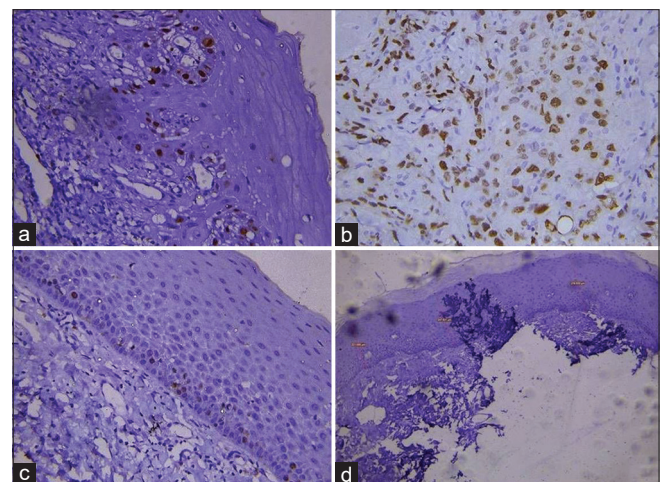**Figure 1:** (a) Oral submucous fibrosis (×40) – Ki-67-positive stained nuclei. (b) Positive control, squamous cell carcinoma (×40) – Ki-67-positive stained nucleus. (c) Oral submucous fibrosis (×40) – Ki-67-positive stained nucleus in mild grade of functional staging. (d) Oral submucous fibrosis (×10) – measurement of epithelial thickness in mild grade of functional staging
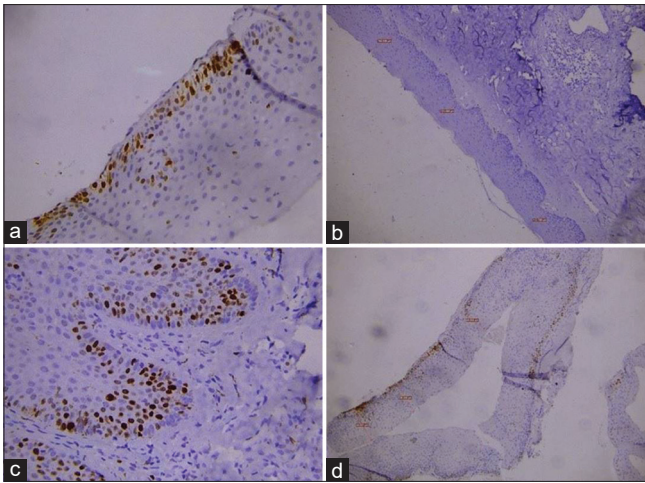
Figure 2: (a) Oral submucous fibrosis (×40) – Ki-67-positive stained nuclei in moderate grade of functional staging. (b) Oral submucous fibrosis (×10) – measurement of epithelial thickness in moderate grade of functional staging. (c) Oral submucous fibrosis (×40) – Ki-67-positive stained nuclei in severe grade of functional staging. (d) Oral submucous fibrosis (×10) – measurement of epithelial thickness in severe grade of functional staging

to study the proliferation potential of an atrophic epithelium in OSMF and correlate with functional staging and epithelial thickness.

However, our study did not show a consistent decrease in epithelial thickness with increasing functional grades of the disease. Each grade of the disease was found to display either hyperplastic or atrophic epithelial changes. Earlier, Pindborg *et al.* and Vilmer *et al.* also suggested that the overlying epithelium is either atrophic or hyperplastic and often hyperkeratotic.[21,22]

Thus, epithelial changes in our study were in agreement with earlier reports.

Furthermore, the microtrauma to oral mucosa and gastrointestinal mucosa caused due to the continuous friction of coarse fibers of areca nut chewing leads to the diffusion of betel quid alkaloids and flavonoids into the subepithelial connective tissue through atrophic and microperforated gastrointestinal and oral mucosa which results in juxta-epithelial inflammatory cell infiltration. Gastrointestinal tract (GIT) with microperforations due to microtrauma is also a feature seen in leaky gut syndrome. The current standard of care paradigm is to treat the symptoms of disease, not the cause of disease, but reversing this paradigm and healing leaky gut syndrome would prevent, reverse, or delay chronic diseases such as OSMF. Extensive research needs to be done to study leaky gut and its cure in OSMF. A leaky gut also reduces the absorptive capacity of the epithelium. Persistent gastrointestinal inflammation in due course disrupts the integrity of the mucosal lining of the gut, and tiny perforations allow undigested molecules larger than usual to pass across this barrier. Both animal studies and recent clinical evidence support this new paradigm of leaky gut syndrome and provide the rationale for innovative

approaches to prevent and treat autoimmune diseases as per Fasano's study.[23]

Pindborg *et al.*, however, considered that the atrophic epithelium first becomes hyperkeratotic and later develops intercellular edema and basal cell hyperplasia, eventually followed by epithelial atypia with moderate epithelial hyperplasia which forms perfect soil for future neoplastic changes. It is generally accepted that an atrophic mucosa is more likely to undergo malignant transformation. However, there is no clear evidence in regard to the development of dysplasia and connective tissue changes.[21] However, Lee SS *et al.* stated that hypoxia could be one of the etiological factors in OSMF which is principally mediated by hypoxia-inducible factor-1 alpha (HIF-1a) expression.[24] Furthermore, HIF-1a is implicated in OSMF and its overexpression leads to changes in cell proliferation, maturation, and metabolic adaptation, escalating risk of malignant transformation of OSF.[25] HIF-1a may thus be essential for both progression of fibrosis and malignant transformation in OSMF.[25,26] The Ki-67 expression in OSMF was significantly higher than that of normal mucosa. Similar findings have been reported by Gonzalez-Moles *et al.* in nonneoplastic epithelium adjacent to OSCC, Gyorgy and Belain in leukoplakia and oral cancer, and Kurokawa *et al.* in epithelial dysplasia.[19,20,27]

We did not correlate Ki-67 expression with degree of dysplasia as most of our cases had atrophic epithelium without much similar significant dysplastic changes in each group.

In the present study, as the functional staging increased, the Ki-67 expression also increased, and it was highest in severe functional staging/severely decreased mouth opening and was least in mild functional staging/mild decrease in mouth opening.

In moderate functional staging, an intermediate result of Ki-67 expression was observed. Similar findings were observed by "Ranganathan *et al.*"[28]

Epithelial atrophy is said to be one of the key features in OSMF. However, the present study did not show a consistent decrease in epithelial thickness with increasing functional grades of the disease which indicates that there was no significant difference in the epithelial thickness according to mouth opening ($P > 0.05$), and these results were in accordance with the study conducted by "Pindborg *et al.*" and "Vilmer *et al.*"[21,22] who suggested that the overlying epithelium in OSMF is not always atrophic. They found hyperplastic and often hyperkeratotic epithelium in their study which was associated with aggressive form of the lesion.

The drawback of the study was that the sample size was small. Similar studies should be performed in the future including other molecular markers. In addition, GIT mucosa should also be analyzed through endoscopy, for microscopic and macroscopic alterations of GIT, caused by constant areca nut chewing.[29]

Conclusion

Thus, there was an increase in Ki-67 expression with increasing OSMF functional grading that causes decreased mouth opening. However, the present study did not show a consistent decrease in epithelial thickness with increasing grades of the disease.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Ranganathan K, Kavitha R. Proliferation and apoptosis markers in oral submucous fibrosis. J Oral Maxillofac Pathol 2011;15:148-53.
2. 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;2:38-46.
3. Chattopadhyay A, Ray JG, Caplan DJ. AgNOR count as objective marker for dysplastic features in oral leukoplakia. J Oral Pathol Med 2002;31:512-7.
4. Mondal K, Mandal R, Sarkar BC. A study of Ki-67 expression and its clinicopathological determinants in nondysplastic oral leukoplakia. Contemp Clin Dent 2016;7:493-9.
5. Tumuluri V, Thomas GA, Fraser IS. Analysis of the Ki-67 antigen at the invasive tumour front of human oral squamous cell carcinoma. J Oral Pathol Med 2002;31:598-604.
6. Lim JJ, Kang S, Lee MR, Pai HK, Yoon HJ, Lee JI, *et al.* Expression of vascular endothelial growth factor in salivary gland carcinomas and its relation to p53, Ki-67 and prognosis. J Oral Pathol Med 2003;32:552-61.
7. Kannan S, Chandran GJ, Pillai KR, Mathew B, Sujathan K, Nalinakumary KR, *et al.* Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: Correlation with expression of ki67. Clin Mol Pathol 1996;49:M170-5.
8. Mani NJ, Singh B. Studies on oral submucous fibrosis: III. Epithelial changes. Oral Surg Oral Med Oral Pathol 1976;41:203-21.
9. More CB, Das S, Patel H, Adalja C, Kamatchi V, Venkatesh R. Proposed clinical classification for oral submucous fibrosis. Oral Oncol 2012;48:200-2.
10. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577-80.
11. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001;411:342-8.
12. Arakeri G, Patil SG, Aljabab AS, Lin KC, Merkx MA, Gao S, *et al.* Oral submucous fibrosis: An update on pathophysiology of malignant transformation. J Oral Pathol Med 2017;46:413-7.
13. Amaral FR, Mateus GC, Bonisson LA, de Andrade BA, Mesquita RA, Horta MC, *et al.* Cell proliferation and apoptosis in ameloblastomas and keratocystic odontogenic tumors. Braz Dent J 2012;23:91-6.
14. Razavi SM, Tabatabaie SH, Hoseini AT, Hoseini ET, Khabazian A. A comparative immunohistochemical study of ki-67 and bcl-2 expression in solid ameloblastoma and adenomatoid odontogenic tumor. Dent Res J (Isfahan) 2012;9:192-7.
15. Hegab A, Shuman M, Abd El-Akher M, Arwlan D. Ki-67 immunohistochemical expression in mandibularameloblastoma: A prognostic indicator for localrecurrence. Open J Stomatol. 2013;3:520-6. doi:10.4236/ojst.2013.39086.
16. Harvey W, Scutt A, Meghji S, Canniff JP. Stimulation of human buccal mucosa fibroblasts *in vitro* by betel-nut alkaloids. Arch Oral Biol 1986;31:45-9.
17. Kamath VV, Satelur K, Komali Y, Krishnamurthy SS. Image analyses of collagen types and thickness in oral sub mucous fibrosis stained with picrosirius red under polarizing microscope. J Orofac Sci 2013;5:123-7.
18. Rajendran R. Benign and malignant tumors of oral cavity. In: Rajendran R, Sivapathasundaram B, editors. Shafer's Textbook of Oral Pathology. 6th ed. Philadelphia: Elsevier; 2009. p. 198-200.
19. Kövesi G, Szende B. Changes in apoptosis and mitotic index, p53 and Ki67 expression in various types of oral leukoplakia. Oncology 2003;65:331-6.
20. Kurokawa H, Matsumoto S, Murata T, Yamashita Y, Tomoyose T, Zhang M, *et al.* 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.
21. Pindborg JJ, Chawla TN, Srivastava AN, Gupta D. Epithelial changes in oral submucous fibrosis. Acta Odontol Scand 1965;23:277-86.
22. Vilmer C, Civatte J. [Oral sub mucous fibrosis. Review of the literature apropos of a case]. Ann Dermatol Venereol 1986;113:107-12.
23. Fasano A. Leaky gut and autoimmune diseases. Clin Rev Allergy Immunol 2012;42:71-8.
24. Lee SS, Tsai CH, Yang SF, Ho YC, Chang YC. Hypoxia inducible factor-1α expression in areca quid chewing-associated oral squamous cell carcinomas. Oral Dis 2010;16:696-701.
25. Tilakaratne WM, Iqbal Z, Teh MT, Ariyawardana A, Pitiyage G, Cruchley A, *et al.* Upregulation of HIF-1alpha in malignant transformation of oral submucous fibrosis. J Oral Pathol Med 2008;37:372-7.
26. Ekanayaka RP, Tilakaratne WM. Oral submucous fibrosis: Review on mechanisms of malignant transformation. Oral Surg Oral Med Oral Pathol Oral Radiol 2016;122:192-9.
27. 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.
28. Ranganathan K, Kavitha R. Proliferation and apoptosis markers in oral submucous fibrosis. J Oral Maxillofac Pathol 2011;15:148-53. doi:10.4103/0973-029X.84478.
29. Misra SP, Misra V, Dwivedi M, Gupta SC. Oesophageal subepithelial fibrosis: an extension of oral submucosal fibrosis. Postgrad Med J 1998;74:733-6.