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Expression profile of Ki-67 in OSMF and its possible correlation with clinical and histological features

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Abstract---Background: Oral submucous fibrosis(OSMF) is a potentially malignant disorder (PMDs) with characteristic epithelial and connective tissue changes and regarded as possessing a high degree of malignant potential. Epithelial dysplasia is an important marker of malignant development from PMDs. Because agreement among oral pathologists is poor regarding lesional diagnosis, Ki-67 as a proliferative marker may have a place in objectively characterizing dysplasia in tissue specimens. Material & Methods: The study groups included 60 patients diagnosed with OSMF based on history and clinical examination. After obtaining the details in regard to habits

and clinical manifestations, the subjects were divided into Group IA and Group IB (very early and early stages-Group I A, moderate and advanced stages-Group I B). 30 subjects without an OSMF-negative control group and 30 patients of SCC-positive control. Biopsy was taken and subjected for H&E staining and IHC analysis of Ki-67. Results: The mean count of Ki-67 was determined in OSMF cases with dysplasia and without dysplasia by using t test, mean value of Ki-67 in with dysplasia (37.41) was higher than that of without dysplasia (28.41) with no significant difference (p value = 0.0810). Cut off values were established by calculating the mean values in the cases of negative control, OSMF cases without dysplasia, OSMF cases with dysplasia, OSMF and positive control, and the values were 25.60, 28.41, 37.41, 32.30 and 71.70 respectively. Conclusion: The determined cut off value of 28.41 for OSMF cases without dysplasia and 37.41 for OSMF cases with dysplasia by using Ki-67. This cut off values may aid in differentiating cases of OSMF without dysplasia from with dysplasia in adjunct with other clinical and histological parameters.

Keywords--Ki-67 antigen, oral submucous fibrosis, carcinoma in situ, staining labeling, connective tissue, hyperplasia.

Introduction

In the process of food consumption oral mucous membrane is exposed constantly to various kinds of stress factors, like heat, cold, microorganisms, chemical and mechanical stimulants. Acute and chronic reactive changes are seen in both epithelial and connective tissue layers of oral mucosa in response to these stimulants.¹ In the Indian subcontinent, a great number of patients report with several oral pathoses following the habit of diverse oral habits, mainly tobacco in both smoking and smokeless forms. Such conditions mainly includes oral leukoplakia, Oral sub mucous fibrosis (OSMF), tobacco pouch keratosis, reverse smokers palate and rarely Squamous Cell Carcinoma (SCC).²

Oral submucous fibrosis (OSMF) is a chronic condition particularly occurring among Indians and lesser extent in other Asiatic people.² It is a potentially malignant condition that has rapidly increased in India, with more than two million cases recorded in the last decade.³ It is a chronic condition affecting oral cavity and oropharyngeal part, marked by inflammation and fibrotic band of mucosal region, leading to restriction in opening the mouth. It is considered as a collagen-related disorder caused by betel quid chewing habit resulting in reduced collagen degradation with an increased production of collagen. The severity of the diseases increases with quantitative accumulation and qualitative alteration of collagen.⁴

It has a high degree of malignant potential.² which is reported to be ranging from 3% to 19%.⁵ The pathogenesis of OSMF is multifactorial. The pathogenesis of OSMF is still not fully understood even after the conduct of research spanning more than four decades on the epidemiology, molecular biology/pathology and

histopathology.⁶ However data available on OSMF from recent studies concluded that OSMF is basically a result of collagen dysregulation and areca-nut play a major role in causing changes at various stages of collagen metabolism.^{6,7} Many studies have centered on reduced collagen degradation and increased synthesis of collagen, as possible mechanisms in the development of OSMF.^{6,8} However, the pathogenesis of OSMF is not well established, and the exact mechanism of areca nut causing OSMF is still being explained.⁹ On the bases of availability of scientific literature, the pathogenesis of OSMF can be considered under broad Theories such as Defective collagen homeostasis theory, Genetic theory, Autoimmunity theory, Nutritional deficiency theory, Role of chillies and spicy food and theory of Salivary pooling. The rate of morbidity in OSMF patient is high because of difficulty in eating and nutritional deficiency, resulting from progressive irreversible inability to open (the) mouth.¹⁰

Alteration of growth rate, commonly reflected as increased cell proliferation is considered as significant characteristics of neoplastic transformation of epithelium but the occurrences and development of dysplasia to malignant lesions seems dubious. A lower degree of dysplasia has less proliferative activity, meaning malignant transformation is slower. Therefore, this project attempts to study the expression pattern of Ki67 in epithelium of OSMF and its possible correlation with various stages and grades of diseased process, further with appropriate statistical methods to establish the cut-off point for dysplasia if exists.

Materials and Methods

A hospital based prospective study was conducted among the patients attending Dental College and Hospital, Karnataka. A total of 60 patients diagnosed as OSMF based on history and clinical examinations were included in the study group. The subjects would be divided into Group 1 and Group 2, were in, very early and early stage would be categorized as Group 1 and moderate and advanced stage as Group 2. Thirty subjects without OSMF, undergoing minor surgical procedures like impaction, periodontal surgery, etc formed the negative control group. The positive control group formed by 30 patients of diagnosed SCC. After obtaining informed consent the details in regard to habits and clinical manifestations of OSMF were recorded on prescribed proforma and under aseptic precautions, buccal mucosal tissue was procured and subjected for both H/E staining, grading and IHC analysis of Ki67.

Sections were obtained from negative and positive control formalin fixed paraffin embedded tissue specimens were subjected to sectioning with a rotary semi-automatic microtome for obtaining sections of 4-5 μ m thickness and were stained with routine H&E stain. Other sections were obtained on super frost slides and subjected to stains with monoclonal Ki-67 antibody using the Standard method. These preparations were counter stained with Haematoxylin, mounted with paramount mounting medium and examined with light microscopy.

Inclusion Criteria

All clinically diagnosed cases of OSMF.

Exclusion Criteria

- The study group presented other associated lesions with OSMF and underlying systemic diseases like iron deficiency anaemia, immunoproliferative disorders and any other malignancy.
- The negative control group was ruled out of innocuous presence of any other lesion.
- The positive control group presenting with OSMF as a precursor lesion were excluded.

Evaluation of Staining

Evaluation of the Haematoxylin & Eosin and IHC stained slides were done by two experienced oral pathologists, blindfolded, quantified the positively stained cells and intensity of staining of epithelium was calculated.

Quantification of Positively Stained Cells

Positively stained cells were counted in four intensely stained areas under high power field (40x)

Clinical Staging

Clinical staging was based on the clinical features present in the oral cavity like stomatitis, vesicles, ulcerations, blanching, pallor, rigidity etc at various intraoral sites while the functional stages were based on the mouth opening of the patients. In the present study, According to the Chandramani Bhagvan More et al.¹¹ clinical classification, 6 patients were in Stage I, 21 in stage II, 31 in stage III and 2 in stage IV (n = 60). According to the functional classification, 19 patients were in M1, 30 in M2, 7 in M3 and 4 in M4.

Histological Grading

The dysplastic features in study groups were categorized according to WHO 2005 classification of dysplasia¹² and histological grading was done according to pindborg et al.¹³ histological grading.

Statistical Analysis

Tukey's multiple post hoc procedures, one way ANOVA and t test were used for statistical analysis and comparison among three groups and between study group cases with dysplasia and without dysplasia. Also, significance is analysed among mean values of different histological grades and stages of OSMF. Significance was set at <0.05.

Results

In the overall samples, wide age group distribution of both study and control groups were observed. OSMF occurred over an age range of 2nd to 5th decade with the peak incidence in the 3rd decade. Mean age of occurrence of study, positive

and negative control groups was 28.2 years, 51.8 and 20.3 years respectively. Almost 75% of the cases were males in both positive and negative control groups. According to pindborg et al.¹³ histological grading, eleven cases showed very early stage (18.33%), thirty-one cases with early stage (51.6%), and eighteen cases with moderately advanced stage (30%) of OSMF. Out of 60 cases, 25 cases showed mild dysplasia, 3 cases with moderate dysplasia and only 2 cases with severe dysplasia (50%) while no dysplastic features were seen in the remaining 30 cases (50%).

In the study group (60 cases of OSMF), Forty-three cases were reported with epithelial atrophy (71.6%), 17 cases showed epithelial hyperplasia, hyperkeratosis and intercellular edema (28.3%). Comparison of mean count of Ki-67 was done in group I (very early and early grade) and Group II (moderate and advanced grade), which showed significant association (p value = 0.0020) (Table 1). Out of 42 cases of Group I, 18 cases showed dysplastic features (42.85%) and 24 cases did not show (57.14 %). Out of 18 cases of Group II, 12 cases showed dysplastic features (66.66%) and 6 cases did not show (33.33%). The mean count of Ki-67 was determined in OSMF cases with dysplasia and without dysplasia. Mean value of dysplasia and without dysplasia found no significant difference (p value = 0.0810)(Table 2).

Comparison of mean count of Ki-67 between study, negative control and positive control was found to be statistically significant but no significant difference was found within the groups (OSMF with and without dysplasia) (Table 3). Mean values were calculated in study, positive control and negative control, and the values were 32.30, 71.70 and 25.60 respectively by using Tukey's multiple post hoc procedures. A significant correlation was found between study and positive controls (p value = 0.0001) and between positive and negative control (p = 0.0001), whereas there was no significant correlation found between study and negative control (p= 0.3263) (Table 4). The cut off values were established by calculating the mean count of Ki-67 positive cells. The highest value was found in SCC and lowest being in normal mucosa.(Table 5).

Discussion

OSMF affects both genders and the male:female ratio varies by region. It occurs more frequently in males than in females. In our study, all the patients were male, (100%). This was in relevance with other studies where a significant predominance of OSMF in men was observed in a study of 1000 cases from central India with male to female ratio of 4.9:1.¹⁴ A male: female ratio of 6: 1 was reported from a clinic-pathological study in Chennai consisting of 75 cases. Another case control study in Chennai, South India, over a period of 3 years revealed a high preponderance of OSMF in males, with male to female ratio of 9.9:1.¹⁵ This can be attributed to the socioeconomic status, and habit practices seen more among males of working class belonging to this decade. ³

OSMF may affect any part of the oral cavity depending on the site of placement of quid. Persistent chewing at specific sites has been suggested as the reason for the diverse distribution of OSMF. It occurs in multiple sites among the unilateral chewers because of the prolonged exposure of the oral mucosa to saliva

containing dissolved products of quid, which occurs when the saliva pools in specific area.⁶ In our study, the most common site of involvement was predominantly buccal mucosa, followed by palate, circum oral region, and tongue. A study on the regional variations of this condition, however, pointed out that the difference in site of involvement depends more on whether the areca nut juice and the quid is swallowed or spat out rather than reflect the general pathogenesis.¹⁶ These investigators found that, the soft palate, palatal fauces and retro-molar areas were significantly more affected.¹⁷ Such pattern of involvement is also attributed to the type of profession or occupation where in social appearance or status of an individual be affected may be posterior regions are safe areas to mask from the public while anterior or lateral spaces were used who apparently didn't speak for long hours.⁶

Dysplastic features like focal budding, altered rete morphology are usually observed in OSMF which was very much illustrated in present study where majority or 50% of cases showed these features. Epithelial atrophy, epithelial hyperplasia, intercellular edema and hyperkeratosis is seen in remaining 50% cases. According to Jayasooriya et al,¹⁶ considering the epithelium with budding rete morphology as dysplastic and is important in the context of OSMF. Histological diagnosis might be improved by quantitative evaluation of more specific diagnostic biomarkers, which could help to precisely identify the potential of premalignant oral lesions. Various proliferative markers such as Ki-67, p53, PCNA, AgNOR, CK 13, 17, and Notch genes have been utilized to identify the rate of proliferation progressing to malignancy.¹⁸

Among these, Ki-67 is a human nuclear protein associated with cell proliferation and maximally expressed in cells with G2 and M phases of the cell cycle but absent in resting cells. Hence, it can be widely used in pathology as a proliferation marker to measure growth fraction of cells in premalignant and malignant lesions, along with normal tissues.¹⁹ Ki-67 positive cells are often correlated with the clinical course of the disease.²⁰ It provides significant information about the degree of aggressiveness and prognosis of OSCC.²¹

In our study Ki-67 expression in all the cases of normal oral mucosa (negative control) was found to be restricted to basal and parabasal layers with intense staining was seen in the parabasal layer of the epithelium(Fig1a&1b). In almost all the cases of OSMF with dysplasia expression of Ki-67 was noted in basal, parabasal including spinous layer of epithelium, two cases showed Ki-67 expression till the superficial layer of the epithelium(Fig2a&2b). The similar expression was also noted by other studies.^{20,22}

The increased number of Ki-67 positive cells till spinous layer was also observed in the previous study of leukoplakia was attributed to increased proliferation in the upper compartment due to the loss of heterozygosity in 3p, 9p and 17p behaving as a marker of precancerous fields. This was also seen in almost all the cases of study groups. Another group also viewed that, this proliferation not only progresses in the superior direction but also downwards towards the basal layer.^{23,24}

Ki-67 positivity in OSCC was located at the periphery of the tumour nests than the centre (which appeared granular) in almost all cases (Fig3a). According to the literature this Ki67 positivity in the periphery of the tumour nests is mainly because of the presence of less differentiated cells and the central cells are highly differentiated with an ability to keratinized, thus no expression of Ki-67 was observed in the central cells of the tumour island.^{23,24,25} Some cases of OSCC showed diffuse Ki-67 expression in the tumour nests(Fig3b). This expression of Ki-67 in both peripheral and part of the central layer indicates that cells were less differentiated in both the areas.

Inter observer variability is known to exist in evaluation and reporting of dysplasia in PMD's. Over the years attempts have been made to overcome this problem by employing quantification and semi quantification studies. In 2007 authors working in this regard established 2.3 as a cut-off to differentiate with and without dysplasia of leukoplakia using AgNoR as a marker. They stated that obtained cut off may be used in conjunction with other histological parameters in differentiating dysplastic from non-dysplastic.²⁶

The available literature on the expression of Ki-67 in Oral sub mucous fibrosis is sparse. Moreover, the study on establishment of cut-off points for dysplasia in OSMF has not been conducted yet. Thus the study was undertaken to propose a cut off value for dysplasia in particular in OSMF so as to improve the assessment and evaluation of dysplasia. So this is the first sincere effort in this regard to determine the cut-off point in OSMF using Ki-67. Statistical method of bootstrap is used in obtaining cut off values for both study and control groups. The Ki-67 count was statistically more in the moderate and advanced stages than compared to very early and early stages, signifying the increased proliferation of epithelial components in proportion to the advanced stages of the underlying connective tissue.

In conclusion our immunohistochemical study of Ki-67 in assessing the expression in OSMF showed high expression in basal, parabasal layer and extending into superior layer in some cases of OSMF. Comparing the count with stages of OSMF, Ki-67 count was seen to be more in moderate and advanced stages than compared to very early and early stages. The determined cut off value of 28.41 for OSMF cases without dysplasia and 37.41 for OSMF cases with dysplasia may aid in differentiating cases of OSMF without dysplasia from with dysplasia. We suggest larger studies of this nature is necessary to determine an effective cut-point. Multiple larger studies will enable accurate determination of an effective meaningful cut-point to distinguish between OSMF without dysplasia and with dysplasia.

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Table 1: Comparison of group 1 and group 2 with respect to number of Ki-67 cells by t test

Group	Mean	SD	SE	Bootstrap				
				Mean difference	Bias	SE	t-value	p-value
Group 1	25.05	17.69	2.73	-24.17	0.36	7.35	-3.9940	0.0020*
Group 2	49.22	28.63	6.75					

Table 2: Comparison of with and without dysplasia with respect to number of Ki-67 cells by t test

Group	Mean	SD	SE	Bootstrap				
				Mean difference	Bias	SE	t-value	p-value
With	37.41	22.70	4.29	10.13	0.01	5.54	1.8664	0.0810
Without	27.28	19.34	3.42					

Table 3: Comparison of three groups (OSMF, Normal, SCC) with respect to number of Ki-67 cells by one-way ANOVA

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	P-value
Between groups	2	39897.83	19948.91	45.7794	0.00001*
Within groups	117	50984.10	435.76		
Total	119	90881.93			

Table 4: Pair wise comparison of three groups (OSMF, Normal, SCC) with respect to number of Ki-67 cells by Tukey's multiple post hoc procedures

Group	OSMF	SCC	Normal
Mean	32.3000	71.7000	25.6000
SD	24.0538	21.0666	11.7109
OSMF	-		
SCC	0.0001*	-	

Normal	0.3263	0.0001*	-
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Table 5: Cut-off point of number of Ki-67 positive cells in various groups

	Cut-off values
OSMF With dysplasia	37.41
OSMF Without dysplasia	28.41
OSMF	32.30
Normal mucosa	25.60
SCC	71.70

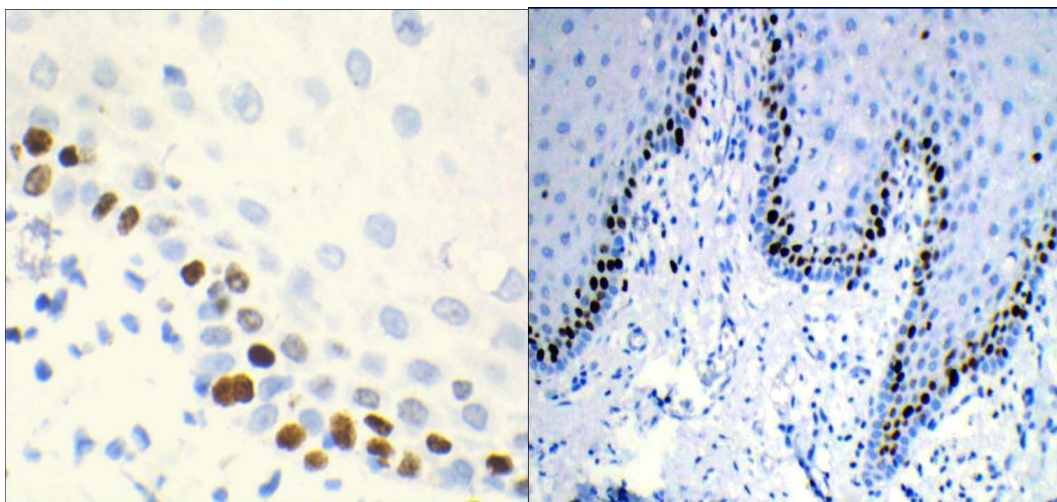


Fig 1a: Ki-67 Expression In Para Basal Layer Of Normal Mucosa (Under 40x) Fig 1b : Ki-67 Expression In Para Basal Layer Of Normal Mucosa (Under 10x)

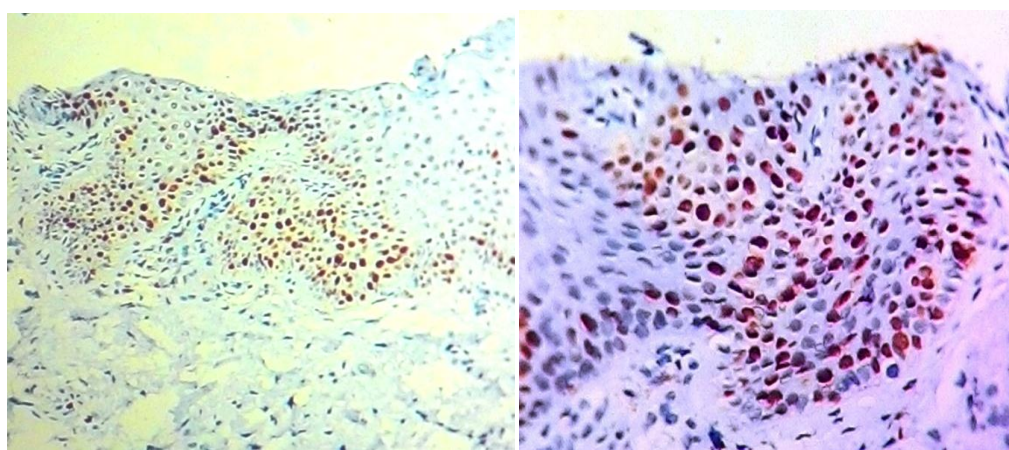


Fig 2a: Ki-67 Expression In Basal, Para Basal, whole length of normal spinous layer in OSMF (Under 10x) Fig 2b : Ki-67 Expression In epithelium in OSMF (Under 40x)

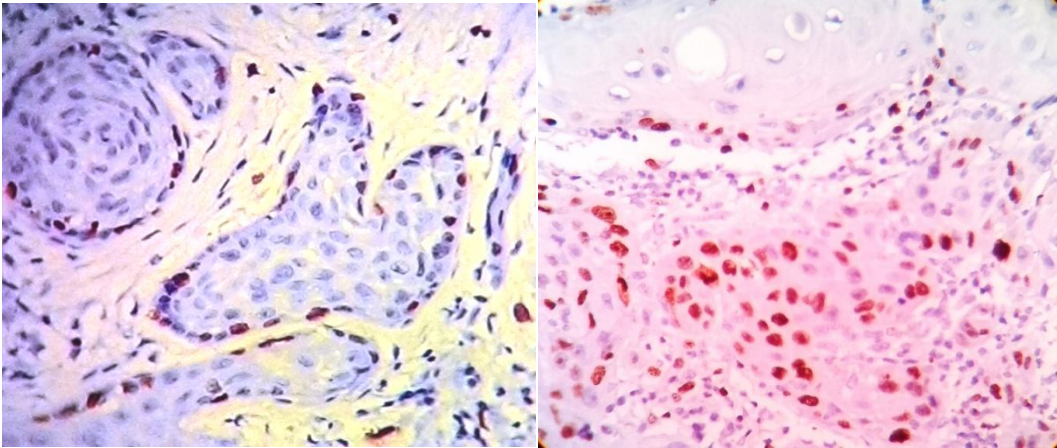


Fig 3a : Ki-67 Expression in the peripheral layer of central and peripheral layer tumor Island (under 40x) of tumor island(under 40x)

Fig 3b: Ki-67 Expression in central and peripheral layer tumor Island (under 40x) of tumor island(under 40x)