

ORIGINAL ARTICLE

Proliferation and apoptosis markers in oral submucous fibrosis

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ABSTRACT

Background: Premalignant/potentially malignant-oral lesions and conditions such as oral submucous fibrosis are known to transform into oral cancer. The malignant transformation is often associated with changes at the genetic level that in turn is reflected by the altered expression of proteins related to cell cycle, proliferation, and apoptosis. **Aim:** To evaluate the expression of p53, Ki67 (MIB), bcl2, and bax in oral submucous fibrosis and oral squamous cell carcinoma. **Materials and Methods:** To assess the immunohistochemical expression of p53, Ki67 (MIB), bcl2, and bax in 50 cases of oral submucous fibrosis (OSF) and ten each of normal and oral squamous cell carcinoma (OSCC). **Results:** The labeling indices (LI) of OSF and OSCC were comparable for p53 and Ki67. The p53 LI ranged from 7.9 to 71.9 in OSF and 65.2 to 85.9 in OSCC, and for Ki67 it ranged from 4.39 to 43.23, 18.35 to 42.33, respectively. **Conclusion:** The p53, Ki67, and bax profiles of OSF and OSCC were altered compared to the normal and these markers could be used as surrogate markers of malignant transformation in OSF.

Key words: Apoptosis, cancer, immunohistochemistry, malignant transformation, oral, proliferation, submucous fibrosis

INTRODUCTION

Oral submucous fibrosis (OSF) is a chronic progressive, precancerous condition of the oral mucosa, which is associated with areca nut chewing habit widely prevalent in India and South-East Asia.^[1,2] Clinical pathognomonic features include burning sensation of the oral mucosa accompanied by pallor and progressive, irreversible fibrosis leading to difficulty in opening the mouth, speech and swallowing.

Histopathological feature of OSF involves increased deposition of collagen in the oral mucosa, due to a collagen-metabolic disorder, resulting from exposure to the areca nut alkaloids. Other histopathological changes in established OSF are reduced vascularity, reduced number of inflammatory cells, and sub-epithelial hyalinization.^[3]

Various etiological factors have been suggested for OSF including capsaicin and spicy food. Epidemiological and

in vitro experimental studies show that chewing areca nut is the foremost etiological agent for the occurrence OSF.^[2,3] Areca nut, is the fourth most commonly abused substance in the world (following nicotine, ethanol and caffeine). 10-20% of the world population chews areca nut in some form and more than 200 million people chew betel quid worldwide.^[3-6]

OSF is a potentially malignant condition. Reports of malignant transformation rate of the surface epithelium in OSF ranges from 3% to 19%, among patients attending hospitals and in the community.^[7,8] Eighty percent of OSCC in Taiwan and India are associated with use of areca nut as quid, along with other ingredients including tobacco.^[9-11] Though extensive data for molecular markers, such as p53, proliferation associated antigens, cytokeratins, Bcl2 group of proteins, exist for other premalignant lesions such as leukoplakia, data for OSF is limited and much work needs to be done, especially in the Indian context.^[8] The aim of our study was to study p53, Ki67 (MIB), bcl2 and bax expression by immunohistochemistry and evaluate their potential as surrogate biomarkers of malignant transformation.

MATERIALS AND METHODS

All the study subjects and tissue samples for this study were from the Departments of Oral Medicine and Oral Pathology, Ragas Dental College and Hospital (RDCH), Chennai, India.

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Institutional Review Board (IRB) approval and patients consent as per the IRB guidelines was obtained for each part of this study. The patients attend RDCH for routine dental treatment and constituted a combination of people from urban and rural areas.

The tissue samples were drawn from archives of Department of Oral and Maxillofacial Pathology, RDCH, Chennai. OSF patients were identified based on well-established clinical criteria.^[2,12]

Fifty OSF cases and ten OSCC with sufficient tissue for all antibodies were selected for immunohistochemical analysis using commercially available antibodies. All the biopsied tissues were fixed in 10% buffered formalin and paraffin embedded using standard procedures. Normal tissues were obtained from the buccal mucosa of ten patients during the surgical removal of the third molar.

Haematoxylin and Eosin staining was done for histopathological diagnosis for all cases. Subsequently, thin sections on APES (3-Aminopropyltriethoxysilane)-coated slides were obtained and immunohistochemical analysis performed. The sections were stained by the modified labeled avidin–biotin technique with DAKO LSAB™ kit (DAKO Corporation, Carpinteria, CA, USA).

Appropriate positive and negative controls were used. p53 and Ki67 exhibit nuclear staining. Bcl-2 and bax exhibit cytoplasmic staining.

For cytoplasm staining antibodies: two investigators examined all the slides separately. Cytoplasmic staining was assessed as follows: – negative, + mild, ++ moderate, and +++ intense. The layers were assessed separately as basal, suprabasal, and keratin layers. Kappa statistics were obtained.

For antibodies staining the nuclei the labeling index was calculated, for this purpose 1000 cells were counted and labeling index (LI) were calculated as follows:

$$LI = \frac{\text{Number of positive cells}}{\text{Number of cells}} \times 100$$

For the purpose of counting the positively staining nuclei an image analysis system – . Microimage™ (Olympus) software with Pro series Capture kit MV™ (Media Cybernetics) – one-chip CCD camera, Olympus BX45 microscope, and Pentium IV PC with frame grabber card was used. Histopathological photographs were taken of representative sections for all the antibodies and stored in Image analysis system.

Statistical analysis

Data were entered and analyzed using SPSS 10.05. One-way ANOVA was used to assess the statistical difference

in the percentage of positive cells between normal, OSF, and OSCC for p53 and Ki67 (MIB). Bonferroni test for multiple comparisons was performed. The chi-squared test was undertaken to analyze the differences between the intensity levels, in normal, OSF, and OSCC. Differences with a probability value of <0.05 were considered statistically significant. Bivariate correlation was done between LI of p53, Ki67, and bax positivity, and Pearson's correlation coefficient (r) calculated.

RESULTS

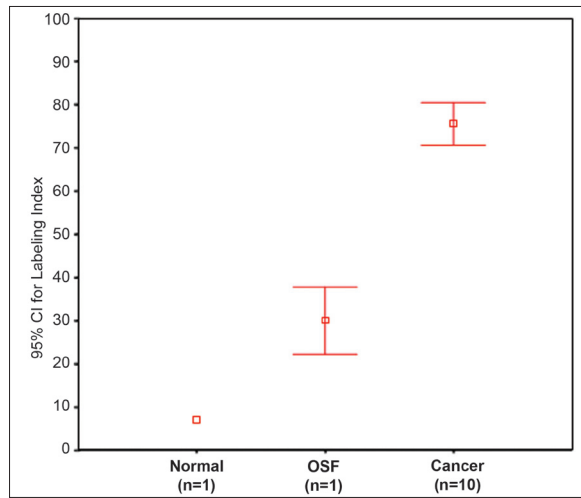
p53 [Graph 1]: in normal, one case was positive for p53. In OSF, 44% were positive and in oral cancer all cases showed positivity for p53. The labeling indices (LI) of the three groups were: in the normal group the LI was 7.1. In OSF, the LI ranged from 7.9 to 71.9 and in OSCC, the LI ranged from 65.2 to 85.9. The LI at 95% interval were 7.1 for normal, 30.0±17.7 for OSF and 75.6±6.9 for oral cancer. The one positive case from the normal group exhibited staining in the basal layer. All ten cases of OSCC exhibited staining that was both basal and suprabasal. In OSF, four cases showed basal staining only, while the rest exhibited both basal and suprabasal staining.

Ki67 [Graph 2]: all the cases in the three groups were positive for Ki67. The LI of the three groups were: 2.05 to 11.03, 4.39 to 43.23, 18.35 to 42.33 in normal, OSF, and OSCC respectively. The LI at 95% interval were 5.33.6 for normal, 19.1± 7.6 for OSF, and 29.1± 8.8 for oral cancer.

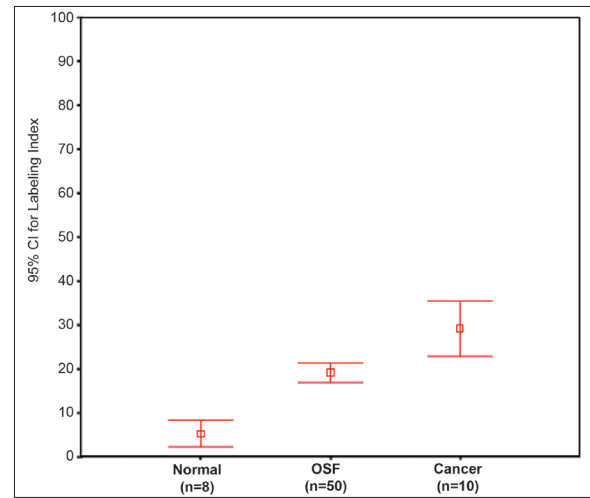
bcl2: all cases of normal, oral cancers, and OSF were stained for bcl2 along with the positive controls. None of the normal epithelium showed bcl2 positivity. One case in the OSF group exhibited isolated positivity in the basal layer. In the oral cancer group, one case exhibited positivity. Isolated positivity in the connective tissue (lymphocytes) was present in a few cases in all the three groups bax [Table 1]: bax positivity was seen in eight normal, 38 OSF cases, and all cases of OSCC. All normal exhibited both basal and suprabasal staining. In OSF 76% showed basal while 44% of these also showed suprabasal expression. In oral cancers all cases showed basal and suprabasal expression of bax. There was however, no statistically significant difference between the three groups. The intensity of staining was compared between the three groups and was as follows.

Basal layer: in normal 40% exhibited moderate and 40% intense staining. In OSF almost equal number of cases exhibited mild (28%), moderate (22%), and intense (26%) staining. In oral cancers 60% of cases exhibited intense staining, 20% moderate, and 20% mild staining. The difference in staining between the three groups was not statistically significant.

Suprabasal layer: normal showed mild (60%) and moderate (20%) staining. In OSF mild (18%), moderate (16%) and intense (10%) staining was seen, while 56% exhibited no bax



Graph 1: p53: comparison of mean labeling index in the study groups



Graph 2: Ki67 (MIB): comparison of mean labeling index in the study groups

Table 1: Bax expression in normal, OSF and OSCC/oral cancer

Epithelium	Cases	+		++		+++		-		P value
		n	%	n	%	n	%	n	%	
Basal layer	Normal (n=10)	0	0	4	40	4	40	2	20	0.142
	OSF (n=50)	14	28	11	22	13	26	12	24	
	OSCC (n=10)	2	20	2	20	6	60	0	0	
Suprabasal layer	Normal (n=10)	6	60	2	20	0	0	2	20	0.001
	OSF (n=50)	9	18	8	16	5	10	28	56	
	OSCC (n=10)	2	20	6	60	2	20	0	0	

expression. In oral cancers, the predominant staining was of moderate (60%) intensity while 20% each of mild and intense was also seen. The difference in the staining intensity between the three groups was statistically significant ($P=0.001$).

When the intensity of staining in the basal and suprabasal layer expression was compared within the group normal and OSF showed a statistically significant difference ($P=0.02$ and $P=0.02$, respectively) while the oral cancer group did not show a statistically significant difference.

When mean LI of p53 and Ki67 were compared with bax positivity, the correlation was low. However, there was a negative correlation ($r=-0.045$) between p53 LI and bax positivity and a positive correlation ($r=0.153$) between Ki67 LI and bax positivity.

DISCUSSION

p53 and Ki67

Mutation of p53 is one of the most common events in oral cancer. Mutant p53 is normally detected in tissues by immunohistochemistry because the mutant type has a longer half-life than the wild type. High incidence of p53 protein expression in betel quid associated potentially malignant

lesions and oral cancers have been reported from India, Taiwan, Sri Lanka, Thailand and China^[6]. It has also been shown that p53 expression in tumor-adjacent mucosa may serve as an important biomarker in assessing the risk of tumor development in carcinogen-exposed normal mucosa^[13].

Expression of p53 in 9/10 cases on malignant conversion from a premalignant lesion (which were p53 negative) has been reported from Indian betel quid chewers by Murti *et al.*,^[14] and 70 % of patients with OSF, from Nepal exhibited increased p53 expression of the epithelium.^[15] Trivedy *et al.*,^[16] in their cohort from India observed 75% (15/20) of OSF and 50% (3/6) of cancer from OSF had p53 expression. In our study of 50 cases of OSF 44% (22/50) OSF and all cases of cancer expressed p53. This is similar to the 47% positivity for p53 in premalignant lesions, reported by Warnakulasuriya *et al.*^[17] The mean LI (95% CI) of OSF was 30.0 between the low LI of 7.1 for the normal and 75.6 for cancer. It has been shown that there is a step-wise increase of p53 positivity in the sequence of progression of normal oral mucosa-potentially malignant lesion –oral cancer.^[18,19] These results might indicate an involvement of p53 in neoplastic transformation as well as in proliferative events.

One normal was positive for p53 and this was seen basally. This could indicate early alterations in epithelium due to

unknown factors; alternatively, it could also be due to the stabilization of wild p53, by other influences such as HPV, rather than mutation. Minimal staining in normal mucosa has also been reported by other investigators.^[20,21]

In our study, except for four cases, all OSF cases had parabasal staining as did OSCC. This pattern of parabasal staining has been reported to have a strong correlation with progression to cancer.^[22-24] Human Ki-67 protein is associated with cell proliferation. During interphase, the antigen is detected only in the nucleus, and the protein is present during all active phases of the cell cycle but is absent from resting cells, making it an excellent marker for determining the growth fraction of a given cell population. Studies using various proliferation markers have shown an association between the proliferative index and tumor progression in oral mucosa.^[25-27]

Studies in leukoplakia have shown that Ki67 expression correlated significantly with the histopathological stage of the tumor and directly correlated with the proliferative status of oral lesions. In one large study involving a group of 239 patients with T1-T3 carcinoma of the oropharynx or oral cavity, the investigators found that high Ki-67 labeling index reflected poor prognosis more accurately than p53 or PCNA status.^[28]

In our study, the LI for OSF was 19.1 ± 7.6 less than that reported by Kannan *et al.*, who showed that premalignant lesions had a LI of 32.6 ± 21.7 . However, this included leukoplakia where the epithelium was of increased thickness and showed dysplasia. While, in the present study the epithelium in OSF was atrophic; the LI of normal and OSCC were 5.3 ± 3.6 and 29.1 ± 8.8 , respectively.^[25]

The LI of Ki67 in OSF was significantly higher than that of normal but less than that of OSCC. Similar high LI for premalignant lesions of the oral cavity have been reported by Miguel A, Gonzalez-Moles *et al.*, in non-neoplastic epithelium adjacent to OSCC, Gyorgy and Belain in leukoplakia and oral cancer and Hideo *et al.*, in epithelial dysplasia.^[29-31] The association between p53 expression and proliferation has been reported by Cox and Walker.^[15] They found a strong association between p53 and PCNA. However, in our study there was no correlation between p53 and Ki67 positivity.

We did not correlate the LI of Ki67 with degree of dysplasia as in most of our cases the histopathology was that of atrophy with similar epithelial changes. It would be useful to follow up these patients and assess the Ki67 LI and its relationship to the clinical evolution of OSF with treatment.

bcl-2 and bax

The balance between cell proliferation and apoptosis determines growth in both normal tissue and in cancer. This

balance is controlled by many factors of which the Bcl-2 family, particularly bcl-2, and bax, play an important role.^[8]

The bcl-2 protein, an anti-apoptotic marker and its over-expression has been reported in several tumors including breast, thyroid, lung and skin carcinomas.^[32] In oral carcinomas, over expression of bcl-2 from 7% to 60% has been reported from developed countries^[33-36] while Ravi *et al.*,^[37] and Kannan *et al.*,^[38] reported 100% and 23% bcl-2 expression respectively, in oral cancers from Southern India. It has been suggested that apoptosis is a barrier against cancer. In our study all normal epithelium was negative for bcl-2 expression. Technical issues in the procedure was ruled out as the positive controls (external control: lymph node; internal control: lymphocyte) exhibited bcl-2 expression. Lack of bcl-2 expression in normal has also been reported by other investigators.^[39,40]

In the OSF group, one case exhibited isolated positivity in the basal layer and in the oral cancer group, one case exhibited positivity. Lack of bcl-2 immunoreactivity in oral epithelial dysplasia has been reported by other investigators also.^[39,40] Though Teni *et al* reported bcl-2 positivity in 16% (6/31; 14 leukoplakias and 17 OSF cases) oral premalignant lesions, all of them showed only mild staining.^[41] Chen Y *et al* showed an inverse correlation between bcl-2 expression and tumor differentiation in oral cancer Staibano *et al* found no significant variation of bcl-2 expression in their series of oral cancer. They suggest that that the bcl-2-induced block of the apoptotic process may be absent, or may not be at a level of immunohistochemical detection.^[42,43] However, Chang *et al*, McAlinden *et al*, and Piffko *et al* observed no significant bcl-2 expression in oral cancer.^[39,40,44] It has been suggested that the lack of bcl-2 expression is a result of methodological constraints, of immunohistochemistry, such as low levels of protein expression, antibody source, or antigen retrieval. In addition, other gene products in the cell cycle regulation, such as *mdm2* may be involved in apoptosis.^[39]

In our study, isolated positivity in the connective tissue (lymphocytes) was present in a few cases in all the three groups. These findings are similar to those of McAlinden *et al*, who suggest that the bcl-2 expression in mature lymphocytes within the locality may be much higher than the epithelial cell component leading to an erroneous under-representation within the epithelia due to the sensitivity limits of the assay.^[40]

Thus, the very sparse bcl-2 staining in our study could be due to the actual absence or low levels of expression that are not detectable by immunohistochemistry.^[36,39,40,44] Alternatively, bcl-2 immunoreactivity may not reflect *m*-RNA activity of bcl-2 as post-transcriptional regulation could be a possible mechanism of its action in oral cancer.^[42] It has also been suggested that alternate mRNA splicing of bcl-2 and caspase may be responsible for the differences in immunoexpressivity in different studies.^[45,40]

Further studies using different antibody clones or more sensitive techniques such as RT-PCR or western blot may help to more clearly define the role of bcl-2, if any, in oral precancer and cancer. It is also possible that apoptosis regulation by mechanisms involving proteins other than bcl-2 may have a role in OSF.

The proapoptotic protein bax plays an important role in defense against cancer as it mediates apoptosis in response to genotoxic stress. It has been shown that bax can bind to bcl-2, resulting in inactivation of the antiapoptotic action of bcl-2.^[46] Bax is expressed in normal epithelium. In our study, the percentage of case positive for bax in normal was 80% and in OSF 76% showed basal while 44% showed suprabasal expression. All the OSCC cases expressed bax both basally and suprabasally. Studies have shown that expression of bax in oral carcinoma ranges from 2.2% to 63%.^[47] There are very few studies of bax in OSF. In one study where p53, bcl-2, and bax were studied in premalignant lesions and oral cancer (63 cancer, 14 leukoplakia, and 17 OSF cases) 39% of OSF were bax positive and bcl-2 negative as compared to 14% oral cancers, also, over expression of bax occurred in the absence of p53 and bcl-2 expression.^[41] Our finding of no correlation between p53, bcl-2, and bax expression was similar to that reported by Teni *et al.*^[41]

It has been reported that patients with oral cancer along with bax expression had a better prognosis than those without bax expression.^[47] In our study, the staining intensity in the suprabasal layer showed significant difference between normal, OSF, and cancer and 56% of OSF cases showed no suprabasal bax immunoreactivity (compared to no expression in 20% in normal and no cases of negative expression in OSCC). The absence of bax in the suprabasal region in such a large series of OSF cases has not been reported earlier and could possibly be an indicator of abnormal proliferation predisposing to malignant transformation.

The findings of this study indicate that p53, Ki67, and bax exhibit altered expression in OSF that is different from normal but similar to that in oral squamous cell carcinoma, and has the potential to be used as surrogate markers of malignant transformation.

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