

ORIGINAL ARTICLE

Immunohistochemical analysis of tenascin expression in different grades of oral submucous fibrosis

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ABSTRACT

Aim: Tenascin, a glycoprotein, is one of the major constituents of extracellular matrix, which may function in organizing the stroma in normal and pathological conditions. The study aimed to correlate the structural organization of tenascin with the pathological progression of disease from early, moderate and advanced changes in oral submucous fibrosis (OSMF). **Study Design:** A retrospective cross-sectional immunohistochemical (IHC) analysis of OSMF cases was performed. Total 70 slide samples were prepared for the study from 35 formalin-fixed paraffin-embedded tissue blocks with 10 each from histologically proven and graded as early, moderate and advanced OSMF and 5 of normal oral mucosa. The IHC sections were analyzed for the intensity and pattern of tenascin expression at the junction of epithelium and connective tissue (ECJ) and deeper connective tissue (CT), as well as presence or absence of staining around inflammatory cells, fibroblast and endothelial cells using anti-human tenascin. **Result:** Most of the OSMF cases showed retention of antigen at ECJ and in deeper CT. Its expression varied in different grades as well as around inflammatory cells, fibroblast and endothelial cells in same tissue section. Highly significant *P* values of 0.001 and 0.003 were obtained for tenascin intensity and pattern, respectively, at ECJ in different OSMF grades. In addition, for the expression of tenascin pattern in deeper CT among different OSMF grades, a significant *P* value of 0.018 was obtained. **Conclusion:** A differential expression of tenascin was observed with the progression of disease. The expression of tenascin as bright and continuous deposition at ECJ in early and moderate stages of OSMF signifies either proliferative organization within the overlying epithelium or an epithelial-mesenchymal interaction. However, a weak immunoreactivity of tenascin at ECJ was observed in advanced stage of OSMF.

Key words: Epithelial-connective tissue junction, extracellular matrix, oral submucous fibrosis, tenascin

INTRODUCTION

Oral submucous fibrosis (OSMF) is a potentially malignant disorder predominantly seen among betel quid chewers in South Asian countries. Substantial amount of research on elucidating the etiology and pathogenesis of OSMF have been focused on changes in the extracellular matrix (ECM)

proteins. In addition to the maintenance of tissue architecture and cellular proliferation, the ECM is indispensable for cell migration, morphogenesis and wound healing.^[1] Furthermore, it has been reported that the components of ECM modulate the activity of certain cytokines. Therefore, a cell not only influences the neighboring cell or distant cell through direct

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cell to cell contact and by secretion of cytokines and hormones but also by creation of particular matrix environment, leading to the concept that the ECM plays a major role in intercellular communications.^[2]

ECM comprises four classes of components namely collagens, glycoproteins, proteoglycans/glycosaminoglycans and elastin. The main constituents of ECM glycoproteins are fibronectin, tenascin and undulin.^[2] Among these glycoproteins, the expression of tenascin in normal human mucosa and a distinct upregulation in inflammation, tissue repair, in certain reactive situation in the stroma of OSMF and in many tumors, suggests that it may function in organizing the stroma in normal and pathological conditions.^[3]

There are several isomers of tenascin, differentially expressed during embryogenesis and tumorigenesis. Based on the previous studies, tenascin is considered to be a product of mesenchymal and glial cells. Recent studies have shown that it is synthesized not only by fibroblast and muscle cells *in vitro* and *in vivo* but also by epithelial cells.^[3]

Despite extensive research into various aspects of this disease, the actual location of the initiation of fibrosis has eluded researchers and remains inconclusive. It is rather prudent to believe that in some cases of OSMF, the plane of initiation of fibrosis is not in the lamina propria but further down in the submucosa. This seems to be a possible reason for some of the early manifestations of disease such as dryness of the mucosa and functional impairment.^[4]

While the factors that determine this histological alteration in cases of OSMF remain elusive and in early clinical stages, the histopathological features are not pathognomonic. Further studies on different components of ECM glycoproteins during the progression of OSMF may throw light on early diagnosis of the disease. Therefore, the present study was aimed to study the tenascin expression in early, moderate and advanced cases of OSMF, in individuals exclusively associated with the habit of chewing areca nut, to characterize its role in progression of the disease.

MATERIALS AND METHODS

A retrospective cross-sectional immunohistochemical (IHC) study was carried out on 35 patients including 30 cases of OSMF and five cases of normal oral mucosa as control group. The samples for the study were collected from 35 formalin-fixed paraffin-embedded tissue blocks, 10 each from histologically proven and graded early, moderate and advanced stage of OSMF according to the grading system given by Utsunomiya *et al.*^[5] as well as 5 of normal oral mucosa.

Total 70 slide samples were prepared for the study with two slides from each tissue block. The paraffin blocks were

sectioned on a rotary semiautomatic soft-tissue microtome into 5 μ thick sections and transferred onto two glass slides including one on 3-aminopropyl triethoxysilane (APES) coated slide. Albumin coated normal slide was stained with routine Hematoxylin and Eosin staining for the confirmation of lesion, while the APES coated glass slide was stained for IHC staining of tenascin. Circles were drawn with a glass-marking instrument around the tissue, so that the antibody was localized in the area of interest. The antibodies and reagents used for IHC technique were obtained from Sigma Aldrich chemicals private limited and Dako cytometry (Denmark).

Endogenous peroxidase blocking was done by dipping the sections in freshly prepared 3% H₂O₂ in methanol for 10 min. The sections were then washed in phosphate buffer saline, 2 changes 5 min each. For optimal antigen retrieval, the sections were digested in 2 mg/ml pepsin in 0.1 M HCl for 30 min at room temperature. Blocking of nonspecific antigens was done by treating the sections with 10% goat serum at room temperature for 30 min. Sections were then incubated with mouse monoclonal primary antibody (anti-human tenascin) prediluted at 37°C temperature (1:400 dilution), for 1 hr and 30 min in a humid chamber. Next, sections were incubated with biotinylated secondary antibody (F_c conjugated goat-antimouse IgG) diluted in a ratio of 1:200 at room temperature for 30 min. Incubation with secondary conjugate was carried out with streptavidin-peroxidase conjugate, diluted at a concentration of 1:200 for 30 min. For visualization of the tenascin positive areas, sections were incubated with diaminobenzidine tetrahydrochloride at a concentration of 1:30 for 5 min. Counterstaining was done by Mayer's hematoxylin for 45 sec.

Using light microscope, evaluation of antigen-expressing cells was made at 10 \times and 40 \times magnifications. Brown color expression was accounted for antigen positive areas. In each case, three fields were randomly selected and evaluated by two independent observers for tenascin expressing areas in different histological grades of OSMF.

Following criteria was used to define tenascin positivity: (a) Intensity of staining: As bright/weak at junction of epithelium and connective tissue (ECJ); (b) pattern of staining: At ECJ as continuous/discontinuous and diffuse/patchy in deeper connective tissue (CT); (c) presence/absence of staining: Around inflammatory cells, fibroblast and endothelial cells.

Kendall's tau-b test was applied for each parameter to minimize the interobserver errors and with this measure of agreement *P* value was nonsignificant. Thus, the observation made by observer 1 was then subjected for further statistical analysis.

STATISTICS AND RESULTS

All normal oral mucosa sections showed expression of tenascin at the ECJ as faint linear continuous brown staining [Figure 1]. Few cells in the basal cell layer also showed positive staining.

In OSMF group, most of the cases showed retention of the antigen. Its expression varied from one case to another and also within the same tissue section.

Among early OSMF cases, 90% sections exhibited a bright and continuous deposition of tenascin at ECJ, like a band [Figure 2 and Table 1]. In these sections, tenascin protein was deposited diffusely in deeper CT in 80% sections with 20% sections showing patchy areas of tenascin positive areas. Surprisingly, tenascin deposition was also observed around endothelial cells, fibroblasts and inflammatory cells in majority of sections [Table 2].

In moderate OSMF cases, 80% sections exhibited a bright and 70% sections showed continuous deposition of tenascin at ECJ, like a band [Figure 3 and Table 1]. In these sections also, tenascin protein was deposited diffusely in deeper CT in 80% sections with 20% sections showing patchy areas of tenascin positive areas. However, the expression of tenascin, around endothelial cells, fibroblasts and inflammatory cells

was noticed in less number of cases than in early OSMF sections [Table 2].

In OSMF sections of advanced grade, 90% sections showed weak, discontinuous tenascin deposition at ECJ [Figure 4 and Table 1]. A patchy deposition of tenascin was

Table 1: Comparison of intensity and pattern of tenascin expression at epithelium and connective tissue among different grades of oral submucous fibrosis

Histologically proven grade	Intensity-ECJ		Pattern-ECJ	
	Bright (%)	Weak (%)	Continuous (%)	Discontinuous (%)
Early	9 (90)	1 (10)	9 (90)	1 (10)
Moderate	8 (80)	2 (20)	7 (70)	3 (30)
Advanced	1 (10)	9 (90)	1 (10)	9 (90)
Chi-square test	$\chi^2=15.833$ $P=0.001$ VHS		$\chi^2=12.189$ $P=0.003$ HS	

HS: Highly significant, VHS: Very highly significant, ECJ: Epithelium and connective tissue

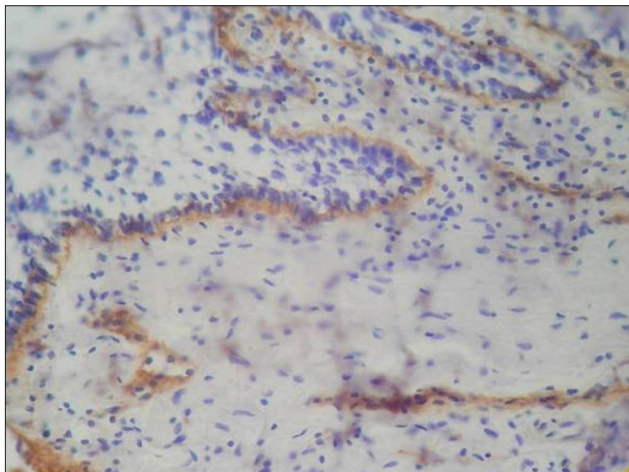


Figure 1: Normal oral mucosal section showing linear deposition of tenascin along the basement membrane (IHC stain, x400)

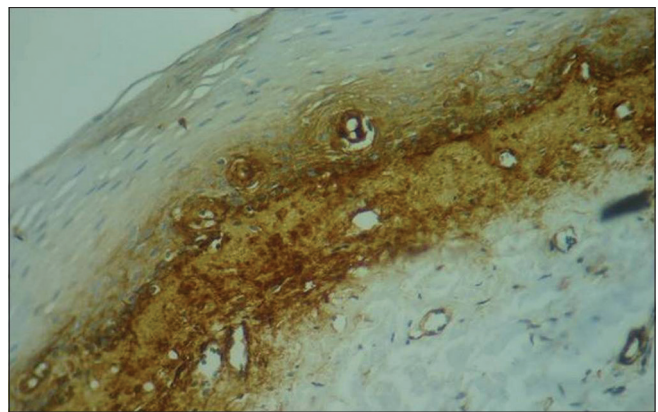


Figure 2: Early oral submucous fibrosis section showing bright immunoreactivity of tenascin at the junction of epithelium and connective tissue with extension into connective tissue (IHC stain, x200)

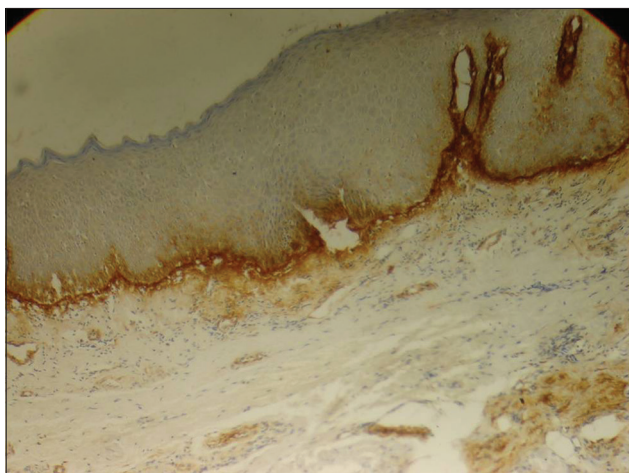


Figure 3: Moderate oral submucous fibrosis section showing bright immunoreactivity of tenascin at the junction of epithelium and connective tissue with extension into connective tissue (IHC stain, x100)

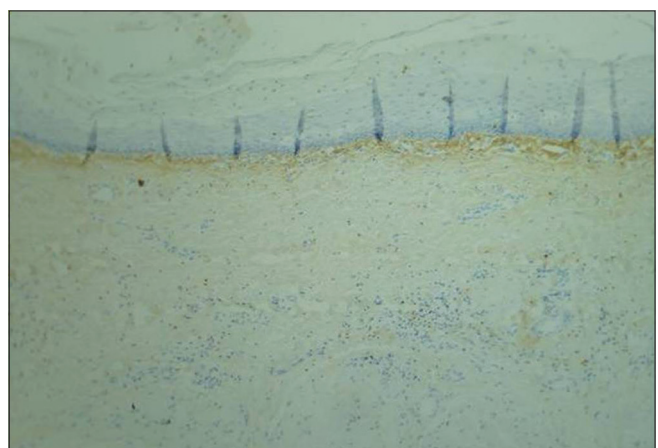


Figure 4: Advanced oral submucous fibrosis section showing weak immunoreactivity of tenascin at the junction of epithelium and connective tissue and patchy deposition in deeper connective tissue (IHC stain, x100)

Table 2: Comparison of pattern of tenascin expression and presence of staining around the cells in connective tissue stroma among different grades of oral submucous fibrosis

Histologically proven grade	Pattern (%)	Inflammatory cells		Endothelial cells		Fibroblasts	
		SE	DCT	SE	DCT	SE	DCT
Early	D - 8 (80)	9+	10+	9+	9+	9+	9+
	P - 2 (20)	1-	0-	1-	1-	1-	1-
Moderate	D - 8 (80)	8+	7+	7+	6+	7+	8+
	P - 2 (20)	2-	3-	3-	4-	3-	2-
Advanced	D - 2 (20)	2+	2+	6+	6+	3+	8+
	P - 8 (80)	8-	8-	4-	4-	7-	2-
Chi-square test	$\chi^2=10.045$	$\chi^2=11.913$	$\chi^2=12.189$	$\chi^2=2.386$	$\chi^2=2.857$	$\chi^2=8.038$	$\chi^2=0.48$
	$P=0.018$ S	$P=0.007$ S	$P=0.003$ S	$P=0.303$ NS	$P=0.24$ NS	$P=0.038$ S	$P=0.787$ NS

S: Significant, NS: Nonsignificant, +: Staining present, -: Staining absent, SE: Subepithelial, DCT: Deeper connective tissue, D: Diffuse, P: Patchy

observed in 80% sections in deeper CT along with variable expression of extracellular deposition mainly around endothelial cells and fibroblasts in subepithelial region [Table 2].

On applying the Chi-square test to tenascin intensity and pattern at ECJ in relation to OSMF grades, P values of 0.001 and 0.003 were obtained, respectively, which were highly significant [Table 1]. When this test was applied for expression of tenascin pattern in deeper CT, a significant P value of 0.018 was obtained [Table 2].

DISCUSSION

OSMF is a potentially malignant disorder predominantly seen among betel quid chewers in South Asian countries. Utsunomiya *et al.* histologically divided the OSMF into 3 categories: Early, moderate (intermediate) and advanced OSMF. In the early stage, large numbers of lymphocytes are seen in subepithelial, CT zone along with myxedematous changes. In the moderate (intermediate) stage, hyalinization appears in subepithelial zone where blood vessels are compressed by fibrous bundles and granulation changes are observed close to the muscle layer. In addition, inflammatory cells are reduced in subepithelial layer. In the advanced stage, inflammatory cell infiltration is hardly seen. Marked fibrous areas with hyaline changes extend from subepithelial to superficial muscle layers. Number of blood vessels is dramatically low in subepithelial zone. Moreover, atrophic degenerative changes start in muscle fibers.^[5]

Although studies have been focused on the molecular mechanisms, especially on the enhanced biosynthesis of collagen by fibroblast, histological investigation on fibrosis, precise staging and progression of the disease and analysis of ECM proteins have not been extensively studied.^[6] Therefore, in the present study, an antibody against the component of ECM was employed in different histological grades of OSMF to observe its role in disease progression.

In control group comprising normal oral mucosa sections, a faint linear continuous immunoreactivity of tenascin was

observed at basement membrane zone and along the basement membrane in vessel walls. An enhanced expression of tenascin was also evident in the basal cells of the oral epithelium. A similar observations had also been demonstrated by previous IHC and immunoelectron microscopic studies by Mathew *et al.*, Anbazhagan *et al.* and Anuradha and Shyamala Devi.^[6-8]

In OSMF group, the expression of tenascin varied from one case to another and also within the same tissue section. Staining was found to be heterogeneous at ECJ, in deeper CT, around inflammatory cells, endothelial cells and fibroblasts, also with variation in pattern and staining intensity.

The immunolocalization of tenascin in 90% sections of early OSMF cases was observed at subepithelial zone-like a band with some extension into underlying CT. This pattern of staining is consistent with previous studies of Becker *et al.*, and Luomanen and Virtanen, wherein the authors have concluded that ECM proteins play an important role in the attachment, regulated gene expression, growth and differentiation of epithelial and mesenchymal cells.^[9,10]

The observations of the present study indicate that probably tenascin possesses an autocrine growth factor-like property and may influence the proliferation associated changes in the subepithelial CT. Further, an enhancement of tenascin immunoreactivity at tips of epithelial ridges in the early stage of the disease in this study was observed which can be attributed to either proliferative organization within the overlying epithelium or to an epithelial-mesenchymal interaction.^[10,11]

The results presented in the study also gave an insight into the CT composition, with varying intensity and distribution pattern (diffuse and patchy) of tenascin. This supplemented the findings of Mighell *et al.* on focal reactive overgrowth of oral mucosa wherein they observed a marked variation in the spatial distribution and intensity of tenascin immunoreactivity. This has been interpreted as areas of active tissue morphogenesis.^[12] However, in the present study, the areas of diffuse staining in the deeper CT observed in early

and moderate stages of OSMF indicate an accentuation of its production by the mesenchymal cells as the disease progress from early to moderate stage.

Utsunomiya *et al.* examined forty biopsied OSMF specimens for expression/deposition modes of eight ECM molecules including tenascin by histochemistry, IHC and *in situ* hybridization methods. The authors observed that in the early stage, tenascin, perlecan, fibronectin and collagen Type III were characteristically enhanced in the lamina propria and the submucosal layer. Furthermore, in the intermediate stage, the ECM molecules mentioned above and elastin were extensively and irregularly deposited around the muscle fibers. The results indicated that the ECM remodeling steps in OSMF are similar to each phase of usual granulation tissue formation.^[5]

Numerous speculations have been put forward to explain the pathogenesis of OSMF, many of which are related to areca nut and its components. The disease process starts from the CT compartment close to the epithelium where the toxic chemical products of areca nut might transform the gene expression of mesenchymal cells.^[13] The observations made in the present study in advanced stage of OSMF provide an additional information that tenascin protein deposited in the deeper region of the CT is most likely related to an altered fibroblast or a genetic modulation in fibroblast as the immunoreactivity of tenascin was weak at ECJ and enhanced in the ECM of deeper CT.

Similar observation has been described by Utsunomiya *et al.* who found that in the advanced stage, the mRNA signals were confirmed in fibroblasts in the submucosal fibrotic areas and the gene expression levels of different ECM molecules varied with progression of fibrosis. In their study, such ECM depositions decreased and were entirely replaced with collagen Type I only leading to restricted mouth opening.^[5]

Tissue specimen showing increased desmoplastic changes in the stroma had enhanced immunoreactivity to tenascin while those accompanied by dense inflammatory cell infiltration had a trace reaction.^[14] The present study showed a distinct correlation between the tenascin expression and the density of inflammatory reaction, the sites of increased immunoreactivity coincided with the sites of intense inflammatory infiltrate both in subepithelial and deeper CT particularly in early and moderate OSMF. These findings are consistent with the studies on dysplasias,^[15] lichen planus^[16] and carcinoma of the cervix.^[17]

Inflammatory mediators have been strongly implicated in increased tenascin protein synthesis. In addition, studies have supported the views that secretion of tenascin is under growth factor regulation, induced by transforming growth factor- β which is produced by T-lymphocytes and is found to stimulate the production of tenascin by mesenchymal cells.^[14]

Further studies on oral lichen planus showed an intense immunoreactivity of tenascin in areas of lymphocytic infiltrate subepithelially. On the contrary, in the present study, few cases showed negative or traces of tenascin immunoreactivity in the CT associated with inflammatory infiltrate in most of the advanced grades of OSMF. This change could be due to the degradation of tenascin by inflammatory cells or disorganized tenascin with production of specific enzymes or to tenascin inhibition factors that remains unanswered. Furthermore, few areas devoid of inflammatory cells and areas with mild inflammation showed weak expression of tenascin. Thus, the results of the study showed lack of relationship between tenascin immunoreactivity and inflammation in advanced OSMF cases. Similar results were also observed in studies on reactive oral mucosal overgrowth and wound healing.^[10,12]

Further in the present study, the tenascin was also found in the vessel walls in moderate and advanced stages and around the plump endothelial cells forming new vascular channel in the early phase of OSMF, an observation made by earlier workers on OSMF. This probably could be through the mediators of inflammation leading to expression of tenascin in vessel walls.^[10,14]

CONCLUSION

A differential expression of tenascin was observed with the progression of disease. The expression of tenascin as bright and continuous deposition at ECJ in early and moderate stages of OSMF signifies either proliferative organization within the overlying epithelium or an epithelial-mesenchymal interaction. Further, its diffuse deposition in deeper CT in most of the sections indicates an accentuation of its production by the mesenchymal cells.

While the weak immunoreactivity of tenascin at ECJ and enhanced expression in the extracellular matrix of deeper CT in the advanced stage of OSMF, provide an additional information that tenascin protein deposited in the deeper region of the CT is most likely related to an altered fibroblast or a genetic modulation in fibroblast cells. Moreover, a lack of relationship between tenascin immunoreactivity and inflammation was observed in advanced OSMF cases.

Tenascin expression was also found in the vessel walls in moderate and advanced stages and around the plump endothelial cells forming new vascular channel in the early phase of OSMF.

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Conflicts of interest

There are no conflicts of interest.

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