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RESEARCH ARTICLE

ASSESSING THE ROLE OF COLLAGEN FIBERS IN THE TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS TO MALIGNANCY- A HISTOCHEMICAL ANALYSIS

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ABSTRACT

Oral sub-mucous fibrosis is a chronic, insidious, debilitating, precancerous condition affecting oral cavity, caused by the habitual use of areca nut. A variety of mediators determine the deposition of collagen that include hormones cytokines and lymphokines. Collagen fibres have been the main focus of study to understand the pathogenesis of the lesion. Picrosirius has the propensity to stain thin fibres also so it has been the most opted stain for study. The main aim of the study here is to compare and correlate the role of collagen fibres and their role in determining the transformation of Oral Submucous Fibrosis (OSMF) to Oral Squamous Cell Carcinoma (OSCC). A total of 40 formalin fixed paraffin embedded tissue (FFPE) blocks were retrieved from the department archives. They comprised of OSMF cases, cases of OSMF associated with OSCC and OSCC. Fresh sections were cut and stained with Picrosirius red to identify the collagen fibers. The content, pattern and distribution of collagen fibers were compared between the different groups. An observable change in stroma was evident as OSMF progressed to oral malignancy

INTRODUCTION

Oral cancer is one of the most fatal health problems faced by the mankind today. Oral cancer in India accounts for about 30% of all new cases annually with highest incidence (More and D'Cruz, 2013). It stands as 6th most common malignancy worldwide and a major cause of cancer morbidity and mortality around the world (Duvvuri, 2009). A high prevalence of smokeless tobacco use has led to an increasing incidence, which in combination with delayed presentation has made oral cancer a major health problem in India. The carcinogenic effects of tobacco acting in synergy with arecanut are well known. Oral Submucous Fibrosis (OSMF) is one of precancerous condition associated with increased risk of transforming into malignancy with 7-13% of OSMF transforming into OSCC (Ekanayaka and Tilakaratne, 2013). OSMF is considered as a chronic debilitating pre-cancerous condition of oral cavity showing juxtaepithelial fibrosis (Arakeri and Brennan, 2013; Rajalalitha and Vali, 2005; Chole *et al.,* 2012). It was first reported in Indian literature at the time of Sushruta as vidari (Chole *et al.,* 2012). It was first defined by Pindborg in 1965 (Issac *et al.,* 2008). This collagen disorder is found to be more prevalent in India and other South East Asian countries in younger age group (Kamath *et al.,* 2013; Tilakratne *et al.,* 2006). In India it accounts for about 0.5% of population (Kamath *et al.,* 2013). Many non-Asian cases have also been reported (Issac *et al.,* 2008). When first identified in 1952 was classified as idiopathic disorder (More *et al.,* 2012). Though OSMF is a condition of multifactorial origin chewing of arecanut is considered to be the prime etiologic factor for the disease (Sudarshan and Annegeri, 2012). Arecoline the main component of arecanut is an alkaloid responsible for fibroblastic proliferation and increased collagen deposition (Khanna and Andrae, 1995). The precancerous nature of OSMF was first described by paymaster. There are many studies with ample evidence proving the role of areca in malignant transformation of OSMF (Ekanayaka and Tilakaratne, 2013).

Collagen-related genes play an important role in the homeostasis of collagen metabolism. There is evidence to suggest that collagen-related genes are altered due to ingredients in the quid. The genes COL1A2, COL3A1, COL6A1, COL6A3, and COL7A1 have been identified as definite transforming growth factor (TGF)-beta targets and are induced in fibroblasts at early stages of the disease. The transcriptional activation of procollagen genes by TGF-beta suggests that it may contribute to increased collagen levels in OSMF (Tilakratne *et al.,* 2006). Studying the structure of collagen has been the main area of interest in OSMF.

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Previously many studies have been done studying the pattern, type, thickness, hue of collagen fibres in various stages of OSMF.

There is a need to answer questions like if OSMF is bilateral then why does OSCC occur unilateral? Very few studies are done to study collagen in OSCC but no study has been done comparing the collagen in OSMF that has transformed into OSCC and OSCC. So the present study was to understand the changes in collagen fibres and correlate them in different stages of OSMF and OSCC. Thus the objective of this study was to identify the collagen hue in various stages of OSMF, OSCC and OSCC associated with OSMF. To compare the fibres in various stages of OSMF, OSCC and OSMF associated with OSCC.

MATERIALS AND METHODS

The study protocol was approved by the ethical committee of the Manipal University. The sample size was determined as per recommendation of Julious (2005). The samples include 10 specimens in OSMF in early and advanced stages, OSCC, OSCC associated with OSMF in respective groups (Table 1).
The Formalin Fixed Paraffin embedded (FFPE) tissue blocks were obtained from the departmental archives. The paraffin-embedded tissues blocks were cut into 4 mm thick sections and were stained using Picrosirius red stain. At first the sections were dewaxed in xylene solutions and hydrated by passing them through a series of alcohol solutions. Later these sections were stained by immersing the sections in .1% Picrosirius red then in haematoxylin solution for 5 minutes. Then these sections were washed in water and differentiated in acid alcohol. The stained sections were examined under a polarizing microscope to observe the polarizing colors of the collagen fibers in the connective tissue.

Statistical analysis

Statistical analysis was done using the SPSS software program 16.0. Fisher Exact Test was done to determine the association between the color of collagen fibers and stage of the diseases. A p value less than 0.05 was considered to be significant.

RESULTS

In this study, collagen subtypes were identified in early and moderately advanced oral submucous fibrosis cases, oral squamous cell carcinoma cases and cases of oral submucous fibrosis associated with oral squamous cell carcinomas. The demographic details are given in Table 1. Out of 40 cases that were studied 14 cases showed reddish orange birefringence of the collagen fibers, 24 cases showed a yellowish to orange birefringence and 2 cases showed a yellowish – green birefringence. Among the 10 cases of early stage OSMF, all the cases showed a yellowish-orange birefringence of collagen fibers Among the 10 cases of moderately advanced OSMF 6 cases showed a yellowish –orange birefringence and 4 cases a reddish –orange birefringence (Fig. 1)

Table 1. The demographic details of the cases studied

Patient	Age	Sex	Site	Lesion	Collagen fibers
1.	42	Female	Buccal mucosa	Early OSMF	Yellow Orange
2	20	Male	Buccal Mucosa	Early OSMF	Yellow-orange
3	58	Female	Buccal mucosa	Early OSMF	Yellow Orange
4	29	Male	Buccal mucosa	Early OSMF	Yellow Orange
5	40	Male	Buccal mucosa	Early OSMF	Yellow Orange
6	58	Female	Buccal mucosa	Early OSMF	Yellow Orange
7	30	Male	Buccal mucosa	Early OSMF	Yellow Orange
8	48	Male	Buccal mucosa	Early OSMF	Yellow Orange
9	38	Male	Buccal mucosa	Early OSMF	Yellow Orange
10	-	Male	Buccal mucosa	Early OSMF	Yellow Orange
11	30	Male	Buccal mucosa	Moderately advanced OSMF	Yellow Orange
12	26	Male	Buccal mucosa	Moderately advanced OSMF	Yellow Orange
13	42		Buccal mucosa	Moderately advanced OSMF	Red orange
14	31	Male	Buccal mucosa	Moderately advanced OSMF	Red orange
15	26	Male	Buccal mucosa	Moderately advanced OSMF	Red orange
16	56	Male	Buccal mucosa	Moderately advanced OSMF	Yellow Orange
17	55	Male	Buccal mucosa	Moderately advanced OSMF	Yellow Orange
18	21	Male	Buccal mucosa	Moderately advanced OSMF	Yellow Orange
19	58	Male	Buccal mucosa	Moderately advanced OSMF	Yellow Orange
20	-	Male	Buccal mucosa	Moderately advanced OSMF	Red orange
21	39	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
22	30	Male	Tongue	OSCC associated with OSMF	Yellow Green
23	45	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
24	60	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
25	40	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
26	34	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
27	46	Male	Buccal mucosa	OSCC associated with OSMF	red orange
28	37	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
29	42	Male	Buccal mucosa	OSCC associated with OSMF	Yellow Orange
30	56	Male	Buccal mucosa	OSCC associated with OSMF	Red orange
31	52	Female	Tongue	OSCC	Red orange
32	72	Female	Buccal mucosa	OSCC	Red orange
33	46	Male	Buccal mucosa	OSCC	Red orange
34	62	Male	Retromolar area	OSCC	Red orange
35	49	Male	Floor of the mouth	OSCC	Yellow Green
36	24	Male	Tongue	OSCC	Red orange
37	32	Male	Buccal mucosa	OSCC	Yellow Orange
38	50	Male	Buccal mucosa	OSCC	Red orange
39	48	Male	Buccal mucosa	OSCC	Red orange
40	44	Male	Buccal mucosa	OSCC	Red orange

OSMF- Oral Submucous Fibrosis, OSCC- oral squamous cell carcinoma

Table 2. Association of the pattern of collagen fibers stain with the disease process

Type of disease	Color of collagen fibers			Fisher- exact value	df	P value
	Red orange	Yellow-orange	Yellow- green			
Early stage of OSMF	0	10	00			
Moderate advance of OSMF	04	06	00	19.0	6	0.004
OSCC with OSMF	02	07	01			
OSCC	08	01	01			

df – degree of freedom p value – 0.004 Fisher exact value – 19

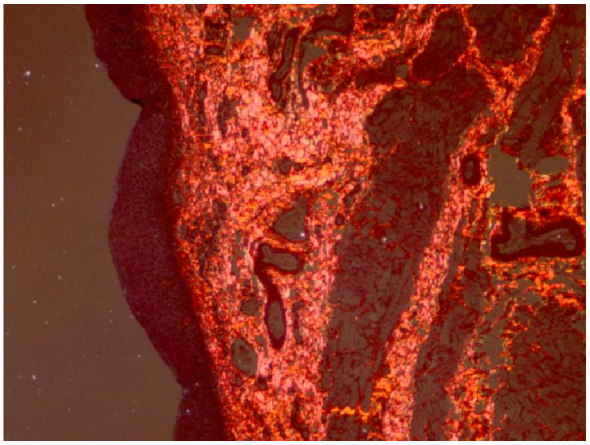

Fig. 1. Picrosirius red staining in OSMF showing reddish-orange birefringence. (Picrosirus stain 10X)

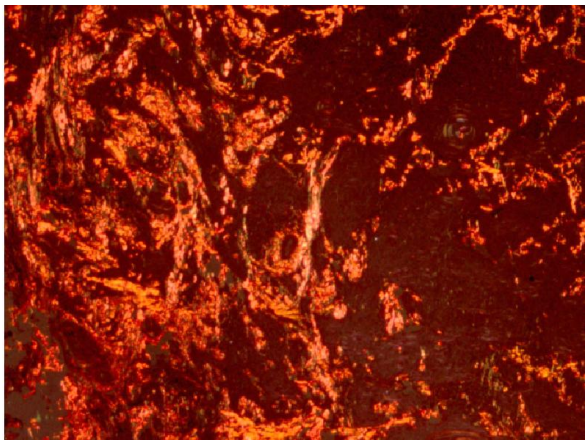

Fig. 2. Picrosirius red staining in OSCC associated with OSMF showing yellow green birefringence. (Picrosirus stain 10X)

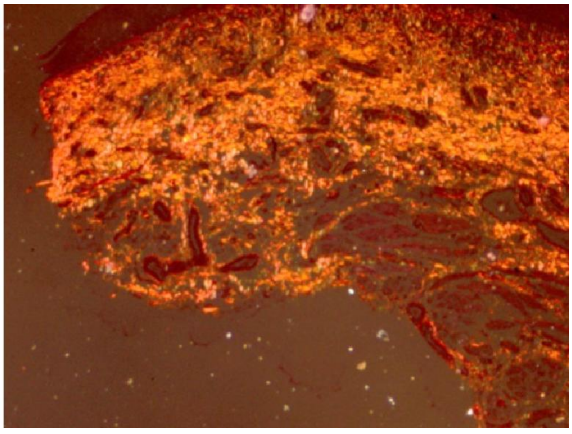

Fig 3. Picrosirius red staining in OSCC showing yellowish- green birefringence. (Picrosirus stain 10X)

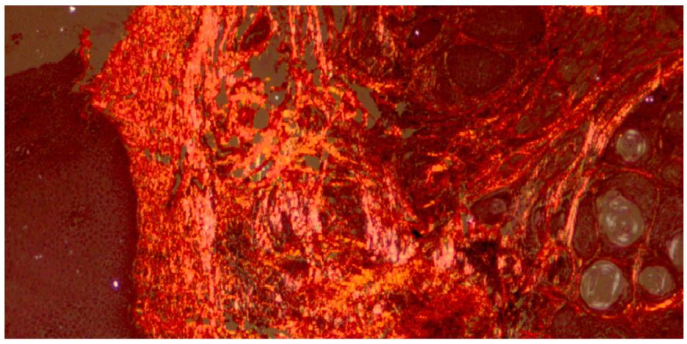

Fig. 4. Picrosirius red staining in OSCC showing reddish-orange birefringence. (Picrosirus stain 10X)

Among the OSMF cases associated with OSCC, 1 case showed yellowish – green birefringence (Fig. 2). 2 cases showed yellowish –orange birefringence and 7 cases reddish –orange birefringence. While, among the OSCC, 1 case yellowish-green birefringence (Fig. 3), 1 case showed yellowish-orange birefringence and 8 cases showed a reddish-orange birefringence (Fig. 4).

In this study we found out the change in colour form yellowish orange to reddish orange in many cases but transformation to yellow green was seen only in two of cases (Fig.1, 2).

A Fischer exact test showed that there is a significant ((p-0.004) change in the structure of collagen fibers as the disease progressed towards malignancy. The observations are tabulated Table 2.

DISCUSSION

In OSMF the equilibrium between MMPs and TIMP is disturbed in such a manner that it ultimately results in increased deposition of ECM. This concept is confirmed by several recent studies (Silvia *et al.,* ?; Ekanayaka *et al.,* ?). The expression of MMP-1 is found to be attenuated in OSF compared to normal oral mucosa. Since MMP-1 is the main human enzyme that degrades fibrillar collagen, this suggests that collagen degradation caused by MMP-1 is down regulated in OSMF.

Arecoline, guvacaine, guvacoline are alkaloids which are known to activate fibroblasts to produce collagen. Flavonoids like catchenins tannins inhibit collagenase, stabilise collagen fibrils and render resistant for degradation. TGF-β is main mediator causing deposition of collagen. This gene cause increase production of collagen by activating procollagen genes up regulating procollagen proteinase and lysyl oxidase activity. They inhibit collagen degradation by production of TIMP

genes and (PAI) plasminogen activator inhibitor. Patients with habits usually expose their oral cavity to various genotoxins, ROS and other irritating agents whose continuous presence leads to impairment of cellular defence, damage the DNA leading to mutations.

Diagnosis of OSMF can be done by using routine H&E stain but to study collagen in specific requires special stains. Here Picrosirius red special stain was used because the Sirius red, a strong cationic dye, stains collagen by reacting with its sulfonic acid and basic groups present in the collagen molecules. Collagen molecules, being rich in basic amino acids, strongly react with acidic dyes.

In present study collagen was identified based on the polarising colours of collagen. The type I and type III collagen gave red and green colour respectively. (Kamath *et al.,* 2013) The different colors and intensities of bifringences displayed by collagen type I, II and III are due to difference their pattern of physical aggregation. Kamath *et al.* in their study tried to quantify and qualify various collagen fibres in different grades of OSMF. They found out that the type I collagen was predominant than type III in increasing grades of OSMF. The mean value of type III collagen was more in stage 2 of OSMF than stage 3. According to studies conducted by Gannepalli *et al.* there was a shift in polarising colours from yellow green to orange red with increasing grades (Ashalata *et al.,* 2012). The varying colours of collagen fibres is due to alteration of optical densities because of difference in thickness of collagen.

In a study conducted by Kalele *et al.* (2014) to study the collagen fibre distribution and hue in various stages of OSCC they found out a change in birefringence from orange red to yellow green as the stage of OSCC advanced. In present study have showed that colour intensity of the collagen fibres in OSMF increases intensity from yellowish green to orange red hue, whereas in OSMF with OSCC change in polarization colors of the thick fibers from yellow-orange to greenish-yellow is considered due to loosely packed fibers which might be composed of procollagens, intermediates or pathologic collagen rather than normal tight packed fibers (Dayan *et al.,* 1989). It is stated that transformation of tissue from preneoplastic state into carcinomas is associated with an increase in collagenolytic enzyme activity. Cancer cells produce collagenases as type I and III collagen are the most abundant component in extracellular matrix of dermal and oral submucosal connective tissue. Hence, it is likely that ability to degrade collagen is essential for invasion and metastasis of neoplastic cells. By increased formation of collagenases, the invading tumor cells are capable of dissolving collagen in connective tissue obstructing its course (Johansson *et al.,* 1997). The loss of tight packing of collagen fibers seen in advanced stages of present study may be, due to increased collagenolytic enzyme activity during transformation of tissue from preneoplastic hyperplasia into carcinoma.

In study gradual change was observed in the polarization colors of thick collagen fibers from initial connective tissue changes of OSF to advanced stages and also from mild, moderate to severe epithelial dysplasia, study of these changes may be used to demonstrate neoplastic changes. As connective tissue changes precede epithelial changes in OSF, the connective tissue changes may give indication of neoplastic change earlier than epithelial changes.

According to Venigella *et al.* (Aparna and Charu, 2010) Type III collagen is present in the advancing front of tumor and Type I collagen increases with advancement of OSMF. So there is a need to study the ECM and stromal content to detect the changes and the stage in which there is transformation of OSMF to OSCC.

Conclusion

OSMF is a common potentially malignant oral lesion in the Indian subcontinent. The role of the stroma in the disease process has been demonstrated. Further studies regarding OSCC associated OSMF have to be done to assist in proper interventional treatment. The question has to be answered. Studies that are undertaken to correlate the collagen changes with epithelial atrophy and basal cell changes is essential to understand the underlying pathogenesis.

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