Original Article

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Dentistry Section

Assessment of Changes in Birefringence and Orientation of Collagen Fibres in Different Grades of Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma using Picrosirius Red and Polarising Microscopy

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ABSTRACT

Introduction: Oral Submucous Fibrosis (OSMF) is a potentially malignant disease with a prevalence of 7-13%. It is characterised by the fibroelastic changes due to excessive deposition of collagen which results in dense fibrous bands and epithelial changes. About 90-95% of oral cancers are Oral Squamous Cell Carcinoma (OSCC). Stroma produced by the invading neoplastic cells are rich in collagen fibres. These collagen fibers have been the main focus of study to understand the pathogenesis of these lesions. Hence, these fibres have been evaluated under polarised microscopy following staining with picrosirius red stain.

Aim: To evaluate changes in birefringence, thickness and orientation of collagen fibres in different histopathological stages of OSMF and OSCC.

Materials and Methods: This observational study involved sections of clinically and histopathologically confirmed cases of OSCC and OSMF from Department of Oral Pathology and Microbiology, Rajarajeswari Dental College and Hospital, Bangalore, India. The study was conducted for a duration of one year from April 2017 to March 2018. The study included total 60 cases among which 20 for each normal mucosa, OSCC and OSMF. Tissue sections were stained with picrosirius red stain and collagen fibres were analysed for colour, thickness and orientation under polarised microscope. Chi-square test was used for statistical analysis. Significance was set at p-value <0.05.

Results: Comparison of birefringence of collagen fibres between OSMF, OSCC and normal mucosa was not statistically significant (p-value=0.37). Orientation of collagen fibres between OSMF, OSCC and normal mucosa was statistically significant with (p-value=0.02).

Conclusion: This study showed a change in colour from yellow orange to orange red in advanced OSMF cases which indicated progression of disease and tightly packing of collagen fibres, suggestive of presence of thick fibres in the extracellular matrix. In OSCC, the colour change from yellow orange to orange red and haphazardly arranged collagen fibres was indicative of transformation of preneoplastic to carcinoma stage.

Keywords: Extracellular matrix, Fibroelastic changes, Malignancy orientation

INTRODUCTION

The word “Collagen” is derived from the Greek, means “glue producing” [1]. It forms the major part of the extracellular matrix [2]. It is the major fibrous glycoprotein present in connective tissue which helps in maintaining the structural integrity of tissues that supports the internal organs and also present in teeth [1]. Collagen Fiber (CF) bundles are referred to as white fibres because of the fact that collection of CF’s appear glistening white in living tissue. Microscopically, they appear as long, wavy, pink fiber bundles after staining with Haematoxylin and Eosin (H&E) [2]. Many histochemical stains have been used to demonstrate CF’s like Van Gieson, Masson’s trichrome, Weigert’s Resorcin Fushsin, modified Movat’s stain, Goldner’s Trichrome method, Wilder Modification of Bielschowsky’s method and picrosirius red [3].

Oral Sub Mucous Fibrosis (OSMF) is a precancerous condition with frequency of malignant transformation reported in the range of 7-13% [4]. It is defined as “an insidious, chronic disease affecting any part of oral cavity and sometimes the pharynx, occasionally preceded by and or associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria with epithelial atrophy, leading to stiffness of the oral mucosa, causing trismus and inability to eat” [5]. OSMF has various aetiological factors which includes ingestion of chilies, arecanut chewing, genetic and malnutrition, nutritional deficiency, iron deficiency and immunological process. Arecanut is considered to be the most important aetiological factor in OSMF which is characterised by deposition of dense CF’s and fibrosis. An alteration of collagen in this disease necessitates in depth understanding of various types of collagen in oral tissues and its intimate relationship to other constituents of the connective tissue components [6].

The OSCC is the most common oral cancer in head and neck region with incidence increasing by 50% in the last decade. The pathogenesis of OSCC is multifactorial associated with cigarette smoke, alcohol and snuff, papilloma virus as well as vitamin deficiencies [7]. The OSCC comprises two discrete compartments, the malignant epithelial cells and the stroma or Extracellular Matrix (ECM). All tissues and organs comprise of a stroma which is acellular that help in cell differentiation, tissue morphogenesis and homeostasis. ECM act as scaffolding for cell adhesion and they influence tumour behaviour. The stroma produced by the invading neoplastic cells are rich in collagen [8].

Collagen demonstrated by picrosirius red stain under polarised microscopy, not only characterises collagenous material specifically and reliably, but also shows orientation of CF’s [9]. Hence, quantitative assessment of birefringence using polarised light microscopy is an important tool to investigate the macromolecular orientation and

organisation of CF's in connective tissues [10]. Examination of CF's by picrosirius red in conjunction with polarising microscope serves as a procedure to differentiate procollagen, intermediate and pathological CF's [11]. The change in polarisation colours and orientation of CF's acts as a prognostic marker indicative of malignant transformation [12]. Determination of the causes and effects of the stromal response will further add on to our understanding of tumor cell interactions with stroma, and also can be prognostic indicators for patients with OSCC and OSMF [13].

The purpose and uniqueness of present study was being that we had assessed connective tissue changes such as birefringence, orientation and thickness of collagen fibres in Early OSMF (EOSMF), Advanced OSMF (AOSMF), Well-differentiated OSCC (WDOSCC), Moderately Differentiated OSCC (MDOSCC) and Poorly Differentiated OSCC (PDOSCC). It would have impact on prognosis because early detection of connective tissue changes are believed to precede neoplastic epithelial changes.

MATERIALS AND METHODS

This was an observational study involving clinically confirmed cases of OSCC and OSMF from Department of Oral Pathology and Microbiology, Rajarajeswari Dental College and Hospital, Bangalore, India, for a duration of one year from April 2017 to march 2018. Informed consent and Institutional Ethical Clearance was obtained with reference no Ref: RRDC&H/129/2015-2016. Clinical examination was done under bright light for OSMF and OSCC.

Inclusion criteria: Tissue sections of clinically and histopathologically confirmed cases of OSMF, OSCC and normal mucosa were included in this study.

Exclusion criteria: Insufficient tissue sample and clinical details cases were excluded.

Sample size calculation: G Power v.3.1.9.2 was used for sample size estimation. Considering the effect size to be measured (f) at 42%, power of the study at 80% and the margin of error at 5%, the total sample size needed was 60, so each study group would comprise of 20 samples.

The total sample size comprised of 60 cases which included 20 cases each of OSMF, OSCC and normal mucosa. Out of 20 cases of OSMF, EOSMF included five cases and AOSMF included 15 cases. Selection of clinical cases of OSMF was done according to Rajendran classification [14]. Out of 20 cases of OSCC, eight cases of WDOSCC, seven cases MDOSCC and five cases of PDOSCC were assessed. OSCC cases were graded based on Broder's grading systems [15]. Total 20 cases of normal mucosa was obtained from patients reported for minor surgical procedures for impacted third molars, which was taken as a control group. Biopsy was taken of the cases to confirm the diagnosis histopathologically and the specimens were preserved in 10% formalin.

Study Procedure

The specimens were subjected for tissue processing and paraffin embedded tissue blocks were prepared. The paraffin embedded tissue blocks were sectioned into 5 micrometer thickness using semiautomatic microtome. The sections were placed on albuminised slides and studied under microscopy after staining with H&E and picrosirius red. All the sections were assessed for orientation, birefringence and thickness of collagen fibres.

- The criteria for assessment of orientation of collagen fibres in relation to epithelium was based on parallel arrangement, haphazardly arranged fibres and mixed (both parallel and haphazard) fibres [12].
- The criteria for assessment of colour of collagen fibres was based on birefringence- greenish yellowish, yellow orange, orangish red and red colour [16].
- The thickness of collagen fibres were based on hue- thick fibres are those who shows dark red to reddish orange colour, thin fibres shows greenish yellow colour and mixed fibres are those who shows yellowish orange to greenish yellow colour [12].

Picrosirius red stained slides were analysed using polarising microscope for colour and orientation of CF's. The image of 10 representative fields in the subepithelial region from each section were captured in a stepwise manner by moving the microscope stage from left to right using Charge-coupled device (CCD) colour camera attached to the research microscope. The images were visualised and stored in a computer for further analysis.

STATISTICAL ANALYSIS

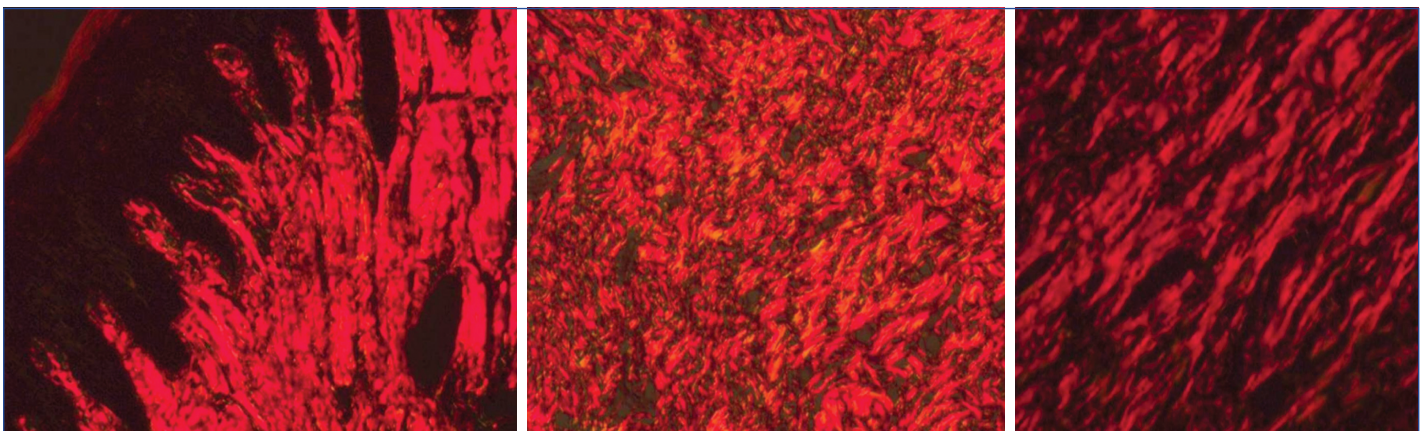
Data was entered in Microsoft excel and analysed using Statistical Package for Social Sciences software (SPSS) version 22.0. Chi-square test was used to compare the distribution of colour, thickness and orientation of CF's between different study groups. Significance was set at p-value <0.05.

RESULTS

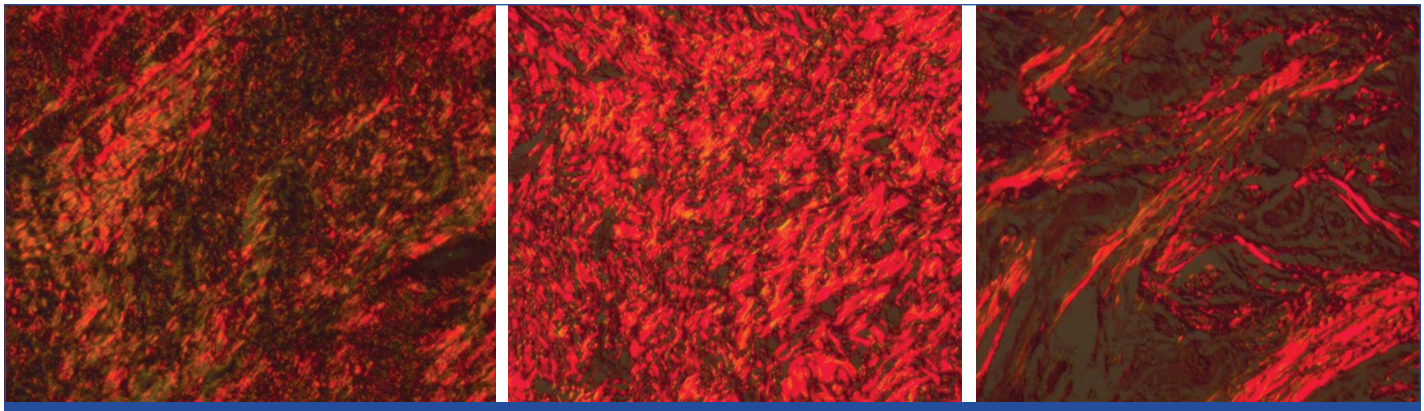
The OSMF and OSCC showed predominantly yellowish orange and orangish red colour and normal mucosa showed predominantly orangish red colour [Table/Fig-1-7].

Colour of collagen fibres	OSMF (N=20)		OSCC (N=20)		Normal mucosa (N=20)		χ^2-value	p-value
	n	%	n	%	n	%		
Greenish yellow	1	5%	1	5%	-	-	6.540	0.37
Yellowish orange	8	40%	9	45%	6	30%		
Orangish red	7	35%	9	45%	13	65%		
Red	4	20%	1	5%	1	5%		

[Table/Fig-1]: Comparison of the colour of collagen fibres in OSMF, OSCC and normal mucosa.



[Table/Fig-2]: Photomicrograph showing predominantly orange red birefringence of collagen fibres in normal mucosa under polarised microscopy (Picrosirius red stain, 40X).
[Table/Fig-3]: Photomicrograph showing yellow orange fibres in OSMF under polarised microscope (Picrosirius red stain, 40X).
[Table/Fig-4]: Photomicrograph showing red birefringence of collagen fibres in OSMF under polarised microscopy (Picrosirius red stain, 40X). (Images from left to right)



[Table/Fig-5]: Photomicrograph showing greenish yellow birefringence of collagen fibers in OSMF under polarised microscope (Picrosirius red stain, 40X).
[Table/Fig-6]: Photomicrograph showing yellow orange birefringence of collagen fibres in OSCC under polarised microscope (Picrosirius red stain, 40X).
[Table/Fig-7]: Photomicrograph showing greenish yellow birefringence of collagen fibres in OSCC under polarised microscope (Picrosirius red stain, 40X). (Images from left to right)

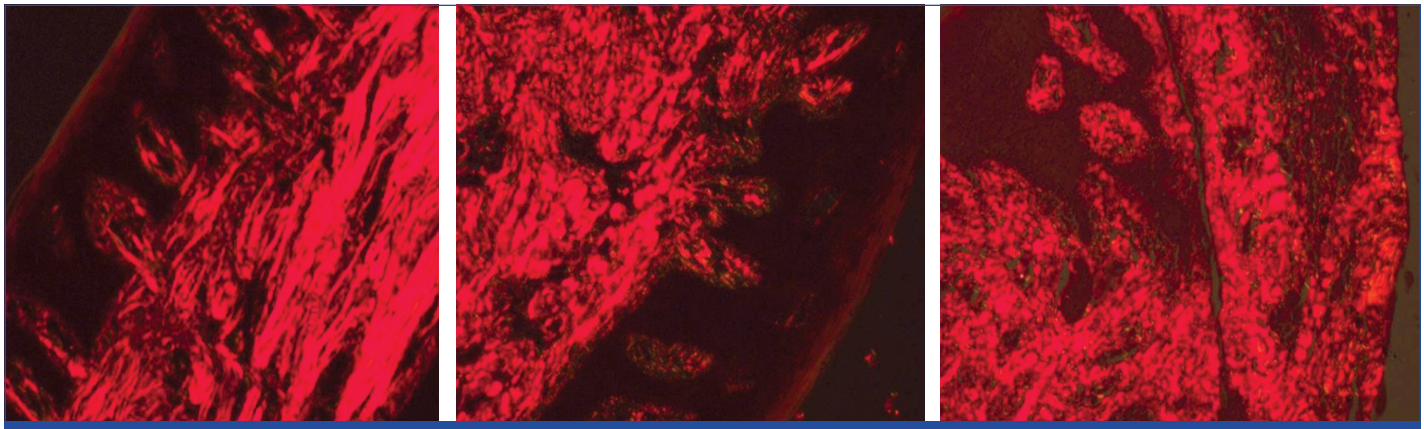
OSMF showed predominantly thick fibres, OSCC and normal mucosa showed predominantly thick and mixed fibres [Table/Fig-8-11]. OSMF showed predominantly parallel orientation, OSCC and normal mucosa showed predominantly haphazard orientation [Table/Fig-12]. EOSMF showed predominantly yellowish orange colour and AOSMF showed predominantly yellowish orange and orangish red colour [Table/Fig-13].

In [Table/Fig-14] EOSMF showed predominantly haphazard pattern and AOSMF showed predominantly parallel orientation.

In [Table/Fig-15] WDSCC showed predominantly orangish red colour, MDSCC showed yellowish orange colour and PDSCC showed predominantly yellowish orange and orangish red colour. In [Table/Fig-16] WDSCC and MDSCC showed predominantly haphazard pattern and PDSCC showed predominantly parallel orientation. Satisfactory

Thickness of collagen fibers	OSMF (N=20)		OSCC (N=20)		Normal mucosa (N=20)		χ^2-value	p-value
	n	%	n	%	n	%		
Thin	1	5%	1	5%	-	-	2.118	0.71
Thick	12	60%	9	45%	10	50%		
Mixed	7	35%	10	50%	10	50%		

[Table/Fig-8]: Showing comparison of thickness of collagen fibers in OSMF, OSCC and normal mucosa.



[Table/Fig-9]: Photomicrograph showing of parallel orientation of collagen fibres in OSMF under polarised microscope (Picrosirius red stain, 40X).
[Table/Fig-10]: Photomicrograph showing of haphazardly arranged orientation of collagen fibres in OSMF under polarised microscope (Picrosirius red stain, 40X).
[Table/Fig-11]: Photomicrograph showing haphazardly arranged orientation of collagen fibres in OSCC under polarised microscope (Picrosirius red stain, 40X). (Images from left to right)

Orientation of collagen fibres	OSMF (N=20)		OSCC (N=20)		Normal mucosa (N=20)		χ^2-value	p-value
	n	%	n	%	n	%		
Parallel	11	55%	7	35%	2	10%	11.996	0.02*
Haphazard	9	45%	9	45%	15	75%		
Mixed	-	-	4	20%	3	15%		

[Table/Fig-12]: Showing comparison of orientation of collagen fibres in OSMF, OSCC and Normal mucosa.
Significance was set at p-value <0.05

Colour of collagen fibres	EOSMF (N=5)		AOSMF (N=15)		χ^2-value	p-value
	n	%	n	%		
Greenish yellow	1	20%	-	-	3.429	0.33
Yellowish orange	2	40%	6	40%		
Orangish red	1	20%	6	40%		
Red	1	20%	3	20%		

[Table/Fig-13]: Showing comparison of colour of collagen fibres in EOSMF and AOSMF.

Orientation of collagen fibres	EOSMF (n=5)		AOSMF (n=15)		χ^2-value	p-value
	n	%	n	%		
Parallel	2	40%	9	60%	0.606	0.44
Haphazard	3	60%	6	40%		
Mixed	-	-	-	-		

[Table/Fig-14]: Showing comparison of orientation of collagen fibres in EOSMF and AOSMF.

Colour of collagen fibres	WDSCC (N=8)		MDSCC (N=7)		PDSCC (N=5)		χ^2-value	p-value
	n	%	n	%	n	%		
Greenish yellow	-	-	1	14.3%	-	-	5.063	0.54
Yellowish orange	3	37.5%	3	42.9%	3	60%		
Orangish red	5	62.5%	2	28.6%	2	40%		
Red	-	-	1	14.3%	-	-		

[Table/Fig-15]: Showing comparison of colour of collagen fibres in WDSCC, MDSCC, PDSCC.

results were obtained on assessment of thickness of collagen fibres in OSMF and OSCC. Also comparison of thickness was not done because of unequal sample size.

Orientation of collagen fibres	WDSCC (N=8)		MDSCC (N=7)		PDSCC (N=5)		χ^2-value	p-value
	n	%	n	%	n	%		
Parallel	2	25%	2	28.6%	3	60%	2.387	0.67
Haphazard	4	50%	4	57.1%	1	20%		
Mixed	2	25%	1	14.3%	1	20%		

[Table/Fig-16]: Showing comparison of orientation of collagen fibers in WDSCC, MDSCC, PDSCC.

DISCUSSION

Extracellular matrix has a crucial role in tumourigenesis. Growth of tumour size beyond a minimal size of 1-2 mm is dependent on stroma. In tumours, quantity of stroma varies from minimal to desmoplastic and also differ from one tumour to another [16].

The OSMF is a potentially malignant disorder resulting from increased production of collagen and there is decreased breakdown, leading to excessive accumulation resulting in juxta-epithelial inflammatory reaction and fibrosis in the oral mucosa. The key pathogenic process here is altered collagen metabolism where early stages of the condition is characterised by excessive deposition of CF's and the advanced stages show dense CF's bundles exhibiting varying degrees of hyalinisation [17].

OSCC is leading malignancy of the Indian population and known for its unpredictable course of progression leading to serious impairment of the tissues involved wherein stromal component influence on the progression of cancer [18]. These stromal elements can be observed using special stains like picrosirius red, Van Geison and Masson's Trichrome, which are used to intensify the colour of collagen fibres [11].

Picrosirius red stain was preferred for the demonstration of collagen and it has provided excellent results consistently due to the fact that the polarising microscopy and the parallel relationship between dye and the collagen, results in an enhanced birefringence and also very thin fibres are detectable [13]. It also shows orientation of CF's very clearly [11].

In the present study, out of 20 cases of OSMF, 8 (40%) cases showed yellowish orange birefringence, 7 (35%) cases showed orangish red birefringence, 4 (20%) cases showed red birefringence and 1 (5%) case showed greenish yellow birefringence. In EOSMF, predominant colour was yellowish orange found in 2 (40%) cases and greenish yellow in 1 (20%) case whereas in AOSMF cases there was a change in colour from orangish red in 6 (40%) cases to red colour in 3 (20%) cases. Hence, suggesting that majority of the CF's in EOSMF showed yellowish orange colour and AOSMF cases showed yellowish orange and orangish red colour. A study done by Ceena DE et al., showed EOSMF showed greenish yellow colour and AOSMF showed orangish red to red colour, suggesting that tight packing of CF's in OSMF progressively increased as the disease progressed [19]. Similarly, study by Ashalata G et al., on optical densities of collagen in OSMF found polarising colour from yellowish green to orangish red as the histopathological grade of the disease increased [20].

In the present study, out of 20 cases of OSMF, 11 (55%) cases showed parallel orientation of CF's and 9 (45%) cases showed haphazardly arranged CF's. In EOSMF 40% (2 cases) cases showed parallel orientation of CF's where as 3 (60%) cases showed haphazardly arranged CF's and in AOSMF 9 (60%) cases showed parallel orientation and 6 (40%) of cases showed haphazardly arranged CF's. Similar findings were seen in study conducted by Smitha BR and Donoghule M, where histologically most of the CF's were parallel to the epithelium, and there was statistically significant difference in orientation between OSMF and control groups. Chronic stimulation of oral mucosa by the irritants could be the reason for unidirectional alignment of clinical fibrous bands. Chronic stimulation might result in scar formation similar to that of wound healing leading to change in the orientation of CF's bundles parallel to the epidermis [21]. Similarly, a study by Ganganna K et al., showed a significant change of CF's arrangement from early stages of OSMF to advanced stages which coincided with the changes found in degrees of epithelial dysplasia. The polarisation colours of thick CF's showed a gradual change from predominantly yellowish orange to greenish yellow in advancing connective tissue stages and degrees of epithelial dysplasia [22].

In a study conducted by Parveen S et al., mild cases of OSMF showed loosely arranged thin yellowish orange coloured CF's, moderate cases OSMF showed thin fibres yellowish orange colour and thick red coloured fibres. In AOSMF, tightly packed bundles of red CF's were seen parallel to the epithelium. These results were in accordance to present study [23].

In the present study, out of 20 cases of OSCC, 9 (45%) cases each showed yellowish orange and orange red birefringence of collagen fibres and 1 (5%) case, each showed greenish yellow and red birefringence. In WDSCC, 3 (37.5%) cases showed yellowish orange colour and 5 (62.5%) cases showed orangish red colour. In MDSCC, predominant colour was yellowish orange with 3 (42.9%) cases, 2 (28.6%) cases showed orangish red and in PDSCC 3 (60%) cases showed yellowish orange and 2 (40%) cases showed orangish red colour. These results suggested that change in colour of collagen fibres in OSCC from yellowish orange to orangish red with increasing severity of OSCC. Similar findings were noticed in study by Rakheja M et al., on CF's in OSCC, which showed a higher preponderance of the orangish red birefringence in the WDOSCC (66.6%) and MDOSCC (77.7%) cases [18]. A study by Kalele KK et al., CF's distribution and hue in various stages of OSCC were assessed and found that birefringence from orangish red to yellowish green as the stage of OSCC advanced [13]. MDSCC cases showed predominantly yellowish orange colour. Similar results was observed in a study by Manjunatha BS et al., showed changes in birefringence specifically in the proximity of tumour islands from yellowish orange to greenish yellow in MDSCC and PDSCC. They observed stromal change stain in different thickness of collagens with the progression of neoplasm with a significant change in the arrangement from the early stage to the advanced stage according to tumourigenesis [24]. The results of present study were further supported by a study by Sharma R et al., which indicated that as OSCC regresses from WDOSCC to PDOSCC. Transformation from mature to immature collagen indicates the change in colour from reddish orange to yellowish orange to greenish yellow [25].

In the present study, 20 cases of OSCC were assessed for orientation of CF's among this WDOSCC 4 (50%) cases were haphazardly arranged, in MDSCC 4 (57.1%) cases were haphazardly arranged and in PDSCC 3 (60%) cases were parallelly arranged. These results were in agreement with a study conducted by Gawande M et al., which showed predominate haphazard arrangement of CF's in OSCC. They concluded that it was indicative of increased collagenolytic enzyme activity during transformation of preneoplastic to carcinoma stage [12].

In the present study, 20 cases each of OSMF, OSCC and normal mucosa were compared for thickness of CF's in which OSMF 12 (60%) cases showed thick fibres, OSCC 10 (50%) cases

S. No.	Author's name and year	Place of study	Number of subjects	Parameters assessed	Conclusion
1	Ceena DE et al., 2009 [19]	Mangalore	40 cases of OSMF 10 cases of normal mucosa	Thickness and colour of CF's in OSMF	Mouth opening was restricted with advancing stages of OSMF
2	Ganganna K et al., 2012 [22]	Tumkur	91 cases of OSMF	Colour of CF's in OSMF	Significant change in birefringence of collagen between connective tissue stages
3	Kalele KK et al., 2014 [13]	Pune	10 cases each of WDSCC, MDSCC,PDSCC	Colour of CF's	Stromal changes at the invading front of the tumor islands and with increasing grade of the tumour can be evaluated more efficiently with the use of Picrosirius red stain
4	Gawande M et al., 2015 [12]	Wardha	15 cases each of OSMF, OSCC,OSMF with OSCC 5 cases of normal mucosa	Orientation and thickness of CF's	Changes in orientation and thickness could act as a indicative of malignant transformation.
5	Modak N et al., 2015 [6]	Navi Mumbai	30 cases of OSMF 10 cases of normal mucosa	Colour and thickness of CF's	Help to assess its role in diagnostic evaluation, to determine the prognosis of the disease as well as to provide useful predictive treatment modalities to them.
6	Present study Ashwini C.P et al., 2017-2018	Bengaluru	20 cases each of OSMF, OSCC and normal mucosa	Colour, orientation and thickness of CF's in OSCC,OSMF and normal mucosa	Statistically significant results were obtained on comparison of orientation of collagen fibres. Changes in colour, thickness and orientation of collagen fibres could act as a prognostic marker indicative of malignant transformation in OSMF and OSCC.

[Table/Fig-17]: Comparison of the orientation and colour, thickness of collagen fibres between different studies [6,12,13,19,22].

showed mixed fibres and normal mucosa 10 (50%) of cases showed mixed fibres. The study indicated that the change in orientation and thickness of CF's in OSMF could acts as a prognostic indicator for malignant transformation. Statistically significant result (p-value=0.02) was obtained for comparison of orientation of CF's in OSMF and OSCC. These changes in polarisation colour, orientation and thickness could be indicative of numerous enzymatic actions, which are taking place as a part of change in connective tissue which occurred during malignant transformation [12]. Various studies for comparison of the orientation, colour, thickness of collagen fibres have been depicted in [Table/Fig-17] [6,12,13,19,22].

Limitation(s)

Comparison of thickness of collagen fibres between histopathological grades of OSMF and OSCC could not be done because satisfactory results were obtained on assessment of thickness of collagen fibres in OSMF and OSCC and unequal sample size.

CONCLUSION(S)

In this study, comparison of CF's in OSMF, OSCC and normal mucosa for colour, orientation and thickness were demonstrated and found that change in colour from yellowish orange to orangish red in AOSMF cases indicated the progression of disease and tightly packing of collagen fibres suggested the presence of thick fibres in the extracellular matrix. In OSCC, the colour change from yellowish orange to orangish red and haphazardly arranged collagen fibres was indicative of increased collagenolytic enzyme activities which were observed during transformation of preneoplastic to carcinoma stage. The distinctive polarising colours produced by the picrosirius red stain under polarising microscope on OSMF and OSCC would unambiguously act as important contrivance in accurate grading and definitely would have impact on prognosis. Further studies with picrosirius red stain with polarisation technique should be supplemented with molecular markers and should be conducted on a larger sample to improve the study.

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