Original Article

A quantitative and qualitative comparative analysis of collagen fibers to determine the role of connective tissue stroma in oral squamous cell carcinoma using special stains and polarized microscopy

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Abstract

Background: Solid tumors such as oral squamous cell carcinoma (OSCC) are composed of malignant epithelial cells and the stroma in which these cells are dispersed. As the tumor progresses, the extracellular matrix undergoes dramatic morphological and architectural changes. Special stains make analysis easy and less erroneous by highlighting the area of interest and can be used to study these changes.

Aim: The aim of the study was to analyze morphological changes in collagen fibers in various histological grades of OSCC using Masson's trichrome (MT) and Picrosirius red (PSR).

Study Design: The study comprised 74 tissue samples, divided into two groups: Group I consisted of 63 cases of histologically proven OSCC (39 cases of well-differentiated squamous cell carcinoma [WDSCC], 17 moderately differentiated squamous cell carcinoma [MDSCC] and 7 poorly differentiated squamous cell carcinoma [PDSCC]) and Group II consisted of 11 cases of normal mucosa as controls.

Materials and Methods: Sections were stained with hematoxylin and eosin, MT and PSR and observed under light and polarizing microscope, respectively.

Statistical Analysis: ANOVA, Tukey's honestly significant difference *post hoc* multiple comparison test, Chi-square test and paired *t*-test were used for the statistical analysis.

Results: As the grade of OSCC progressed, collagen fibers became thin, loosely packed and haphazard. The mean area fraction also decreased. They exhibited orange–red hue and strong birefringence in WDSCC, yellowish-orange hue and strong birefringence in MDSCC and greenish-yellow hue and weak birefringence in PDSCC.

Conclusion: Initially, there is a reorganization of the collagen fibers in an attempt to prevent the invasion of tumor cells, but as cancer progresses, the stromal change enhances movement of the tumor cells within it, leading to metastasis.

Keywords: Collagen, masson's trichrome, oral squamous cell carcinoma, picrosirius red, polarizing microscopy, tumor microenvironment

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INTRODUCTION

Oral cancer is one of the most formidable health problems in terms of morbidity and mortality that humankind is facing today.[1] Oral and pharyngeal cancers grouped together are the sixth most common cancers in the world.[2] Globally, about 500,000 new oral and pharyngeal cancers are diagnosed annually, and three-quarter of these are from the developing world, including 65,000 cases from India.[3]

Formation of a clinically relevant tumor requires the support of the surrounding normal stroma, referred to as the tumor microenvironment (TME). In cancer, the malignant cells are known to create their own TME, which crucially affects both the malignant cells themselves and all other cells within the extracellular matrix (ECM).[4] The ECM is composed of proteoglycans, glycoproteins, water, collagen and elastic fibers.[5] As the tumor develops and progresses, the ECM undergoes dramatic morphological and architectural changes.[6] Collagen is the major structural protein of the connective tissue and is synthesized by fibroblasts which play a vital role in maintaining structural integrity and in determining tissue function.[7] Collagen regulates tumor-associated immune infiltration and is essential for tumor angiogenesis.[8,9]

Special stains being less expensive and less time consuming when compared to immunohistochemistry can be routinely done to understand the stromal changes. They make the analysis easy as well as less erroneous by highlighting the area of interest.[10]

The aim of the study is to analyze the morphological changes in collagen fibers in various histological grades of oral squamous cell carcinoma (OSCC) using Masson's trichrome (MT) and Picrosirius red (PSR).

Objectives

1. To compare the distribution and pattern of collagen fibers in terms of fiber arrangement, orientation and packing in various grades of OSCC using both PSR and MT stain
2. To compare the nature of collagen fibers in terms of hue and birefringence and to compare the mean area fraction occupied by collagen fibers in various grades of OSCC and normal mucosa using PSR stain
3. To compare the results of both the stains PSR and MT in terms of collagen fiber thickness in various grades of OSCC and normal mucosa.

MATERIALS AND METHODS

The study comprised 74 tissue samples, divided into two groups: Group I consisted of 63 cases of histologically proven OSCC and Group II consisted of 11 cases of normal mucosa as control. Of the 63 cases, 39 cases were of well-differentiated squamous cell carcinoma (WDSCC), 17 moderately differentiated squamous cell carcinoma (MDSCC) and 7 poorly differentiated squamous cell carcinoma (PDSCC). The tissue specimens for the case and control groups were retrieved from the archives between the years 2015 and 2017.

Formalin-fixed paraffin-embedded tissues were sectioned at 5 μm and three sections were prepared. One section was stained with hematoxylin and eosin, observed under a light microscope and graded according to the Broder's classification. The second section was stained with MT and observed under the light microscope. The third section was stained with PSR and observed under a polarizing microscope.

Parameters for evaluation and evaluation procedure

1. Fiber arrangement and orientation: Collagen fibers were categorized as parallel or haphazard based on their appearance in relation to the tumor islands. The evaluation was performed in the five selected fields at ×100 magnification[11]
2. Packing of fibers: The collagen fibers were categorized as "dense" or "loose" based on their appearance in the five selected fields at ×100 magnification in the immediate vicinity of tumor islands[11]
3. Fiber thickness: The images of both PSR and MT-stained slides were obtained at magnification ×400 (in the normal tissues, collagen fibers from lamina propria were studied, while in OSCCs, collagen fibers around tumor islands were studied) and entered into image analysis software (Image J, version 1.46r, developed by National Institute of Health and Laboratory for Optical and Computational Instrumentation, University of Wisconsin, United States). In each section, two separate high-power fields with at least 50 fibers of each size (25 each of thick and thin fibers) were examined.[7] Collagen fibers of thickness 2–10 μm were considered as thick Type I fibers and fibers 0.5–1.5 μm in diameter were considered as thin Type III fibers[12]
4. Hue and Birefringence: The PSR-stained slides were evaluated at five random high-power fields in the connective tissue stroma at ×400 magnification (in the normal tissues, collagen fibers from lamina propria were studied, while in OSCCs, collagen fibers around tumor islands were studied). Predominant hue exhibited by collagen fibers was noted as orange–red (OR), yellowish orange (YO) and green or greenish yellow (G/GY) and birefringence as strong or weak[11,13-16]
5. Mean area fraction: The images of PSR-stained sections at a magnification of ×400 were fed into image analysis software and the percentage of area occupied

by collagen fibers in a given field was calculated for each grade of OSCC and also for normal mucosa.[11]

Statistical analysis was done by IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) and JMP 10 of SAS 9.3 (SAS Inc., Cary, NC, USA) in two-way randomized control study layout followed by Tukey's honestly significant difference (HSD) *post hoc* multiple comparison test. Association/correlationship of attributes was studied by the Chi-square test of Association.[17]

RESULTS

Arrangement and orientation of collagen fibers around the tumor islands

Picrosirius red

The collagen fibers exhibited predominantly parallel orientation in most of the cases of WDSCC (53.85%), but it changed gradually to a haphazard pattern; 76.47% cases of MDSCC and all the cases of poorly differentiated (100%) showed haphazard orientation as shown. The results were found to be statistically significant ($P = 0.008$).

Masson's trichrome

The results were similar to those of PSR. In 53.85% of the WDSCC cases, the collagen fibers showed a parallel orientation, while in 70.59% of the MDSCC and 100% of PDSCC cases, the fibers exhibited haphazard orientation ($P = 0.015$), as shown in Figures 1-3.

Packing of collagen fibers in the immediate vicinity of tumor islands

Picrosirius red

The collagen fibers were found to be densely packed in 100% cases of normal mucosa and in most of the cases of WDSCC (64.10%), while they were loosely packed in most of the cases of MDSCC (58.82%) and in all the cases of PDSCCs (100%). The results were found to be statistically significant ($P = 0.005$).

Masson's trichrome

The results were similar to those of PSR ($P = 0.016$). WDSCC (58.97% cases) showed dense packing, while MDSCC (58.97% cases) PDSCC (100% cases) showed loose packing of collagen fibers. The packing of collagen fibers around tumor islands changed from densely packed to loosely packed on progression from well to poorly differentiated OSCC, as shown in Figures 1-3.

Fiber thickness in different grades of oral squamous cell carcinoma

Picrosirius red

In WDSCC and normal mucosa samples, the collagen fibres appeared predominantly as bundles of thick fibers having an average thickness of 2.94 ± 0.92 μm and 2.72 ± 1.02 μm, respectively. The average thickness of collagen fibers in MDSCC samples was 1.98 ± 0.82 μm and in PDSCC samples was found to be 1.36 ± 0.50 μm. The thickness of fibers gradually decreased as the carcinoma progressed from well to poorly differentiated. The two-way ANOVA test revealed a significant difference in fiber thickness among the different grades of OSCC, ANOVA $F(3,32)$ statistic = 6.27; $P = 0.002$.

The *post hoc* multiple comparisons of different groups using Tukey's HSD test found that the thickness of collagen fibers of normal mucosa was significantly different ($P < 0.05$) with PDSCC, but there was no difference ($P > 0.05$) with WDSCC and MDSCC.

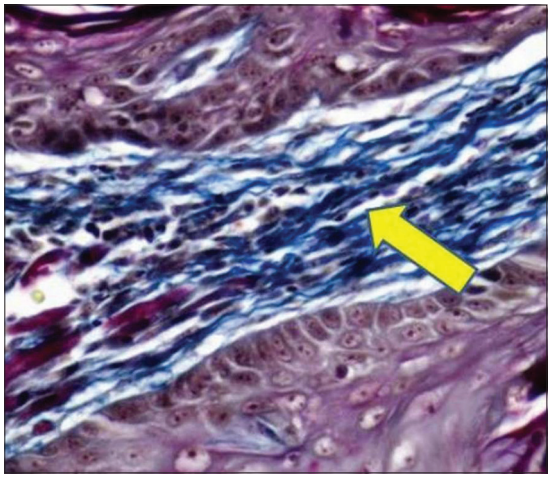

Figure 1: Histopathological image of Masson's trichrome-stained section of well-differentiated oral squamous cell carcinoma showing densely packed collagen fibers (arrow) exhibiting parallel arrangement (×400)

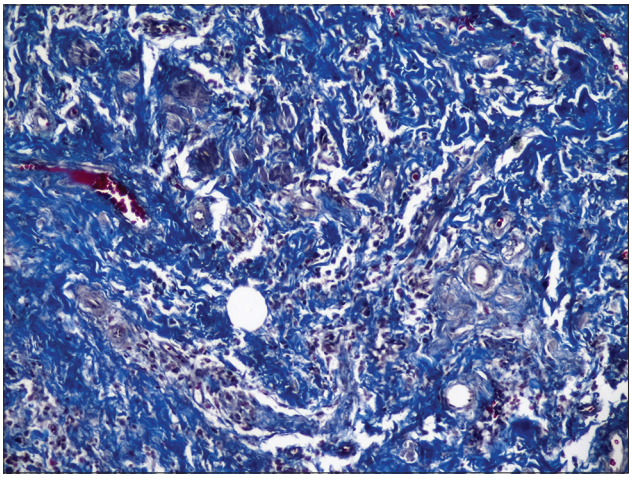

Figure 2: Histopathological image of Masson's trichrome-stained section of moderately differentiated oral squamous cell carcinoma showing haphazardly arranged loosely packed collagen fibers (arrow) at ×400

Masson's trichrome

The average thickness of collagen fiber bundles or individual fibers was highest in WDSSC (2.61 ± 0.75 μm) followed by normal mucosa (1.75 ± 0.53 μm) and MDSCC (1.75 ± 0.64 μm) and PDSCC (0.98 ± 0.34 μm). The two-way ANOVA *F*-test found significant difference in OSCC grades with respect to fiber thickness ($P < 0.001$).

The *post hoc* multiple comparisons of different groups using Tukey's HSD test in revealed WDSSC were significantly different with MDSCC, PDSCC and normal mucosa. MDSCC and PDSCC were statistically at par with normal mucosa.

The comparison between the results of fiber thickness of PSR and MT by means of paired *t*-test was found to be statistically insignificant ($P = 0.0658$).

Hue and birefringence exhibited by collagen fibers

In majority of the samples of normal mucosa and WDSCC, the collagen fibers exhibited predominantly OR birefringence. In most of the cases of MDSCC, the fibers exhibited predominantly YO birefringence, and in majority of the PDSCC cases, the fibers exhibited predominantly GY birefringence. The results were found to be highly significant ($P = 0.008$), as shown in Table 1 and Figures 4-6.

The collagen fibers, in majority of the control tissues, WDSCC and MDSCC samples, exhibited strong birefringence. The fibers in majority of the PDSCC samples exhibited a weak birefringence, as shown in Figures 4-6. The results as shown in Table 2 were statistically highly significant ($P = 0.001$)

Mean area fraction

The mean area fraction was 31.46% ± 3.48% in the normal mucosa samples, 25.14% ± 4.21% in WDSCCs, 19.02% ± 3.49% in MDSCCs and 10.60% ± 3.18% in PDSCCs. The two-way ANOVA *F*-test found a significant difference in different groups with respect to mean area fraction ($P < 0.001$). The *post hoc* multiple comparisons of different groups using Tukey's HSD test revealed that WDSSC, MDSCC, PDSCC and normal mucosa were significantly different with each other.

The mean area fraction occupied by collagen fibers in a given field decreased gradually as the OSCC progressed from well to poorly differentiated.

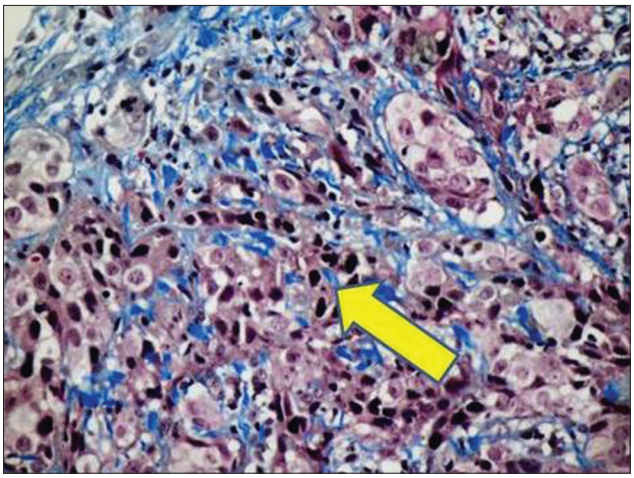

Figure 3: Histopathological image of Masson's trichrome-stained section of poorly differentiated oral squamous cell carcinoma showing haphazardly arranged loosely packed collagen fibers (arrow) at ×400

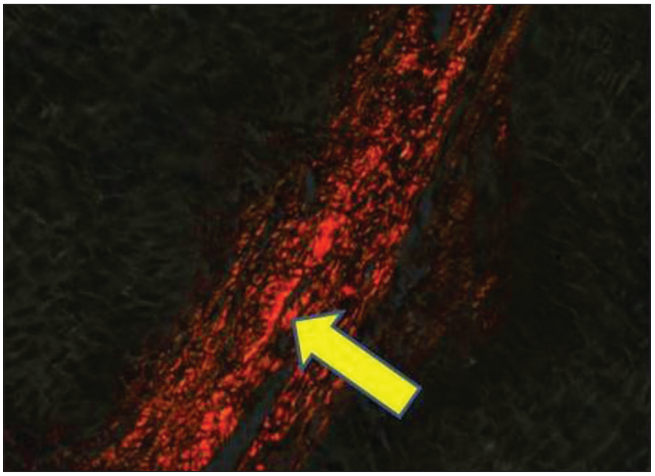

Figure 4: Histopathological image of Picrosirius red-stained section of well-differentiated oral squamous cell carcinoma showing densely packed collagen fibers (arrow) exhibiting parallel arrangement and orange–red birefringence (×400)

Table 1: Polarizing colors observed in control tissues and different grades of oral squamous cell carcinoma

Group	Hue (%)			Total (%)	χ^2	df	*P*
	OR	YO	GY				
Normal mucosa	6 (54.55)	4 (36.36)	1 (9.09)	11 (100)	22.162	6	0.001**
WDSCC	24 (61.54)	11 (28.21)	4 (10.26)	39 (100)			
MDSCC	6 (35.29)	9 (52.94)	2 (11.76)	17 (100)			
PDSCC	0 (0)	2 (28.57)	5 (71.43)	7 (100)			
Total	36 (48.65)	26 (35.14)	12 (16.22)	74 (100)			

**Significant at $P (<0.01)$. OR: Orange-red, YO: Yellowish orange, GY: Greenish yellow, WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma

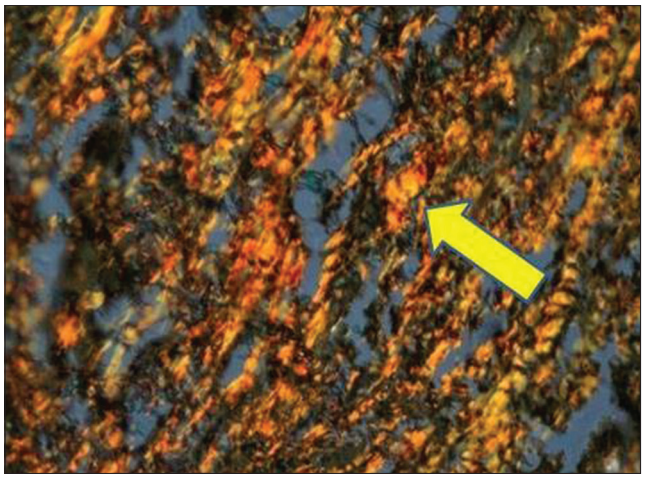

Figure 5: Histopathological image of Picrosirius red-stained section of moderately differentiated oral squamous cell carcinoma showing haphazardly arranged loosely packed collagen fibers (arrow) exhibiting yellowish-orange birefringence (×400)

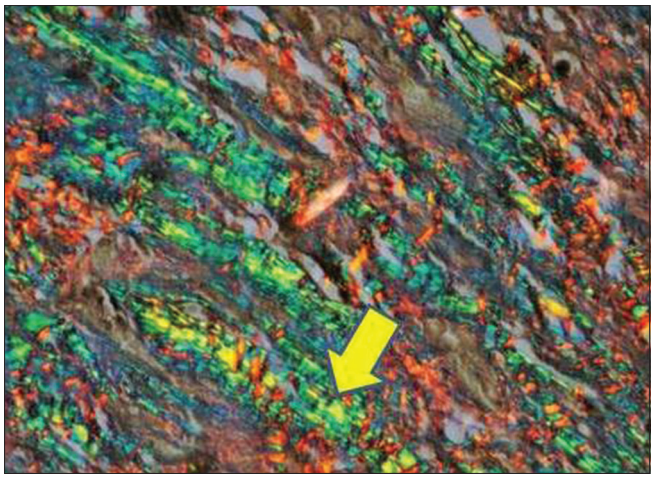

Figure 6: Histopathological image of Picrosirius red-stained section of poorly differentiated oral squamous cell carcinoma showing haphazardly arranged loosely packed collagen fibers (arrow) exhibiting greenish-yellow birefringence (×400)

Table 2: Nature of birefringence observed in control tissues and different grades of oral squamous cell carcinoma

Group	Birefringence		Total	χ^2	df	*P*
	Strong	Weak				
Normal mucosa	10 (90.91)	1 (9.09)	11 (100)	15.418	3	0.001**
WDSCC	35 (89.74)	4 (10.26)	39 (100)			
MDSCC	13 (76.47)	4 (23.53)	17 (100)			
PDSCC	2 (28.57)	5 (71.43)	7 (100)			
Total	60 (81.08)	14 (18.92)	74 (100)			

**Significant at *P* (<0.01). WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma

DISCUSSION

As the grade of OSCC progressed, the orientation of collagen fibers changed from parallel to haphazard, similar to other studies.[11,16,18] In normal stroma, collagen fibers appear curly and anisotropic. During early cancer progression, as in WDSS, the amount of collagen in the stroma increases, and collagen fibers appear straighter and are aligned parallel to the tumor border. With the progression of cancer, the arrangement and pattern changes, the collagen fibers become bundled and are oriented perpendicularly to the basement membrane.[19]

Cancer-associated fibroblasts reorganize the stroma by secreting ECM and enzymes that covalently cross-link the collagens fibers by physically pulling on the collagen network. As a result, the stromal network becomes stiffer. The dramatic reorganization of the stroma in invasive cancers is likely to contribute to changes in the migratory properties of tumor cells that lead to later metastasis.[19]

Packing of collagen fibers changed from densely packed to loosely packed on progression from WDSCC to PDSCC. These findings were similar to that of another study.[11] The dense arrangement of collagen fibers is due to the increased synthesis and increased cross-linking of fibrillar collagen by activated fibroblasts or myofibroblast.[20] With the progression of cancer, there is an increased degradation of the stroma, making it loosely packed.[14,21]

Similar to other studies, the thickness of the collagen fibers decreased with the progression of OSCC from WDSCC to PDSCC.[14,22] However, the thickness of the fibers of WDSCC and normal mucosa was comparable. Increase in the number of thin collagen fibers and decrease in a number of thick collagen fibers on progression from lower to higher grade of OSCC could, initially, be due to the initial fibroproliferative response and in later stages, due to abnormal collagen production and defective maturation, which may promote the neoplastic growth.[14] Thick fibers are type I collagen composed of closely packed thick fibrils, whereas thin fibers are type III collagen made up of loosely disposed thin fibrils.[23,24]

Strong birefringence of the OR hue of control tissue and WDSCC appears to be related to the higher amount of thick/Type I collagen fibers. The weak birefringence of GY hue in PDSCCs could be either because of increase in the number of thinner fibers (Type III collagen fibers that could be identified as reticulin fibers) or it could be the result of abnormal/pathological collagen formed by the tumor cells or stroma. The difference in interference colors and intensities of birefringence can also be due to distinct patterns of physical aggregation, degree of polymerization and three-dimensional organization of the collagen fibers.[12,25] These findings are also supported by nuclear resonance studies on the physical aggregation of

the collagen fiber.[26] Our results were in accordance with those of other studies.[11,13-15,22,27]

Similar to other studies, the mean area fraction occupied by collagen fibers decreased gradually on progression from WDSCC to PDSCC.[1,28] This could be attributed to matrix metalloproteinases-1, which causes degradation of Type I collagen, causing a decrease in the mean fraction area.[28] In our study, the mean area fraction was determined only in PSR-stained samples and was not performed in samples stained with MT. Trichrome stains fail to reveal very thin collagen fibers, a disadvantage which can, under certain circumstances, lead to a substantial underestimation of collagen content.[29-31]

In our study, the results of PSR and MT were found to be similar. However, MT-staining procedure is more technique sensitive than that of the PSR. In some of the samples, MT staining was hazy and not very effective in delineating very thin fibers.

CONCLUSION

The results of this study show that in OSCC, invasion of epithelial cells into the connective tissue brings about a massive architectural change in the underlying stroma. During early cancer progression, this reorganization and change in the structure of collagen fibers is an attempt to prevent further invasion of tumor cells, but as cancer progresses, the stromal change enhances the movement of these cells within it, leading to metastasis. Many factors play a role in deciding whether the tumor cells will be facilitated in further progression or stopped. The interplay of these factors decides the fate of the tumor.

Based on the above study, it is concluded that MT is a simple, cost-effective method for evaluation of collagen fibers. PSR should not supplant MT but rather be viewed as a distinct stain with complementary properties.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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