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Research Article

A HISTOCHEMICAL STUDY OF COLLAGEN IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

To decrease the morbidity and mortality of oral cancer, detection at an early stage is of utmost important. There is abundant evidence that tumour stroma plays a critical role during carcinogenesis by giving strength and a skeleton to the tumour cells. Many studies have been done to evaluate the nature of connective tissue stroma in different grades of OSCC but there is paucity of information regarding characteristics of the stroma in relation to the invading malignant epithelial cells and interdependence between stroma and tumor cells. The objective of the study was to analyze and compare the nature of connective tissue stroma in the vicinity of invading tumor islands in different grades of oral squamous cell carcinoma.

Subjects and Methods: The study included thirty cases of different grades of histologically diagnosed OSCC and ten sections of normal buccal mucosa as a control group. Nature of collagen was analyzed using picrosirius polarization method. The results were tabulated and analyzed statistically. **Results:** Normal oral mucosa showed predominantly reddish birefringence. All cases of well differentiated OSCC showed reddish orange color. Nearly 70% moderately differentiated cases showed yellowish orange (YO) and 60% of poorly differentiated cases, showed greenish yellow (GY). **Conclusion:** Tumor cells have a role in determining the nature of the collagen fibers in tumor stroma of OSCC, probably with opposing effects on stromal behavior and hence both tumor cells and the nature of collagen fibers are significant in predicting prognosis.

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INTRODUCTION

Squamous cell carcinoma of the oral mucosa comprises 94% of all oral malignancies. Microscopically oral squamous cell carcinomas(OSCC) are composed of two discrete, independent compartments, the malignant epithelial cells and the stroma in which they are dispersed. The collagenous tissue in the stroma gives strength to a tumour by giving a skeleton to the tumour cells. The characteristics of the stroma in relation to the invading malignant epithelial cells and interdependence between stroma and tumor cells are always a matter of discussion.[1] Now it is understood that the tumour stroma plays a critical role during carcinogenesis; to grow beyond a minimal size of 1-2 mm, the tumour requires stroma. Stroma not only provides the vascular supply for nourishment, gas exchange and waste disposal, but also limits the influx of inflammatory cells, thus providing a barrier to immunological rejection. However, the quantity of stroma differs from one tumour to another; some tumours are desmoplastic while others have minimum stroma.

Picrosirius red (PSR), a strong anionic dye, stains collagen by reacting, via its sulphonic acid groups, with basic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fiber in such a way that their long axes are parallel. This parallel relationship between dye and collagen results in an enhanced birefringence. The collagen fibers stained with PSR and observed under polarized light microscope show a color that depends on the thickness of the fibers from thin(type III collagen) to thicker fibers(type I collagen) the color varies from green to yellow, then to orange and finally red.[2] A thorough literature survey has revealed only few studies that had been done on stromal change of oral sqamous cell carcinoma using PSR stain.

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Aims and Objectives

In this study, we analyzed and compared the nature of connective tissue stroma in the vicinity of invading tumor islands in different grades of oral squamous cell carcinoma. The present study throws more light to our understanding about the interaction between tumor cells and stroma.

SUBJECTS AND METHODS

Formalin fixed paraffin embedded tissue sections of OSCC from the archives of the Department of Oral Pathology and Microbiology were used for the study, after getting approval from the Institutional Ethics Committee. Retrospective analysis of the cases of OSCC diagnosed and reported from the department was done. A total of thirty cases of histologically diagnosed OSCC (ten each of well, moderate, and poorly differentiated) with the adequate size of tissues were selected. In addition, ten sections of normal buccal mucosa were studied as a control group. Paraffin embedded tissues of OSCC cases with adequate size ≥5 mm size were included in this study. Tissues with inadequate sizes, cases without adequate clinical details, and malignancies other than OSCC were excluded from the study. From each selected cases, two serial sections of 3–5 μ were prepared. One section was subjected to hematoxylin and eosin stain (H and E) and the other with PSR stain (Direct Red 80, Sigma Aldrich). Modified PSR staining was done as described by Junqueira *et al*., 1979.[3]

H and E stained sections of OSCC were evaluated according to Broder's grading system to confirm the histological grade of the lesion**.** Before evaluating the PSR stained slides for nature of connective tissue slides were coded to avoid bias in interpretation. The sections were then examined in detail under polarized microscope to analyze the polarizing colors of the collagen fibers. Nature of collagen was analyzed in five selected areas in each case and findings were recorded. Based on color observed, nature of collagen was recorded as three categories as proposed by Venigella and Charu [Table 1].[4] Of five different fields, the predominant polarizing color was taken into consideration while deciding the nature of stroma for that particular case. Accordingly, the nature of stroma was recorded for each case. To remove the inter observer and intra-observer bias, all the slides were evaluated by two different observers at two different intervals by the same examiners. The results observed were analyzed using statistical tool Fisher's exact test.

Table 1 Categories of collagen based on color noted after picrosirius staining

Category 1: Reddish, Reddish Orange
Category 2: Yellowish, Orange, Yellowish, Green
Category 3: Greenish Yellow, Greenish

RESULTS

When the nature of the collagen was analyzed using picrosirius red stain under polarizing microscope, normal oral mucosa showed predominantly reddish birefringence [Figure 1]. All cases of well differentiated OSCC showed reddish orange (RO) color [Figure 2]. Twenty percent of the moderately differentiated showed RO color, and 10% showed yellowish green (YG) with majority, i.e., 70% showed yellowish‑orange (YO) [Figure 3]. Of ten poorly differentiated OSCC cases, 60% showed greenish‑yellow (GY), 20% RO, and another 20% YO [Figure 4]. Comparison between the natures of collagen in different grades of OSCC showed that the difference was statistically highly significant ($P \leq 0.001$) between well, moderately, and poorly differentiated OSCC. The comparison was done based on color change of collagen in different grades of OSCC [Table 2 and Figure 5].

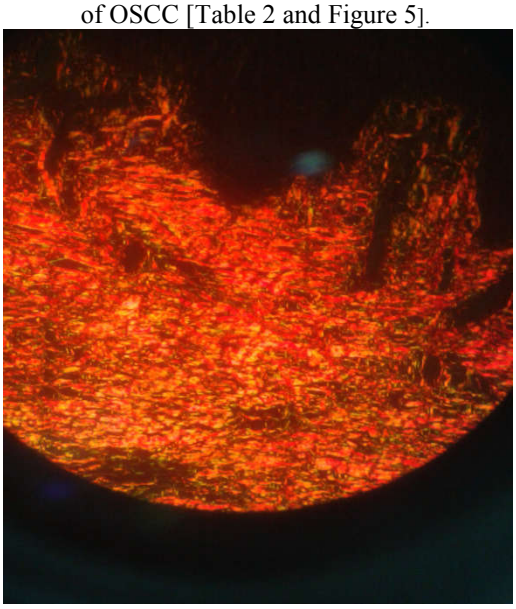


Figure 1 Photomicrograph of normal buccal mucosa showing predominantly red birefringence (PSR stain, 40X)

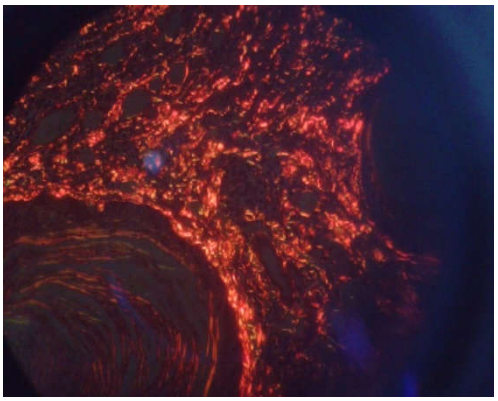


Figure 2 Photomicrographs of well differentiated squamous cell carcinoma showing reddish orange birefringence around the tumor islands (PSR stain, polarised microscopy 40X)

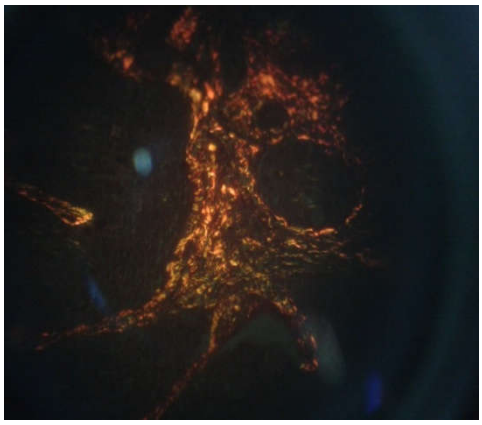


Figure 3 Photomicrographs of moderately differentiated squamous cell carcinoma showing yellowish orange to greenish yellow birefringence (PSR stain, polarised microscopy 40X)

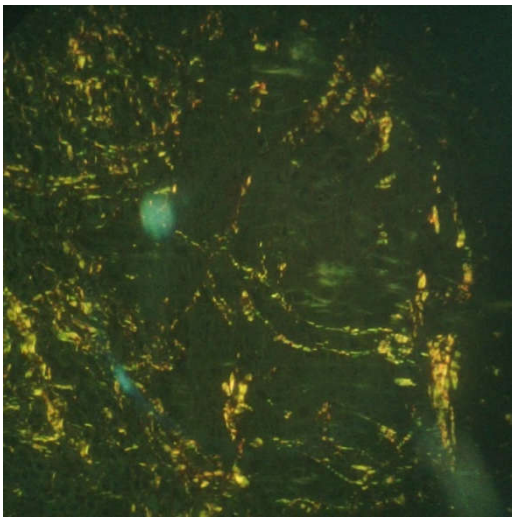
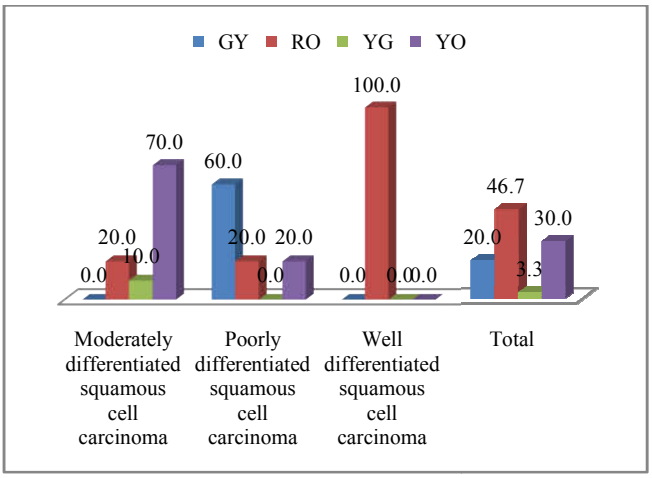


Figure 4 Photomicrograph of poorly differentiated squamous cell carcinoma showing greenish yellow birefringence (PSR stain, polarised microscopy 40X)



(GY- Greenish yellow, RO- Reddish orange, YG- Yellowish green, YO- Yellowish orange)

Figure 5 Graph showing comparison of nature of collagen in different grades of OSCC

Table 2 Nature of collagen in different grades of OSCC

		Grades			
		Moderately differentiated squamous cell carcinoma	Poorly differentiated squamous cell carcinoma	Well differentiated squamous cell carcinoma	Total
Nature of collagen	(GY)	0 .0%	6 60.0%	0 .0%	6 20.0%
	(RO)	2 20.0%	2 20.0%	10 100.0%	14 46.7%
	(YG)	1 10.0%	0 .0%	0 .0%	1 3.3%
	(YO)	7 70.0%	2 20.0%	0 .0%	9 30.0%
Total		10 100.0%	10 100.0%	10 100.0%	30 100.0%

Statistical Analysis: Fishers exact test. GY- Greenish yellow, RO- Reddish orange, YG- Yellowish green, YO- Yellowish orange.

DISCUSSION

Oral Squamous cell carcinoma is the sixth leading cancer by incidence worldwide.[5] The annual estimated incidence is around 275,000 for oral cancer worldwide.[6] The most frequent cancer among males in India is Oral cancer. Among Indian females, is the third most frequent cancer.[7] In spite of many advancement in diagnostic technology and identification of diagnostic markers histopathological evaluation of the lesional tissue remains the gold standard for diagnosis and predictor of prognosis. [8]

Interactions between invading tumor cells and the extracellular matrix of the host is a critical events that occur during the invasion and metastatic processes and expression of cell surface receptors for extracellular matrix molecules and the synthesis/expression of extracellular matrix molecules, themselves, on the surface of malignant tumor cells are thought to underline the events that are significant.[9] Liotta *et al*., have demonstrated that *in vivo*, tumor cells can produce collagenase.[10] Based on conflicting reports regarding the role of the fibrotic stromal response in cancer development, namely, that a desmoplastic reaction can favor either the host or the tumour, it is clear that the role of the stromal response is varied and critical in prognosis of lesion. Earlier reports showed a significant difference in the nature of collagenous stroma in different grades of Squamous cell carcinoma.[11] However the role of the host response in relation to invading tumor cells which is an important determining factor of nature of microenvironment was not correlated. Therefore, in this study we have attempted to analyze and compare nature of stroma in different grades of oral squamous cell carcinoma and correlate the observations with associated host response.[12]

Various methods and techniques have been employed to detect, quantify, and analyze collagen and stromal tissue. Nevertheless, stains historically employed to detect collagen (Mallory, Masson, and Van Gieson methods) have disadvantages, principally a poor specificity for thin fibers. In the present study we used histochemical technique Picrosirius-polarization method, which has the capability to detect thin fibers, to differentiate between thick and thin fibers to the fullest extent.[13]

Collagen molecules, being rich in basic amino acids, strongly react with acidic dyes. Sirius Red (strong acidic azo dye) which reacts with collagen and promotes an enhancement of its normal birefringence due to the fact that many dye molecules are aligned parallel with the long axis of each collagen molecule (Junqueira *et al*., 1979a). The enhancement of birefringence promoted by the Picrosirius – polarization method is therefore specific for collagenous structures composed of aggregates of orientated molecules.[14] Therefore in the present study this technique was used.

In the present study, the relationship between stromal collagenous components and the differentiation of invading tumor cells were correlated. We observed statistically significant difference with respect to different histological grades. All well-differentiated cases showed RO, 70% moderately differentiated showed YO and 60% of poorly differentiated showed GY birefringence. The previous study had attributed distinct difference in stroma in different histological grades of OSCC to the mere influence of invading tumor cells. Our findings are consistent with this observation.[4] In addition, there are other reports available on ECM changes in OSCC using other collagen stains.[13,15] All these authors have suggested a possible alteration in stroma associated with invading tumor cells that may be related to factors released from the lymphocytes or tumor cells, and this stromal change may alter the biological aggressiveness of oral cancer, and

therefore, valuable in predicting the biological behavior of these tumors.[16] In agreement with these investigators, we also have noted alteration in stroma associated with different grades of OSCC.

Junqueira *et al* were pioneers in using Sirius Red stained sections with polarization microscopy for studying the nature of collagen. These researchers suggested from their initial studies that different polarization colors can be used to differentiate types of collagen i.e. collagen type I present a yellow, orange or red color while collagen type III appear green and collagen type II, a variable color according to the tissue and the species, always permitting clear distinction between collagens type I and type III.[2] However the same authors later reported that different polarization colour of collagen depends on thickness of collagen rather than collagen macromolecules. Therefore collagen typing to I, II & III by means of polarization colour which indicate fibril diameter morphometry is likely to remain a controversial issue, since recent report claims that type III collagen can be present on banded collagen fibrils regardless of fibril diameter. (Keene *et al*., 1987). Therefore from our observation we would like to suggest that in well differentiated carcinoma the collagen fibers were thicker in nature compared to thinner fibers of poorly differentiated cases. As the gradation of colour was shifting from RO to YO to GY with change in differentiation from well to moderate to poorly differentiated cases, it can be interpreted that the nature of collagen fibers is changing from thicker to thinner which could be related to factors released from the lymphocytes or tumor cells and the limiting effect on the tumor by ECM remodeling.

CONCLUSION

According to our study, we conclude that there is highly significant diversity in natures of collagen between well, moderate, poorly differentiated OSCC. The nature of collagen fibers is changing from thicker to thinner with change in differentiation from well to moderate to poorly differentiated cases. The observations of the present study also indicate that Picrosirius red stain is an adjunct to the routine staining for studying stromal changes at the invading front of the tumour islands and this, in turn, aids in predicting tumour behaviour.

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