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ORIGINAL ARTICLE

Evaluation of collagen in different grades of oral squamous cell carcinoma by using the Picrosirius red stain- a histochemical study

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ABSTRACT

Oral squamous cell carcinoma is a major public health problem accounting for 94% of all oral malignancies. In this tumour, cells invade the stroma in the form of islands, strands or sheets, which are embedded in or surrounded by an extracellular matrix, thus producing reactive changes in the stroma. Collagen was studied histochemically in different grades of squamous cell carcinoma by staining tumour sections with Picrosirius red and examining them with polarizing microscopy. Polarizing colours of the collagen fibres were recorded and a gradual change from reddish orange to greenish yellow was observed from well to poorly differentiated squamous cell carcinoma, thus indicating that as the tumour progresses, there is a change from the mature form of collagen to an immature form.

Key words: Squamous cell carcinoma, Collagen, Picrosirius red stain

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The term oral cancer encompasses all malignancies that originate in the oral tissues. However, squamous cell carcinoma of the oral mucosa and lips comprises 94% of all oral malignancies. Furthermore, the intraoral location differs in different population groups [1]. Solid tumours like oral squamous cell carcinoma are composed of two discrete independent compartments, the malignant epithelial cells and the stroma in which they are dispersed [2].The collagenous tissue in the stroma gives strength to a tumour by giving a skeleton to the tumour cells. Some studies have reported that the invading tumour cells induce an abundant collagenous or desmoplastic stroma [3].

Further, Junqueira *et al* [4] in their study using Picrosirius red staining followed by polarizing microscopy, selectively demonstrated collagen with an observable difference in the polarizing colours caused by fibre thickness as well as by the packing of the collagen. The examination of collagen fibres by this method served as a procedure to differentiate procollagens, and intermediate and pathological collagen fibres [5],[6],[7].
In the present study, an attempt has been made to observe the nature of the collagen fibres in various grades of Oral Squamous cell carcinoma by determining the polarizing colours in the Picrosirius red stained sections.

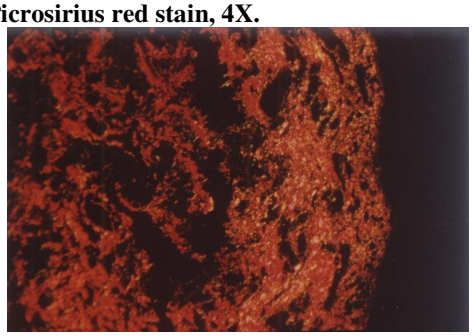
Aims and objectives

The early diagnosis and prevention of oral squamous cell carcinoma depends on the understanding of its development and progression. An attempt has been made in this study to know the biochemical changes in collagen during the progression of Oral squamous cell carcinoma and also, as an adjunct to the routine Haematoxylin and Eosin (H and E) staining. To observe these changes in different grades of Oral squamous cell carcinoma, the Picrosirius red stain has been employed.

Materials and methods

A total of 30 cases of histologically diagnosed

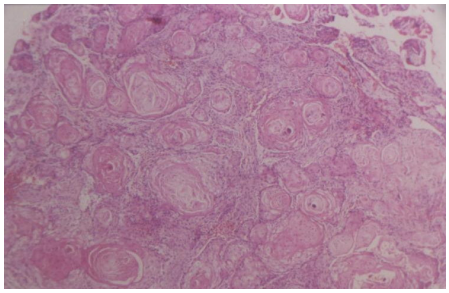
(Table/Fig1) Photomicrograph of normal buccal mucosa showing predominantly red birefringence in Picrosirius red stain, 4X.



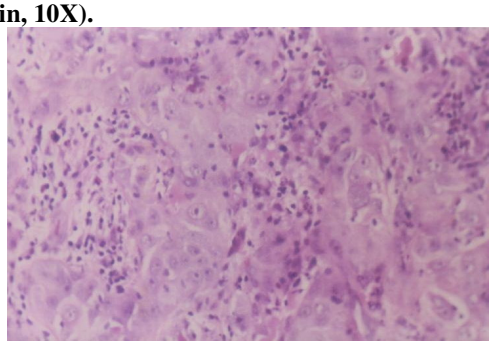
squamous cell carcinoma, 10 each of well, moderate and poorly differentiated squamous cell carcinomas and two sections of normal buccal mucosa as the control (Fig.1) were retrieved from the archives of the Department of Oral Pathology, MCODS, Manipal.

The sections were stained with Haematoxylin and Eosin (Fig. 2 to 4) and also by the modified Picrosirius red stain[8]. Following deparaffinization and hydration in distilled water; the sections were incubated in 0.1% (w/v) Sirius red F3B (C.I.35780) in saturated Picric acid solution for 1 hour at room temperature.

(Table/Created by 2) Photomicrograph of Well differentiated squamous cell Carcinoma (H&E stain, 4X).

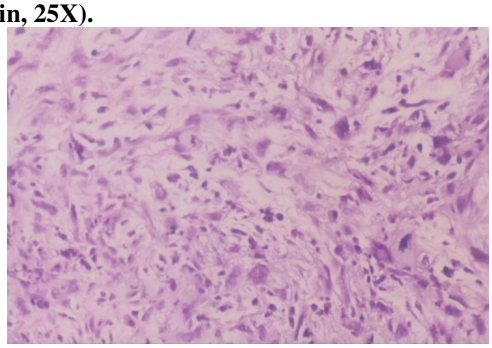


(Table/Fig 3) Photomicrograph of Moderately differentiated squamous cell Carcinoma (H&E stain, 10X).



This was followed by rinsing with distilled water, staining with Mayer's haematoxylin, differentiation in 1% HCl, alkalinization with tap water, dehydration and mounting. The sections were examined with a polarizing microscope. Differences in the polarizing colours of the collagen fibres in different grades were analyzed.

(Table/Fig 4) Photomicrograph of Poorly differentiated squamous cell Carcinoma (H&E stain, 25X).



Results

In the present study, 2 controls from normal buccal mucosa were stained with Picrosirius red stain and they showed predominantly reddish or yellowish orange birefringence in the lamina propria. (Fig.1). In the three grades of squamous

cell carcinoma which were studied, the collagen fibres in well-differentiated carcinoma revealed

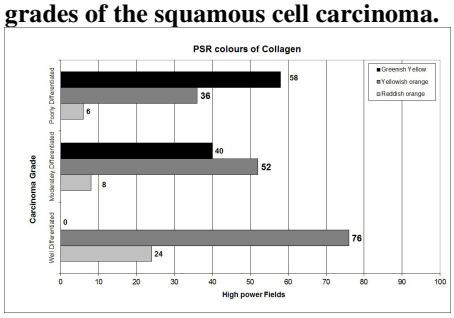
(Table/Fig 1) Showing the polarizing colours observed in 10 fields each in 10 samples of the three grades of the squamous cell carcinoma.

Histopathological Grades of Oral Squamous cell carcinoma	Colours observed (100 HPF)			Statistical analysis
	Reddish orange	Yellowish orange	Greenish yellow	
Well differentiated	24	76	0	χ^2 **84.1569**
Moderately differentiated	8	52	40	**df 4**
Poorly differentiated	*6*	36	58	**P< 0.001 Very Highly significant**
Total	38	164	98	**Kappa 0.174**

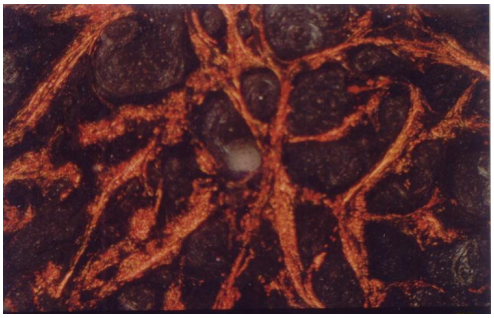
HPF- High Power Field; χ^2 Chi square; df – degrees of freedom; P – probability value

polarizing colours of reddish orange to yellowish orange around the tumour islands in the majority of the fields which were scanned (Table.1 /Graph.1/ Fig.5).

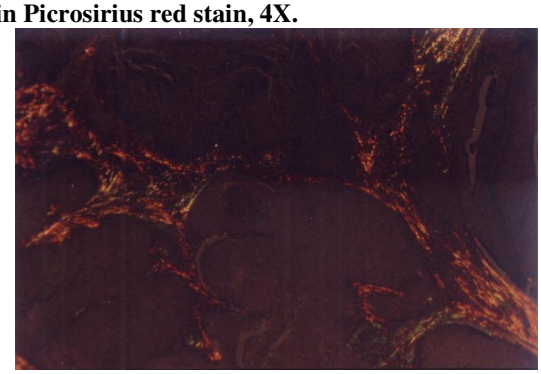
GRAPH 1: Graph depicting different polarizing colors observed in 100 fields, in each of the three grades of the squamous cell carcinoma.



(Table/Fig 5) Photomicrograph of Well differentiated squamous cell carcinoma showing reddish orange to yellowish birefringence around the tumor islands in Picrosirius red stain, 4X.

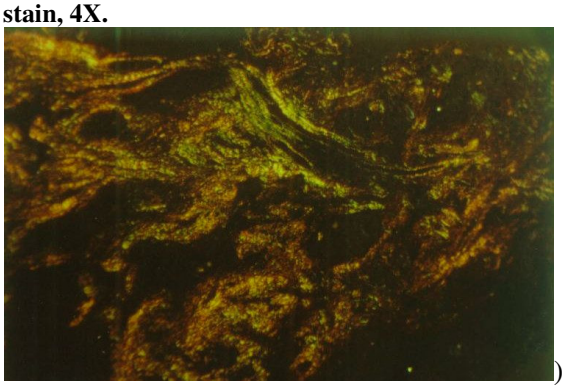


(Table/Fig 6) Photomicrograph of Moderately differentiated squamous cell Carcinoma showing yellowish orange to greenish yellow birefringence in Picrosirius red stain, 4X.



A gradual change in the polarizing colours from yellowish orange to greenish yellow was observed in moderately differentiated carcinoma (Table.1 /Graph.1/ Fig.6). However, in poorly differentiated squamous cell carcinoma, the predominant polarizing colour which was observed was greenish yellow with weak birefringence around the tumour islands (Table-1 /Graph.1/ Fig.7.

(Table/Fig 7) Photomicrograph of Poorly differentiated squamous cell Carcinoma showing greenish yellow birefringence in Picrosirius red stain, 4X.



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The results were subjected to statistical analysis. The Chi square (χ^2) value was 84.1569 with a p value of <0.0001. The findings were very highly significant between different gradations of squamous cell carcinoma.

Discussion

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 [2]. However, the quantity of stroma differs from one tumour to another; some tumours are desmoplastic while others have minimum stroma.

In the present study, with respect to the relationship between the collagenous components in the stroma and the invading tumour cells, there have been some observable changes in different histological grades of Oral squamous cell carcinoma. In well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma, distinct deposits of collagen showed reddish orange to yellowish orange birefringence, which was mainly concentrated around the tumour islands. This may be due to the deposition of collagen fibres which were in the form of thick bands and composed of closely packed fibrils, this feature being consistent with the concept of Junqueira et al [9] and Montes et al [10] . They stated that the thick fibres were Type I collagen fibres and exhibited an intense birefringence of red, orange and yellow colour by polarizing microscopy and a weak birefringence of green when the fibres were thin fibrillar, thus constituting Type III collagen.

Further, nuclear resonance studies on the physical aggregation of the collagen fibres by Sharf et al [11] have also revealed a colour profile of orange to red, which corresponded to the well packed fibres and the green to greenish yellow to poorly packed fibres. However, this peculiar change in colour with respect to the type of collagen fibres adjacent to the tumour cells remains unresolved. This collagen may be from the tumour cell in origin, thus benefiting the tumour by reducing the access to the host lymphocytes. Alternatively, the collagen could be of stromal cell origin, thus benefiting the host by walling off the invading tumour. These statements are supported by studies on cell lines which were derived from various neoplasms, to synthesize different types of collagen, predominantly the types I, III and IV ~~collagen~~ [12], [13], [14]. In a majority of connective tissues, the major type of collagen which is present is of type I, although some amount of type III and Type V are also present [15] Picrosirus red stains the collagen types I, II and III and the C1q complement component [16].It also stains the amyloid and the keratohyalin granules of the cornified epithelia and the mucous glands of some species and fish hearts [4].

A majority of the fields in moderately differentiated and poorly differentiated squamous cell carcinoma in the present study, showed a gradual change in the polarizing colour from yellowish orange to greenish yellow, particularly in the immediate vicinity of the invading tumour islands. These could be Type III collagen fibres (Junqueira et al [9] and Montes et al [10]) Furthermore, this difference in birefringence in the present study could be due to [17]

1. The action of enzymes such as collagenases or the metalloproteinases secreted by tumour cells.
2. An abnormal disintegration of the matrix by the tumour cells.
3. An uninhibited proliferation of the dedifferentiated tumour cells with the secretion of their abnormal matrix
4. Disorganized or abortive stroma.

The above are further supported by the presence of a delicate meshwork (reticular) of Type III collagen at the invading front of the tumour islands in increasing gradations of skin cancer (Stenback et al) [18]. Few workers have also reported a qualitative change in Type I collagen with an embryonic phenotype adjacent to the tumour islands [19].

Thus, the colour changes observed in the present study clearly indicate some alterations in the stromal tissue around the tumour cells, which in

turn may be related to the carcinogenic agents that are involved in tumourigenesis. The above results are further supported by Brekken et al,[20]. who stated that the extracellular matrix can influence tumour progression. This concept is reinforced by a recent mathematical study on the mechanics of capsule formation, which predicts that a more robust extracellular matrix and capsule result in the slower growth of the tumour [21] .

Oral squamous cell carcinoma is not the only malignant tumour that has shown such changes in the collagen of the stromal tissue. Studies on breast cancer [22] have also shown that an increase in the collagen content of the extracellular matrix increases the mechanical stiffness and transport resistance of the tumours. In a similar manner, the observations of follicular thyroid carcinomas have revealed a higher frequency of yellow-green collagen fibres than orange-red fibres at the sites of invasion, whereas orange-red fibres significantly predominated at non-invaded sites [23]. Allon et al [24], in their study on stromal differences in salivary gland tumours, found that 50% of the collagen fibres in polymorphous low -grade adenocarcinomas and adenoid cystic carcinomas were greenish yellow, whereas in pleomorphic adenoma, only 13% of them were greenish yellow. A study on human osteosarcomas by Junqueira et al [25] indicated the presence of both type I and III collagens in the fibroblastic areas of the tumour: Type III in the anaplastic areas and type II in the chondroblastic areas of osteosarcomas. Kreig T et al [14] stated that a continuous cell line derived from human embryonal rhabdomyosarcoma synthesizes predominantly type III procollagen with a small amount of type IV.

Conclusion

In the present study, an observable stromal change with the progression of the neoplasm was evinced with Picrosirius red stain. In well differentiated squamous cell carcinoma, there was a deposition of collagen in the form of thick bands adjacent to the neoplastic epithelial cells/islands. The staining was more distinct with Picrosirius. In moderately and poorly differentiated squamous cell carcinomas, the fibres were reticular/ fibrillar and more disorganized. This definitively indicates the contribution of the stromal constituents in the progression of the neoplasm; in particular, these stromal changes may enhance the movement of the tumour cells towards the blood vessels or the lymphatic vessels. The observations of the present study 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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