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RESEARCH ARTICLE

STROMAL COLLAGEN AS A PROGNOSTIC INDICATOR OF ORAL SQUAMOUS CELL CARCINOMA - A NEW WAY FORWARD

***Neelam Shivani, Shivani Neelam, Hitasree, B. N., Malathi Manne, Syed Afroz Ahmed, Charu Suri, Shahela Tanveer and Shravan Kumar**

Sri Sai College of Dental Surgery, Vikarabad

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) encompasses all malignancies originating in the oral cavity and globally it is the most common cancer. It is a fact that tumor stroma plays a vital role in tumor progression as it contains significant stromal glycoprotein and collagen. Therefore analysis of collagen fibers is of immense value which in turn aids in predicting the biological behavior of the tumor.

Aim: The aim of the present study was to evaluate birefringence pattern of collagen fibers and to determine varying proportions of stromal collagen in different grades of OSCC using picrosirius red and polarizing microscopy.

Materials and Methods: A total of 28 cases, which included 22 histopathologically diagnosed cases of OSCC, of which 8 were of well differentiated, 8 moderately differentiated and 6 poorly differentiated OSCC. 6 cases of control group were also retrieved. The sections were stained with both Haematoxylin and Eosin (H&E) and picrosirius red and they were examined under polarizing microscope.

Statistical analysis: The statistical differences were analyzed by one way ANOVA.

Result: It was observed that thick collagen fibers decrease and thin collagen fibers increase with dedifferentiation of OSCC and also polarization colors of thick and thin fibers changes from reddish orange (RO) to yellowish orange (YO) to greenish yellow (GY) from well to moderate to poor grades of OSCC respectively.

Conclusion: Thus the present study suggested that there was a change in polarization colors of thick fibers from RO to GY, which could be due to change in mature form of collagen into immature form as the tumor progress from well to poorly differentiated OSCC. The significance of this study lies in birefringence of stromal collagen fibers observed around tumor islands in different grades of OSCC.

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INTRODUCTION

Oral cancer is the sixth most common cancer worldwide. (Feller and Lemmer, 2012) Approximately, 94% of all oral malignancies are OSCC. In India its incidence is high, the morbidity is further increased due to its tendency for invasion and metastasis. (Agarwal *et al*., 2011) OSCC is a malignant epithelial neoplasm composed of two interdependent components, tumor epithelial cells and the stroma in which they are dispersed. (Aparna and Charu, 2010) Stromal components are the key factors for provision of nutrition and growth to any tumor and they also act as a barrier for the spread of tumor. One of the prime aspects of tumor cell invasion and metastasis is the interaction between cancer cells and extracellular matrix (ECM) component. Collagen is the basic skeleton of ECM which undergoes proteolytic remodelling resulting in abundant changes in the collagenous stroma promoting tumor progression. The method to detect and evaluate the nature of collagen in the stroma is significant in this regard. Routine stains employed to stain collagen have disadvantage of poor specificity for thin fibers. In contrast, picrosirius red has the capability to detect thin fibers and also to differentiate mature and immature fibers. (Kalele *et al*., 2014) The purpose of this study was to enlighten the role of stromal fibers in tumor progression through the phenomenon of birefringence, when stained with picrosirius red. Thus, thorough understanding of the stromal changes helps us to target our existing treatments more effectively which in turn improves the prognosis of the individual.

Aims and Objectives

Prime objective of this study was to evaluate birefringence pattern of collagen fibers using polarizing microscopy in different grades of OSCC stained with picrosirius and to

**Corresponding author: Neelam Shivani,*
H.No:11-25-155, M.G.M Road Kothawada, Warangal, India

observe variation in thickness of collagen fibers between different grades of OSCC.

MATERIALS AND METHODS

A total of 22 cases of histologically diagnosed OSCCs, 8 each of well, moderate and poorly differentiated and 6 sections of normal buccal mucosa as control were retrieved from the Department of Oral Pathology, SSCDS, Vikarabad. The sections were stained with H and E and also by the picrosirius red stain. Following deparaffinization and hydration in distilled water, the sections were stained with weigertshaematoxylin a nuclear stain followed by sirius red in saturated picric acid solution for 1 hour at room temperature. This was followed by wash in two changes of acidified water then dehydrated, cleared and mounted. The sections were examined under polarizing microscope. Differences in the polarizing colors of the collagen fibers in different grades were analyzed under 10X and 40X.

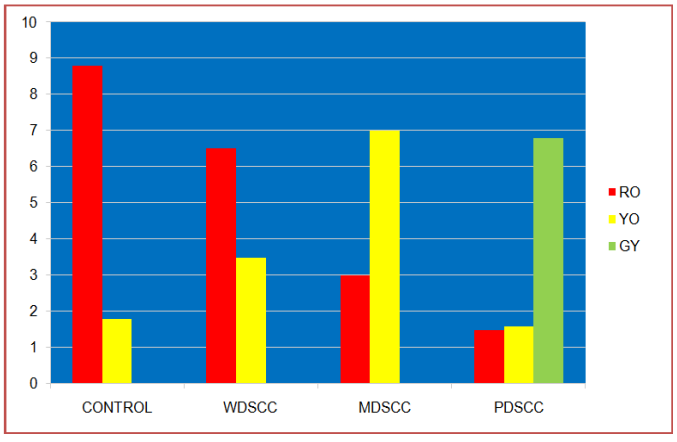
RESULTS

In the present study it was observed that interstitial collagen displayed different birefringence of colors with different intensities. Collagen fibers in control group demonstrated RO, which were much brighter than well differentiated OSCC, suggesting thick fibers were more in normal mucosa compared to well differentiated OSCC. In well differentiated OSCC cross hatchet pattern arrangement of collagen fibers was also appreciated. (Fig.1) Collagen fibers in moderately differentiated exhibited YO (Fig.2) and in poorly differentiated GY (Fig.3)suggesting that as the tumor dedifferentiate thin immature fibers increased implicating poor prognosis (Table 1/Graph1).

Table 1. Mean values of polarizing colors observed in ten fields under 40 X in control and study groups

Groups	RO	YO	GY	F-Value	P-Value
Control	8.1±0.75	1.8±0.75	.00	291.61	.000**
Well differentiated OSCC	6.5±0.92	3.5±0.92	.00	148.16	.000**
Moderately differentiated OSCC	3.0±0.92	7.0±0.92	.00	172.66	.000**
Poorly differentiated OSCC	1.5±0.54	1.6±0.81	6.8±0.75	107.93	.000**

One way ANOVA with Tukey's Post-hoc**p<0.05 (Significant)



Graph 1. Polarizing colors expressed by picrosirius red in control and study groups

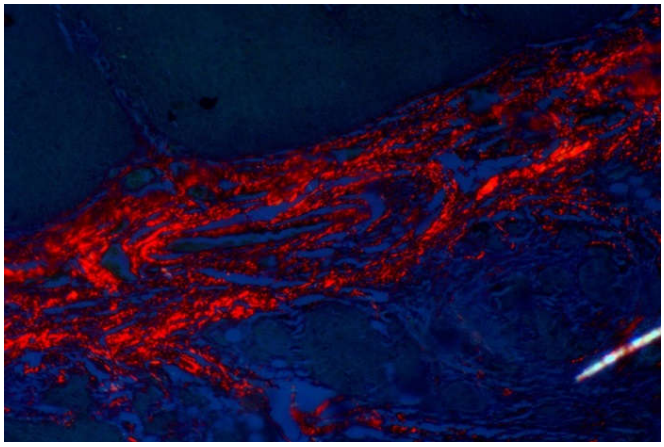


Fig.1. Photomicrograph of well differentiated OSCC stained with picrosirius red stain demonstrates cross hatchet arrangement of YO collagen fibers under 10X

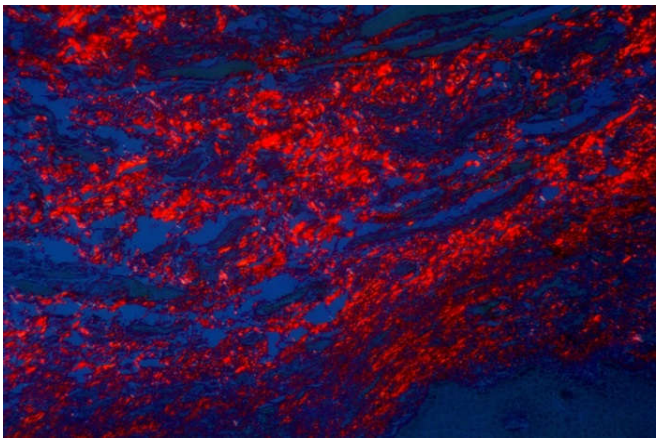


Fig.2. Photomicrograph of moderately differentiated OSCC stained with picrosirius red stain demonstrates YO collagen fibers under10X

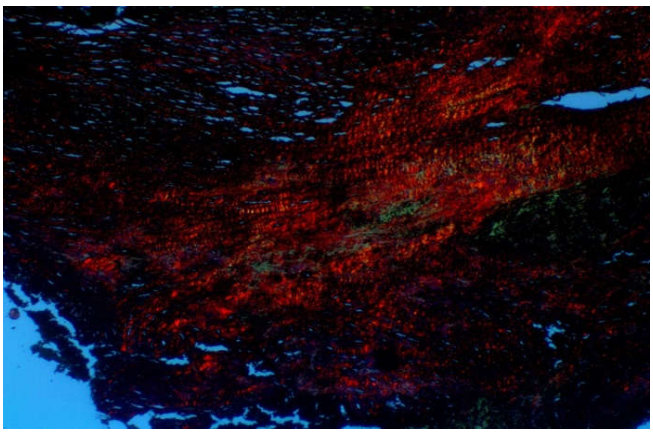


Fig.3. Photomicrograph of poorly differentiated OSCC stained with picrosirius red stain demonstrates GY collagen fibers under10X

DISCUSSION

The development of OSCC is believed to occur in a piecemeal fashion, beginning with oral epithelial dysplasia progressing to carcinoma-in-situ and finally to OSCC. An essential step in tumor progression is to invade the surrounding stroma, as the tumor requires its stroma to grow beyond 1-2 mm size. The stroma acts as a mixed blessing, it provides the environment for nourishment and also restricts the influx of inflammatory cells

for the neoplastic cells. (Rashi Sharma *et al*., 2015) The quality of ECM is mainly dependent on its collagenous content and the presence of collagen is considered as a main barrier to be cleared away during invasion, thus making room for infiltrating cell mass. Matrixmetalloproteinases (MMP) are a group of proteolytic enzymes which degrade the components of ECM. The ECM mainly consists of Type I collagen which is about 90% and Type III collagen which is about 8-10%. Electron microscopic studies demonstrate Type I collagen fibers as thick fibrils, whereas type III collagen fibers composed of loosely dispersed thin fibrils. (Gopinathan *et al*., 2015) Collagen of ECM is a triple helical structure rich in amino acids, having strong affinity with the acidic dyes. Sirius red is a strongly acidic azo dye which is effectively used to stain collagen. It is an elongated dye molecule which reacts with collagen and amplifies its normal birefringence. This is because the dye molecules are aligned parallel with the long axis of each collagen molecule. (Kalele *et al*., 2014) The present study showed a statistically significant ($P < 0.05$) change in the colors of bundles of collagen fibers which varied from RO in well‑differentiated OSCC to YO in moderately differentiated OSCC to GY in poorly differentiated OSCC. However, the control samples predominantly exhibited RO color. Our results were consistent with the study done by Kardam *et al*. (2016) who also suggested that there was a gradual change in the polarizing color along with the advancing grade of tumor from the RO to GY. (Kardam *et al.,* 2016) One of the causes for this can be linked to the type of collagen fibers, where the Type I collagen fibrils were thick and they exhibit birefringence of red, orange and yellow color, whereas Type III fibrils were thin and they showed the birefringence of green. (Kalele *et al*., 2014)

Various authors have studied the birefringence property of collagen fibers. The reason for this color change occurring predominantly in the vicinity of tumor islands is still not clear. Van den Hooff (1988) suggested that this difference in birefringence of color surrounding the tumor islands could be firstly due to the action of enzymes such as collagenases or the metalloproteinases secreted by tumor cells. Second, there could be an abnormal disintegration of the matrix by the tumor cells. Third, the dedifferentiated tumor cells could be secreting an abnormal matrix. Finally, there could be a formation of disorganized or abortive stroma around the tumor islands. (Kardam *et al*., 2016) According to Junqueira *et al*. and Montes *et al*. (1980) in their study on human osteosarcoma, the color changes can be due to the carcinogenic action of stromal MMPs. They stated that the thick type I collagen fibers exhibited intense birefringence of red, orange and yellow by polarising microscopy. A weak birefringence of green was observed, when the fibers were thin fibrillar, thus constituting type III collagen. Thus the changes in birefringence inferred that in osteosarcoma, anaplastic areas showed type III fibrils and chondroblastic areas showed type II collagen fibers predominantly. (Aparna and Charu, 2010) Sharf *et al*. (1997) also revealed a color change of orange to red which corresponds to well packed fibers and the green to greenish yellow for poorly packed fibers in his nuclear resonance studies on the physical aggregation of collagen fibers, which signifies well packed fibrils as Type I fibers and poorly packed as Type III fibers. (Gawande *et al*., 2015) According to Koren *et al*. (2001) in their study on stromal differences in salivary gland tumors, it was observed that stromal collagen fibers differ significantly in pleomorphic adenoma from those in polymorphous low grade adenoid cystic carcinoma (PLGA) and adenoid cystic carcinoma (ACC). The less well packed collagen fibers in ACC and PLGA inferred that thin Type III collagen fibrils facilitated further invasion into adjacent tissues and development of metastases. (Allon *et al*., 2006)

Conclusion

Thus the present study concludes that picrosirius red is an adjunct to conventional staining techniques for studying stromal changes at the invading front of tumor islands. The importance of matrix changes has to be understood in the overall perspective of tumor biology, which indicates the propensity of tumor cells to infiltrate and metastasize, hence the present study focused on stromal collagen. It is said that 'Without biologic understanding of the oral cancer it can't be hoped for a real and lasting solution of the problem'.

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