Qualitative Analysis of Connective Tissue Stroma in Different Grades of Oral Squamous Cell Carcinoma: A Histochemical Study

Abstract

Background: Detection of oral cancer at an early stage is of utmost importance to decrease morbidity and mortality. Tumor stroma plays a critical role during carcinogenesis. There is lack of information regarding the characteristics of the stroma in relation to the invading malignant epithelial cells and the interdependence between stroma and tumor cells in different grades of oral squamous cell carcinoma (OSCC). Aim: The present study was aimed to analyze and compare the nature of stroma in the vicinity of invading tumor islands in different grades of OSCC, using a histochemical technique picrosirius-polarization method. The present study also evaluated and correlated the possible role of inflammatory response in determining the nature of the stroma. 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, and intensity of inflammatory cell infiltrate was recorded using ImageJ software (1.42q, NIH, USA). 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). The mean inflammatory cell count was highest in well-differentiated group. There was shift to YO and GY collagen when the cell differentiation and inflammatory cell count decreased in moderate and poorly differentiated cases. Conclusion: Both inflammatory cells and 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 are significant in predicting prognosis.

Keywords: Collagen, connective tissue stroma, lymphocytes, oral squamous cell carcinoma, picrosirius red stain

Introduction

Squamous cell carcinoma of the oral mucosa comprises 94% of all oral malignancies. It is the sixth leading cancer by incidence worldwide. The annual estimated incidence is around 275,000 for oral cancer worldwide. Oral cancer is the most frequent cancer among males in India. Among Indian females, oral cancer is the third most frequent cancer. Histopathological evaluation of the lesion is the gold standard for the diagnosis and predictor of prognosis.

Microscopically oral squamous cell carcinoma (OSCC) is composed of two discrete, independent compartments – the stroma and the malignant epithelial cells which are interspersed in the stroma. Interactions between tumor cells and surrounding tissue stroma critically determine the development of any given solid malignancy. However, the role of different components of stroma in progression of tumor is not clear. The collagenous tissue in the stroma gives strength to tumor by providing a skeleton to them. 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 and interest.

Picrosirius red (PSR) is a strong anionic dye that stains collagen. The sulfonic acid groups of PSR react with the basic groups of collagen molecule. The elongated PSR molecules glue to the collagen fiber with their long axes arranged parallel to them. This parallel association 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

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thickness of the fibers. As the collagen fibers change from thin to thicker, the color alters from green to yellow, then to orange, and finally red. Hence, the dye has capability to detect thin collagen fibers and also to differentiate between thick and thin fibers to the fullest extent.

**Aims and objectives**

In this study, we evaluated the nature of connective tissue stroma in different grades of OSCC and correlated between the inflammatory components and differentiation of invading malignant cells. 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 case, 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.[7]

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 and inflammatory component, 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].[8] 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.

Inflammatory component in the same fields was noted and graded on the basis of nature and density of inflammatory cells in vicinity of invading tumor islands. For this purpose, photomicrographs were taken under high-power field ×40 light microscope [Figures 1-3]. The inflammatory cells were counted manually using ImageJ software (1.42q, NIH, USA). The mean value of five examined areas was considered as the final value.

All the slides were evaluated by two different observers and at two different intervals by the same examiner to remove the interobserver and intra-observer bias. The results observed were analyzed using statistical tool Fisher’s exact test, ANOVAs F test, Bonferroni multiple comparison test, and Kruskal–Wallis test.

**Results**

When the nature of the collagen was analyzed, normal oral mucosa showed predominantly reddish birefringence [Figure 4]. All cases of well-differentiated OSCC showed reddish-orange (RO) color [Figure 5]. 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 6]. Of ten poorly differentiated OSCC cases, 60% showed greenish-yellow (GY), 20% RO, and another 20% YO [Figure 7]. Comparison between the natures of collagen in different grades of OSCC showed that the difference was statistically highly significant (P ≤ 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 8].

The inflammatory cell count was recorded for all three groups including well, moderately, and poorly differentiated OSCC. The inflammatory cell count in well-differentiated group ranged from 247.93 to 465.43 cells/field with mean value of

**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 |
|-------------------------------------|--------------------------------------------|-------------------------------------|

Figure 1: Photomicrographs of well-differentiated oral squamous cell carcinoma (PSR stain, light microscopy, ×40)
356.68 ± 152.03 cells/field. The inflammatory cell count in moderately differentiated OSCC ranged from 93.07 to 214.61 cells/field with a mean value of 153.84 ± 84.95 cells/field. The inflammatory cell count in poorly differentiated OSCC ranged from 78.27 to 118.77 cells/field with mean value of 98.52 ± 28.31 cells/field. Using statistical analysis ANOVA F-test, inflammatory cell count in all the study groups (OSCC) was statistically highly significant $P \leq 0.001$, [Table 3 and Figure 9]. Multiple comparisons between the groups done using Bonferroni test showed statistically highly significant ($P \leq 0.001$) difference between well differentiated to moderately and poorly differentiated OSCC. However, no significant difference was observed between moderately differentiated to poorly differentiated OSCC [Table 4].

![Figure 2: Photomicrographs of moderately differentiated oral squamous cell carcinoma (PSR stain, light microscopy, ×40)](image1)

![Figure 3: Photomicrograph of poorly differentiated oral squamous cell carcinoma (PSR stain, light microscopy, ×40)](image2)

![Figure 4: Photomicrograph of normal buccal mucosa showing predominantly red birefringence (PSR, polarized microscopy, ×40)](image3)

![Figure 5: Photomicrographs of well-differentiated squamous cell carcinoma showing reddish-orange birefringence around the tumor islands (PSR, polarized microscopy × 40)](image4)

**Table 2: Nature of collagen in different grades of oral squamous cell carcinoma**

| Nature of collagen | Grades                              | Total          |
|--------------------|-------------------------------------|----------------|
|                    | Moderately differentiated            |                |
|                    | squamous cell carcinoma              |                |
| (GY)               | 0 (0.0%)                            | 6 (20.0%)      |
| (RO)               | 2 (20.0%)                           | 2 (20.0%)      |
| (YG)               | 1 (10.0%)                           | 0 (0.0%)       |
| (YO)               | 7 (70.0%)                           | 2 (20.0%)      |
| Total              | 10 (100.0%)                         | 10 (100.0%)    |

Statistical analysis: Fisher’s exact test. GY=Greenish-yellow, RO=Reddish-orange, YG=Yellowish green, YO=Yellowish-orange

$P \leq 0.001$, [Table 3 and Figure 9].
In the present study, we also correlated the nature of stroma based on color change of collagen with number of inflammatory cells per field. In relation to RO color collagen, highest count of inflammatory cells was noted which ranged from 74.4 to 637.8 cells/field with the mean value of 281.61 ± 176.93. With YO and GY collagen, values were 61.2–193.4 cells/field with mean 128.04 ± 47.4 and 79.6–171.8 cells/field with mean 105.7 ± 34.69, respectively. When the results were statistically compared using Kruskal–Wallis test, significant difference ($P \leq 0.021$) was noted [Table 5 and Figure 10].

**Discussion**

In India, oral cancer is one of the major causes of death. Around 80,000 new cases are diagnosed each year. Early detection of oral cancer is of high importance to decrease the associated morbidity and mortality. Fibrotic stroma may be the result of the attempt of host to contain malignant cells, or it may help the cancer cells through neovascularization and by acting as a physical barrier against lymphocytes, macrophages, and other immune cells of the host, thus exhibiting opposite biological behaviors. Metastatic potential of malignant cells has been detected to be inversely proportional to the capability of tumor cells to bring about a host stromal response.

Interactions between invading tumor cells and the extracellular matrix (ECM) of the host are critical events that occur during the invasion and metastatic processes. Expression of cell surface receptors for ECM molecules and synthesis/expression of ECM molecules on the surface of malignant tumor cells are thought to highlight the phenomenon that is significant. Liotta et al. have demonstrated that in vivo tumor cells can produce collagenase. Contradictory reports are observed in the literature regarding whether stroma promotes cancer or acts as a barrier to the development of cancer. It could well be considered that response of stroma differs and is important to predict the prognosis of the tumor. Earlier study showed a significant difference in the nature of collagenous stroma in different grades of OSCC. However, the role of the host response in relation to invading tumor cells, which is an important determining factor to modulate nature of microenvironment was not
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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.

In addition, there are other reports available on ECM changes in OSCC using other collagen stains. 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. In agreement with these investigators, we also have noted alteration in stroma associated with different grades of OSCC.

Junqueira et al. 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, and always permitting clear distinction between collagens type I and type III. However, the same authors later reported that different polarization color of collagen depends on the thickness of collagen rather than collagen macromolecules. According to our observation, in well-differentiated carcinoma, the collagen fibers were thicker in nature compared to thinner fibers of poorly differentiated cases. As the gradation of color 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 or it can also be due to ECM modeling which can have limiting effect on tumor.

While evaluating inflammatory cell component in different grades of OSCC with the objective of analyzing the host response, we have observed that predominant cell type was lymphocytes. Well-differentiated group had highest inflammatory cell count when compared to moderately and poorly differentiated groups. Immune cells of the host react to OSCC by promoting lymphocytic infiltration. However, immune cell functions may be compromised by OSCC cells. Signaling abnormalities, spontaneous apoptosis, reduced proliferation and function of circulating dendritic cells, tumor-infiltrating lymphocytes, T-cells, and natural killer cells have been detected in oral cancer patients. Immunosuppressive actions are believed to be mediated by both soluble factors and contact-mediated factors of the cancer cells, which inhibit normal functioning of immune cells and promote cancer growth and progression. Transcription factors nuclear factor kappa B and signal transducer and activator of transcription 3 and their functions are observed to be raised in cancer cells. These transcription factors are specifically implicated in suppressing immune system in tumor microenvironment. Also observed is that tumor-specific T-cell recruitment is suppressed if there is increased accumulated infiltration of CD4(+)CD25(+) regulatory T cells in the tumor environment. These CD4(+)CD25(+) regulatory cells are a subset of CD4(+) T-cells. All these scientific evidence support our observation of reduction in inflammatory component from well to moderate to poorly differentiated OSCC.

In the present study, we have also correlated the inflammatory responses with nature of stroma based on change in color of collagen with respect to number of inflammatory cells per field. In relation to RO color collagen, highest count of inflammatory cells was noted when compared to those of YO and GY collagen with statistically significant difference. The association between OSCC and inflammatory component is considered as one of the criteria for determining the differentiation of the tumor. In addition, there is sufficient evidence in the literature that inflammatory cells, particularly lymphocytes modify the nature of stroma by releasing lymphokines that regulate the biosynthesis of collagen and proliferation of fibroblasts. Mononuclear cells (MNL), when they are activated by inflammatory stimulus, produce various inhibitory and stimulatory regulatory mediators. The balanced productions of these mediators are required to regulate fibroblastic activity, for a normal fibrotic response to occur. Studies also demonstrate that activated MNLs regulate fibroblast growth, fibroblast proliferation, and collagen biosynthesis.

Therefore, we would like to link our observation of thicker collagen fibers in well-differentiated OSCC, which also showed high count of lymphocytes, to increased production of stimulatory mediators by these mononuclear inflammatory cells. The thinner fibers associated with poorly differentiated cases indicate lack of stimulatory effects due to significantly less number of inflammatory cells and may also be due to the production of proliferation inhibitory lymphokines by the available cells. At the same time, role of tumor cells in modulating the stroma also cannot be neglected. It is possible that in poorly differentiated cases, the tumor cells are least differentiated and highly aggressive and aberrant in behavior which may also be reflected in increased production of destructive enzymes that can modify the collagenous component of microenvironment while this may not be a prominent feature in well-differentiated cases, as the tumor cells are well differentiated and less aggressive. In short, it can be appreciated that various processes may occur independently during growth and progression of OSCC, with the processes occurring simultaneously at various sites of the same tumor. These processes ultimately determine the composition of collagen fibers and stromal connective tissue. Probably, the inflammatory cells and tumor cells have opposing effects on stromal behavior and therefore both are significant in predicting prognosis. To understand such complex communications and relationship between tumor cells and the surrounding ECM, it is necessary to carry out further research on the role of T-cells. Further studies in this direction are recommended.

Conclusion
According to our study, we conclude that there is highly significant diversity in natures of collagen between well,
moderate, and poorly differentiated OSCC. The nature of collagen fibers changes from thicker to thinner with change in differentiation from well to moderate to poorly differentiated cases. There is highly significant discrepancy in inflammatory cell infiltrate, between different grades of OSCC. Among the different OSCC grades, inflammatory cells are predominantly lymphocytes. Well differentiated group show RO color (thicker collagen) with highest inflammatory cell count compared to moderate and poorly differentiated OSCC which show YO and GY (thinner collagen). Both the inflammatory cells and tumor cells have role in determining the nature of the collagen fibers in tumor associated stroma of squamous cell carcinoma, probably opposing effects on stromal behavior and therefore both are significant in predicting prognosis.

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Conflicts of interest
There are no conflicts of interest.

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