Original Article

Tumor - stromal crosstalk in oral squamous cell carcinoma: A histochemical study

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ABSTRACT

Background: Tumor invasion involves complex interactions between tumor and stromal cells and is bidirectional and such a mutual support allows for the progression of malignancy. The Aim of the study was to predict the biological behaviour of tumors by evaluating the changes in the connective tissue i.e. stromal response in different histopathological grades of oral squamous cell carcinoma (OSCC). **Materials and Methods:** A total number of '30' cases of oral squamous cell carcinoma were examined using Connective Tissue Special Stains and Immunohistochemical Staining. **Result:** All the 3 grades of OSCC's were noted for staining intensity of α-SMA(alpha smooth muscle), collagen, neutral mucins and acidic mucins around tumor islands and within connective tissue. **Conclusion:** Understanding cancer by stromal cell genomic and histochemical analysis, provide more comprehensive and meaningful data, as this surrounding stroma plays an important role in the progression of cancer. The cancer associated with a reactive stroma is typically diagnostic of poor prognosis. So this study confirms that characterizing the stromal cells and their reciprocal interaction with tumor cells will provide supportive evidence that stromal therapy can be a rewarding approach for cancer prevention and intervention.

Key words: Extracellular matrix, transforming growth factor-beta, tumor microenvironment

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a disfiguring, potentially fatal disease, with a significant impact on quality of life. It continues to rise in incidence among younger and older people alike accounting for approximately 94% of all the oral malignancies.[1,2] In spite of advances in the last three decades in combined chemotherapy, radiotherapy, and targeted therapy and with the expanding field of oncology, that have helped us to explore molecular pathogenesis, the annual incidence and the mortality rate continue to rise in the incidence, with survival rates remaining in range of 50–59%. Hence, the prognosis of OSCC is poor due to aggressive local invasion and metastasis thus leading to recurrences. Therefore, OSCC is still a challenging disease in the field of head and neck cancer.This prevention could be achieved by recognizing the prominent modifiers of cancer initiation and progression.[3]

OSCC is a malignant epithelial neoplasm with varying degree of squamous differentiation and epithelial mutation. Migration of these oncogenetically mutated epithelial cells through the basement membrane and invasion into the underlying connective tissue stroma causes dynamic changes in its microenvironment, which can be seen as radical changes in the stroma.[4,5]

Connective tissue stroma has a role in maintaining not only the normal epithelial tissues by their tumor-suppressing abilities, but they also orchestrate with the cancer cells to regulate the disease progression. Stroma constitutes the extracellular matrix (ECM), which consist of three basic components, i.e., fibrous structural proteins, proteoglycans, and glycoproteins and is a dynamic, constantly remodeling

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macromolecular complex which regulates the proliferation, movement, and differentiation of the cells living within it.[4,5] Ironically, in cancer, the neoplastic cells invade the healthy underlying stromal tissue, evokes a fibrotic response by causing degradation of ECM, which results in the release of growth factors and cytokines such as transforming growth factor-beta (TGF-β), which further signal for the progression and leading toward the malignancy.[4-8]

TGF-β is known to have been involved in transdifferentiation of the fibroblasts to myofibroblasts (MFs), producing an activated MF rich stromal microenvironment which is considered to be the hallmark of myofibroblastic phenotype with subsequent increased deposition of the collagen, contributing to increased mechanical stiffness.[7-9]

Fibrous structural protein Type IV collagen is considered to be one of the most affected molecules as its degradation is seen during tumor invasion and is mediated by metalloproteinases secreted by the neoplastic cells. In advanced stages of invasion, the capacity of degradation increases, associating the discontinuity of the basal lamina with an increased probability of metastasis, and a poor prognosis.[6,10]

Regressive changes are seen with respect to the glycoprotein and glycosaminoglycans, which is too mediated by matrix metalloproteinases (MMPs), resulting in the disintegration of the stromal components, thus providing space for the tumor cells to proliferate as well as migrate. Thus, they act as a scaffold for infiltration of the inflammatory cells. Lysis also results in the release of the growth factors and cytokines enhancing the growth of tumor cells.[6,10,11]

Henceforth, analyzing the complexities of surrounding stromal tissue, and not only focusing on heterogenicities of the tumor cells, a more comprehensive and meaningful data on the tumor biology will be achieved which enhance one's knowledge in understanding tumors behind the progression of malignancy. Such cancer cells are typically diagnostic as diagnosis does not depend notably on the appearance of the tumor cells in recapitulating the appearance of primordial cells from which they arise but also on specific stromal changes. Embodying these notions into a prognostic system may help in predicting the tumor behavior and clinical outcome.[8,11,12]

Therefore, the aim of our study was to predict the biological behavior of tumors by evaluating the stromal changes in different histopathological grades of OSCC using connective tissue stains as well using immunohistochemical analysis.

MATERIALS AND METHODS

A total of histologically confirmed 30 cases of well-, moderate- and poorly-differentiated OSCC were taken from the archives of the Department of Oral and Maxillofacial Pathology and Microbiology, I.T.S. Dental College, Muradnagar, Ghaziabad, Uttar Pradesh, India. The special stains used for the study were periodic acid–Schiff (PAS), Alcian blue-PAS (AB-PAS), Van Gieson's, and Picrosirius red stain.

In addition, immunohistochemistry using alpha-smooth muscle actin (α-SMA) was done on all three grades of OSCC, so as to access the role of MF in the progression of the lesion. Staining intensity was evaluated and graded as [Table 1]:

Sections were then deparaffinized in xylene and hydrated through decreasing grades of alcohol and taken to water. Consequently, the sections were stained with connective tissue-specific stains using specific protocols for each stain. All the three grades of OSCC's were evaluated for staining intensities of acidic mucins, neutral mucins, and collagen using AB-PAS, PAS, and Picrosirius red and Van Gieson's stains, respectively [Table 2] and were scored as [Table 3]:

Analysis of collagen

Interpretation and comparison of collagen fiber hue using Picrosirius red stain were done under a polarizing microscope.

Assessment criteria based on hue [Table 4]:

Table 1: Frequency distribution of alpha SMA in different grades of OSCC

Staining specificity	Staining intensity
0	Mild
+1	Moderate
+2	Severe

Table 2: Techniques used for staining connective tissue components

Staining technique	Positive color	Components stained
PAS stain	Deep red/magenta	Neutral mucins
AB-PAS stain	Acid mucins-blue Neutral mucins-magenta/ deep red	Acid mucins Neutral mucins
Picrosirius red stain	Color variance seen along with advancing grades of tumor	Mature and immature collagen fibers
Van Geison's stain	Red	Collagen

PAS: Periodic acid–Schiff, AB-PAS: Alcian blue-periodic acid–Schiff

Table 3: Frequency distribution of connective tissue special stains in different grades of OSCC

Staining specificity	Staining intensity
0	Weak or absent
1	Moderate
2	Bright

Statistical analysis

Results of the following stains (PAS, AB-PAS, and Van Geison's) and the differences in the presence of MFs between the groups were statistically analyzed using the Pearson's Chi-square test. $P < 0.05$ was considered to be statistically significant.[5,7]

RESULTS

Stains used in this study are

Van Gieson's stain was performed on all the three grades of OSCC to examine the staining intensity of collagen fibers. Collagen fiber has taken red color while the other fibers have taken yellow color and nuclei has taken black color. Staining intensity was high in well- differentiated squamous cell carcinoma and gradually decline with increasing grades of OSCC [Figure 1a-c].

Further analysis of staining pattern for neutral mucins was identified in the disease progression from well-differentiated carcinomas to poorly differentiated carcinomas, using PAS and maximum intensity was noted in poorly differentiated squamous cell carcinoma, which is demonstrated as bright magenta color [Figure 2a-c].

Histopathological sections were stained using combined AB-PAS to differentiate acidic mucins from neutral mucins in a tissue section. This stain is used as a broad means of detecting mucins, and accessing their role in the disease progression. The cells are stained varying shades of purple to blue-purple. In the most protocol, this stain stains neutral mucins as deep red-magenta and stains acidic mucins (sialomucins, sulfomucins) as blue and maximum staining for acidic mucins is noted in well-differentiated OSCC of our study groups [Figure 3a-c].

Next stain used was Picrosirius red stain for the assessment of nature of fibers, the fiber hue as well as the spatial distribution of different colors in various grades of OSCC and correlate it with the tumor progression. It was found that the birefringence of collagen fibers changes with the different grades of OSCC as the collagen fibers in well-differentiated squamous cell carcinoma revealed the polarizing color of reddish orange, in moderately differentiated carcinoma revealed yellowish orange, and in poorly differentiated carcinoma revealed yellowish green. The purpose of using this stain is because it can detect thin fibers as the other routine stains employed to stain collagen have poor specificity for thin fibers [Figure 4a and b].

On application of Chi-square test, high significant *P* value was noted for neutral mucins and collagen, and *P* value for acidic mucins was not significant [Tables 5-7].

Table 4: Assessment of polarization colors of collagen fibers

Collagen type	Fiber color
Thick	Normal yellowish-orange to orange red
Thin	Green to greenish-yellow
Thick pathological	Green to greenish-yellow

Table 5: Periodic acid–Schiff

Chi-square test	Value	df	*P*
Pearson Chi-square	11.111	4	0.025
Number of valid cases	30		

Table 6: Alcian blue-periodic acid–Schiff

Chi-square test	Value	df	*P*
Pearson Chi-square	8.667	4.070	
Number of valid cases	30		

Table 7: Van Geison's

Chi-square test	Value	df	*P*
Pearson Chi-square	15.333	4	0.004
Number of valid cases	30		

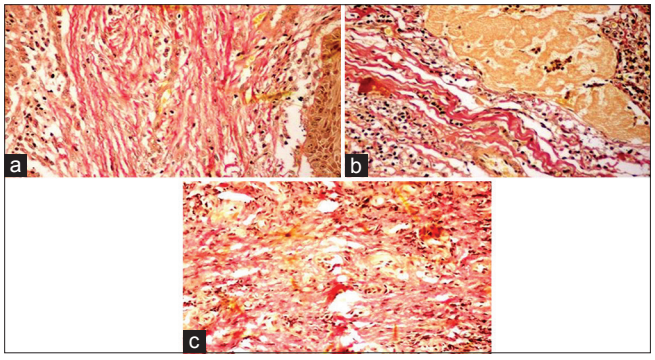

Figure 1: (a) Histopathological section showing Van Gieson's stain in connective tissue stroma in well-differentiated oral squamous cell carcinoma, ×10, (b) histopathological section showing Van Gieson's stain in connective tissue stroma in moderately differentiated oral squamous cell carcinoma, ×10, (c) histopathological section showing Van Gieson's stain in connective tissue stroma in poorly differentiated oral squamous cell carcinoma, ×10

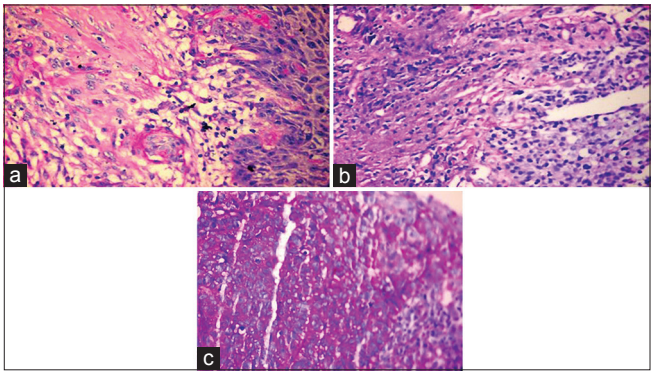

Figure 2: (a) Histopathological section showing periodic acid–Schiff stain in connective tissue stroma in well-differentiated oral squamous cell carcinoma, ×10, (b) histopathological section showing periodic acid–Schiff stain in connective tissue stroma in moderately differentiated oral squamous cell carcinoma, ×10, (c) histopathological section showing periodic acid–Schiff stain in connective tissue stroma in poorly differentiated oral squamous cell carcinoma, ×10

Further analysis includes immunohistochemical staining, where α-SMA was checked in nonendothelial, and noninflammatory stromal spindle cell and the positive reaction for α-SMA were observed, and these cells were regarded as MFs. MFs were found intimately surrounding the tumor cells, and as we move on to poorly differentiated carcinomas, it is more expressed in the stroma of well-differentiated OSCC [Figure 5].

DISCUSSION

Solid tumors are composed of two discrete components that are malignant epithelial cells and the stroma in which they are dispersed (includes inflammatory cells, endothelial cells, and fibroblasts). It has been reported that this ECM produced by the transformed cells differs from that produced by the normal cells as the invading tumor cells induce an abundant collagenous, or desmoplastic stroma.[13,14] There is an increase in production of elastic fibers to limit the invasion. Changes in the ECM are noticeable in our study also, which indicate the propensity of tumor cells to infiltrate and metastasize and can be used as one of the prognostic indicators.[15]

On analyzing collagen using Van Gieson's stain, it was found to be more abundant in the stroma of well-differentiated squamous cell carcinoma attributed to the increased deposition of the thick bands of collagen fibers that are Type I collagen. While going to poorly differentiated carcinomas, the disintegration of collagen fibers is seen as thin immature Type III reticulin fibers, attributed to the action of MMP's, resulting in an abortive stroma, which acts as a scaffold for the migration of tumor cells.

Picrosirius red stain shows a gradual change in polarizing color, along with advancing grade of tumor. Strong birefringence of red, orange, and yellow color in well-differentiated squamous cell carcinomas are indicative of deposition of Type I collagen fibers (thick band of densely packed fibers) while a weak birefringence of green is visible in poorly differentiated carcinomas due to deposition of Type III collagen which is thin fibrillary protein. This is attributed to carcinogenic events, taking place such as the action of MMP's, resulting in the pathological breakdown of ECM by tumor cells, thereby promoting tumor progression. Hence, collagen profiling can be effectively used to correlate qualitative nature of collagen, to progression, and clinical behavior of OSCC.[10,16]

PAS positivity was high in the connective tissue of poorly differentiated carcinomas indicating increased deposition

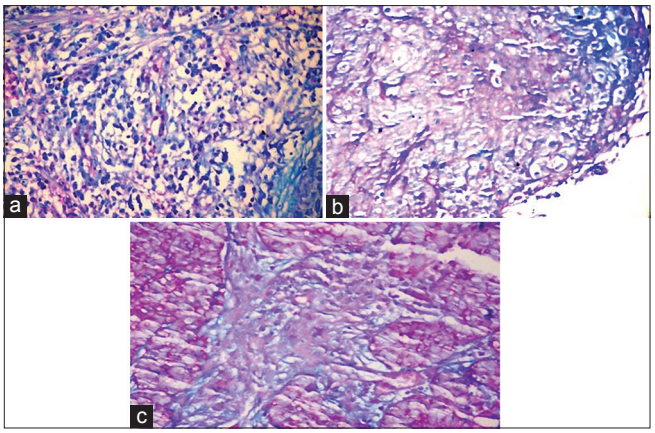

Figure 3: (a) Histopathological section showing Alcian blue-periodic acid–Schiff stain in connective tissue stroma in well-differentiated oral squamous cell carcinoma, ×10, (b) histopathological section showing Alcian blue-periodic acid–Schiff stain in connective tissue stroma in moderately differentiated oral squamous cell carcinoma, ×10, (c) histopathological section showing Alcian blue-periodic acid–Schiff stain in connective tissue stroma in poorly differentiated oral squamous cell carcinoma, ×10

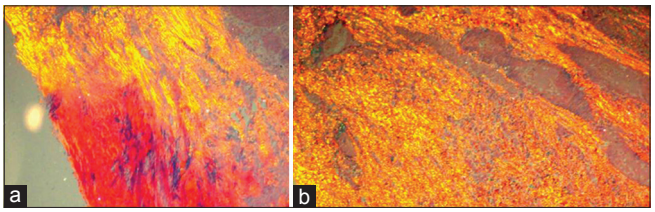

Figure 4: (a) Photomicrograph of well-differentiated oral squamous cell carcinoma showing reddish birefringence in Picrosirius red stain, ×10, (b) photomicrograph of moderately differentiated oral squamous cell carcinoma showing yellowish red birefringence in Picrosirius red stain, ×10

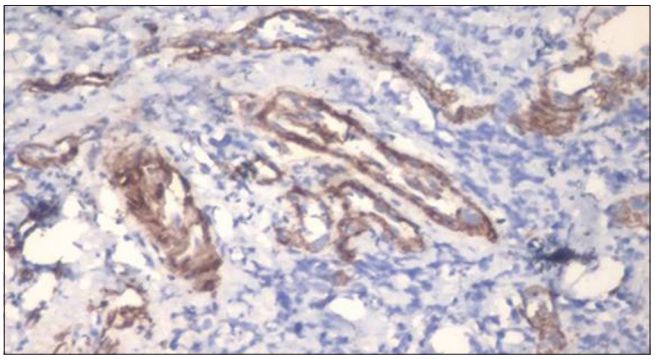

Figure 5: Alpha-smooth muscle actin expression in poorly differentiated oral squamous cell carcinoma, (IHC, ×10)

of neutral mucins secretion. Maximum intensity was seen in poorly differentiated carcinomas. This is attributed to their secretion by epithelial cells as evident by their presence adjacent to the tumor cells. They can also be a product of stromal cells produced in response to invading heterogenous tumor cell population, which act probably as a scaffold around tumor cells.[5,9]

Acidic mucins were scanty in all the grades, and maximum was in well-differentiated carcinoma. This suggests that

these sulfated mucins to be the product of stromal cells. This is attributed to the regressive changes taking place in the connective tissue, more in poorly differentiated carcinomas. Hence, less expression of acidic mucins was seen in poorly differentiated carcinomas.[5,9]

As mentioned earlier, TGF-β can induce transdifferentiation of fibroblasts into MFs, causing increased collagen deposition by inhibiting the induction of nitrous oxide synthetase pathways and hence promotes survival of MF by providing protection against apoptosis. However, no very significant myofibroblastic reaction was seen in our study groups as there is an established fact that there exists a casual role of MF in transition from noninvasive to invasive phenotype.[6-8]

CONCLUSION

In spite of the development in recent targets of molecular strategies, analyses of the underlying changes in the connective tissue fibers at the biochemical level will be exorbitant. The findings of our study infer that there exists a co-evolution of the tumor cells and the stromal cells. The abnormal interplay and the active crosstalk result in the permanent alterations in the stromal cell phenotype. These stromal changes can be concluded as the drivers of invasive cancer growth, which can help for targeting stroma for various treatment strategies.[5,6]

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Conflicts of interest
There are no conflicts of interest.

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