Morphological analysis of collagen and elastic fibers in oral squamous cell carcinoma using special stains and comparison with Broder’s and Bryne’s grading systems

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ABSTRACT

Objectives: Morphologic detection of connective tissue fiber changes in oral squamous cell carcinoma (OSCC) using special stains remains less documented. The aims of the present study were to study the collagen and elastic fibers in different stages of OSCC and to correlate these changes with two grading systems - Broder’s and Bryne’s.

Study Design: Forty-eight cases of OSCC were studied using hematoxylin and eosin, Verhoeff’s - Van Gieson stain for elastic fibers and picrosirius red stain for collagen fibers. The changes were compared with all the grades of carcinoma. Normal mucosa was taken as control.

Results: Statistical analysis using Chi-square and ANOVA, showed significant association between the grades of carcinoma and extracellular matrix changes. Greenish-yellow collagen fibers were found to be significantly increased in the poorly differentiated/Grade 3 cases ($P < 0.0001$) where as well-differentiated/Grade 1 cases showed predominantly reddish-orange and yellowish-orange birefringence of collagen fibers. Chi-square analysis showed a significant amount of fragmented pattern of elastic fibers in poorly differentiated OSCC ($\chi^2 = 104.45, P = 0.009$)/Grade 3 OSCC ($\chi^2 = 94.81, P = 0.016$).

Conclusion: The study of the connective tissue stromal changes can be used as an adjunct to histological grading and thereby helping the surgeon to determine the amount of marginal clearance.

Key words: Broder’s grading, Bryne’s grading, collagen fibers, elastic fibers, oral squamous cell carcinoma, picrosirius red staining, Verhoeff Van-Gieson stain

Oral cancer is the sixth most common cancer. Oral squamous cell carcinoma (OSCC) is the malignant tumor of epithelial cells of the oral cavity and constitutes more than 90% of oral cancers.[1]

OSCC comprises two discrete compartments, the malignant epithelial cells and the stroma or extracellular matrix (ECM).[2] The ECM is the noncellular component present within all tissues and organs, and initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation, and homeostasis.[3,4] ECMs act as scaffolding for cell adhesion and they influence tumor behavior.[5] It has also been reported that the ECM produced by the transformed cells differs from normal cells.[6] Some studies have suggested that the invading tumor cells induce an abundant collagenous or desmoplastic stroma.[7] The studies on lung carcinoma and breast carcinomas[8,9] have shown elastosis. Hence, there can be an increased production of elastic fibers in OSCC, which can limit the invasion.

Studies have shown that the behavior of mesenchyme in tumors can indicate the ability of the tumor cells to invade
and metastasize. These extracellular matrix changes can thus be used as one of the prognostic indicators.\[^{6}\] Even though many markers are available to study the tumor-stroma interface and the ECM, morphologic detection on hematoxylin and eosin (H and E) and special stains for connective tissue fibers are cost-effective compared to the molecular markers and available at most of the hospitals and can be used to advise the surgeon regarding the propensity for invasion and metastasis.\[^{10}\] Sutton et al. in their study found high correlation between various histological indicators of aggressive disease and close or involved surgical margins. Hence, a pathologist guidance is necessary to ensure marginal clearance.\[^{11}\]

Collagen and elastic fibers are the major structural proteins in ECM. The ECM in the oral cavity is in the form of a delicate layer of connective tissue - the lamina propria composed of a few elastic and collagenous fibers and the submucosa of loosely arranged connective tissue. The elastic fibers, which are present in the ECM of oral cavity, are oxytalan fibers and do not stain with the routinely used stains for elastic fibers as they do not contain the protein elastin. Changes in ECM and ECM matrix proteins have been studied in the metastatic progression of colon, lung, prostate, and cervical cancer.\[^{6}\]

Research on the relationship between advanced dysplastic grading systems and the stromal reaction in oral lesions is fragmentary\[^{12,13}\] and most previous reports focus on epithelial cells.\[^{14,15}\] Earlier studies have attempted to prognosticate OSCC based on conventional and invasive front grading systems.

Broder proposed a grading system for squamous cell carcinomas based on the percentage of cells showing incomplete differentiation. OSCC being composed of a heterogeneous population of cells, the neoplastic nature of the tumor cells differs in the superficial part and the advancing front (Bryne, 1998). The tumor-host interface (invasive front) shows various molecular events of importance in tumor spread, such as the gain and loss of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation, and initiation of angiogenesis.\[^{16}\] Bryne’s classification introduced the concept of invasive front in 1992 and according to this classification, there is a lower degree of cellular differentiation at the invasive front compared to other areas.\[^{17}\]

Hence, in this study, the changes of ECM regarding the changes in the collagen and elastic fibers were compared to both Broder’s grading system and Bryne’s invasive front grading system.

**SUBJECTS AND METHODS**

Specimens were obtained from the archival tissue as well as the cases reported during the study in our institution. Tissues with adequate connective tissue thickness were included in the study. Cases of anaplastic carcinoma were excluded from the study. The normal tissues for control were obtained from the Department of Oral and Maxillofacial surgery, during surgical procedures such as impaction and orthognathic surgery.

Three sections were taken from each paraffin block. H and E staining was done for the study sections and was graded according to Broder’s and Bryne’s grading system. Picrosirius red (PSR) staining was done for observing the polarization colors of collagen,\[^{18}\] and Verhoeff’s – Van Gieson (VVG) special stain was done for assessing the morphological pattern of elastic fibers\[^{19}\] as described earlier. The PSR stained slides were observed under polarized microscopy. The nature of collagen fibers thin or thick depending on the polarizing colors varying from reddish-orange, yellowish-green, green was observed. Five fields under a magnification of 20 X adjacent to the tumor islands were observed for the polarizing color changes and tabulated under the corresponding grades of carcinoma for both Broder’s and Bryne’s grading system. The frequency of occurrence of each polarizing color based on the total fields was examined and it was expressed as percentage. The slides stained by VVG were examined under light microscopy and the morphology of elastic fibers adjacent to the tumor cells was tabulated as-normal, clumped fibers, and short/fragmented fibers. Five fields under a magnification of 20 X were observed for the morphological changes, which were tabulated under the corresponding grades of carcinoma for both Broder’s, and Bryne’s grading system. The frequency of occurrence of morphological variation of elastic fibers based on the total fields was analyzed and it was expressed as percentage.

The relevance of connective tissue fiber changes and its association with the widely accepted grading systems – Broder’s and Bryne’s was evaluated. For arriving at the statistical significance between connective tissue fiber changes and the different grades of OSCC Chi-square test using 3 × 3 contingency table was performed.

The association of each polarizing color of collagen fibers and morphological changes of elastic fibers, across normal mucosa and different grades of OSCC were analyzed using ANOVA. Significance was derived using Kruskal–Wallis test by comparing the median percentage of particular polarizing color of collagen fiber/morphological pattern of elastic fiber across normal and different grades of OSCC. Dunn’s multiple comparison test was performed between each groups and differences in rank sum was compared. P value was considered statistically significant if <0.05.

**RESULTS**

**Samples**

This study was done on histopathologically diagnosed cases of OSCC. A total of 48 cases were taken and graded
according to the Broder’s grading system and Bryne’s grading system. Of the 10 normal cases (3-labial mucosa, 3-buccal mucosa, 1-hard palate, 2-soft palate, and 1-tongue) all of them showed reddish- and orange-red birefringence when the PSR stained slides were observed under polarized microscopy. VVG stained slides of normal mucosa showed all the three different morphological patterns - normal long fibers, clumped fibers, and fragmented fibers, but long normal looking fibers were in majority, other two patterns in sparse.

After grading according to Broder’s grading system, the number of cases was distributed as follows:

Well-differentiated squamous cell carcinoma - 17 cases, moderately differentiated squamous cell carcinoma - 16 cases, poorly differentiated squamous cell carcinoma - 15 cases. According to Bryne’s grading system, the distribution was changed as follows: Grade 1-15 cases, Grade 2-15 cases, and Grade 3-18 cases.

The frequency of occurrence of different polarizing colors of collagen fibers (reddish-orange, yellowish-orange, greenish-yellow)/different morphological patterns of elastic fibers (long fibers, clumped, and fragmented) was expressed as a percentage for each case and was later was compared among the different grades of OSCC using ANOVA [Tables 1 and 2].

Chi-square analysis showed significance in the changes of polarizing colors of collagen fibers as well as the morphological changes in elastic fibers across different grades of OSCC, according to both Broder’s conventional grading system and Bryne’s invasive front grading system [Tables 3 and 4].

Both grading systems showed that there was a significant change in the amount of reddish-orange fibers in moderately and poorly differentiated OSCC when compared to the normal mucosa and there was significant difference in the relative amount of yellowish-orange fibers between moderately differentiated and poorly differentiated cases as well as between well differentiated and poorly differentiated (P < 0.0001) [Table 3].

The distribution of greenish-yellow fibers was significantly increased in poorly differentiated when compared to well-differentiated, moderately differentiated cases of OSCC, and normal mucosa (P < 0.0001) [Table 3]. Figure 1a-d shows different polarizing colors of collagen fibers (PSR stain).

Table 1: Frequency of morphological changes of collagen and elastic fibers according to broder’s grading system

| Grade of OSCC (Broder’s grading) | Reddish orange birefringence (%) | Yellowish orange birefringence (%) | Greenish yellow birefringence (%) | Normal elastic fibers (%) | Clumped elastic fibers (%) | Fragmented elastic fibers (%) |
|----------------------------------|----------------------------------|-----------------------------------|----------------------------------|--------------------------|---------------------------|------------------------------|
| Well differentiated OSCC (cases-17) | 41 | 59 | 0 | 11.76 | 23.5 | 64.7 |
| Moderately differentiated OSCC (cases-18) | 12.5 | 43.75 | 43.75 | 33.3 | 10.6 | 56.1 |
| Poorly differentiated OSCC (cases 15) | 0 | 0 | 100 | 0 | 30 | 70 |

Table 2: Frequency of morphological changes of collagen and elastic fibers according to bryne’s grading system

| Grade of OSCC (Bryne’s grading) | Reddish orange birefringence (%) | Yellowish orange birefringence (%) | Greenish yellow birefringence (%) | Normal elastic fibers (%) | Clumped elastic fibers (%) | Fragmented elastic fibers (%) |
|---------------------------------|----------------------------------|-----------------------------------|----------------------------------|--------------------------|---------------------------|------------------------------|
| Grade 1 OSCC (cases-15) | 46.6 | 40 | 13.3 | 13.3 | 26.6 | 60.0 |
| Grade 2 OSCC (cases-15) | 13.3 | 33.3 | 53.3 | 20 | 20 | 60 |
| Grade 3 OSCC (cases-17) | 0 | 0 | 100 | 0 | 27.3 | 72.7 |

Table 3: Polarizing color changes of collagen fibers according to both grading systems. (N=no: of cases x 5 fields)

| Grading system | Histopathological Grades of OSCC | Colors observed (20x) | Statistical analysis |
|----------------|----------------------------------|-----------------------|----------------------|
| Broder’s grading system | Well differentiated (N=85) | 34 | 37 | 14 | df=4 |
| | Moderately differentiated (N=80) | 10 | 33 | 37 | Chi-square=104.45 |
| | Poorly differentiated (N=75) | 1 | 4 | 70 | P=0 |
| | Total (N=240) | 45 | 74 | 121 | |
| Bryne’s grading system | Grade 1 (N=75) | 32 | 31 | 12 | df=4 |
| | Grade 2 (N=75) | 11 | 34 | 30 | Chi-square=94.81 |
| | Grade 3 (N=90) | 1 | 13 | 76 | P=0 |
| | Total (N=240) | 44 | 78 | 118 | |
Similarly, there was a significant difference in the distribution of normal elastic fibers between normal mucosa and all grades of OSCC, with a significant $P$ value ($< 0.001$) on comparison with poorly differentiated cases. Multiple comparisons of clumped elastic fibers between different groups were not significant. Poorly differentiated cases were having a fragmented pattern of elastic fibers [Figure 1d] when compared to well differentiated, moderately differentiated cases of OSCC, and normal mucosa ($P < 0.01$). Figures 2a-d shows morphological changes of elastic fibers (VVG stain).

**DISCUSSION**

Recent advancements have brought forward many prognostic markers for OSCC. However, in most of the medical centers, the histopathology report guides the clinician to plan further treatment (radiotherapy or chemotherapy) and to assess prognosis according to the grade of tumor and adequacy of resection margins.$^6$ ECM influences tumor behavior, inappropriate synthesis, or degradation of any ECM molecule can alter cell physiology and help in the progression of disease.$^{1,2}$ There is a lacuna in the current literature regarding the ECM changes in different grades of SCC using histochemical special stains. Studies analyzing the morphological pattern of elastic fibers in OSCC have been found to be sparse in the literature. Hence, in this study, the changes in the collagen and elastic fibers, components of the ECM were studied special stains in 48 cases of OSCC.

This study show changes in the polarizing colors of collagen from reddish-orange to greenish-yellow across different grades of OSCC, greenish-yellow being significantly increased in poorly differentiated/Grade 3 carcinomas. In addition, there was a significantly increased amount of fragmented/short elastic fibers in poorly differentiated/Grade 3 OSCCs when compared to normal mucosa.

Of the total 48 cases of OSCC taken for the study, 17 were classified as well differentiated, 16 as moderately, and 15 as poorly based on the degree of keratinization and lymphoplasmacytic infiltration (Broder’s grading system). When all the 48 cases were subjected to Bryne’s grading system, 43 cases showed the same grade as that of the Broder’s grading system. A total of 2 cases of well-differentiated OSCC

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**Table 4: Morphological patterns of elastic fibers according to both grading systems. ($N$=no:of cases x 5 fields)**

| Grading system | Histopathological Grades of OSCC | Elastic fiber morphology (20x) | Statistical analysis |
|----------------|---------------------------------|-------------------------------|---------------------|
| Broder’s grading system | Well differentiated ($N=82$) | 22 | 19 | 41 | $df=4$ | Chi-square=13.31 |
| | Moderately differentiated ($N=75$) | 25 | 7 | 43 | |
| | Poorly differentiated ($N=50$) | 5 | 12 | 33 | |
| | Total ($N=207$) | 52 | 38 | 117 | |
| Bryne’s grading system | Grade 1 ($N=75$) | 22 | 18 | 35 | $df=4$ | Chi-square=12.13 |
| | Grade 2 ($N=75$) | 22 | 6 | 47 | |
| | Grade 3 ($N=60$) | 9 | 14 | 37 | |
| | Total ($N=210$) | 53 | 38 | 119 | |

OSCC=Oral squamous cell carcinoma

**Figure 1:** (a) Reddish-birefringence of collagen fibers in normal mucosa (PSR, photomicrograph 20×) (b) reddish-orange birefringence of collagen fibers in oral squamous cell carcinoma (PSR, photomicrograph 20×). (c) Yellowish-orange birefringence of collagen fibers in oral squamous cell carcinoma (PSR, photomicrograph 20×). (d) Greenish-yellow birefringence of collagen fibers in oral squamous cell carcinoma (PSR, photomicrograph 20×)

**Figure 2:** Elastic fibers are stained black. (a) Elastic fiber morphology in normal mucosa long fibers (VVG, photomicrograph 20×). (b) Elastic fiber morphology in oral squamous cell carcinoma - long fibers adjacent to tumor islands (VVG, photomicrograph 20×). (c) Elastic fiber morphology in oral squamous cell carcinoma - clumped elastic fibers adjacent to tumor islands (VVG, photomicrograph 20×). (d) Elastic fiber morphology in oral squamous cell carcinoma - fragmented elastic fibers adjacent to tumor islands (VVG, photomicrograph 20×)
were grouped under Grade 2 of Bryne’s grade and 3 cases of moderately differentiated cases of OSCC were grouped under Grade 3 of Bryne’s grading system. The degree of keratinization and lymphocytic infiltration being common criteria in both grading systems, the difference in grading was mainly due to the amount of nuclear pleomorphism and pattern of invasion which was taken into consideration in Bryne’s grading system. Another reason is that only the most invasive front was taken for the assessment in Bryne’s grading which showed less degree of cellular differentiation when compared to other parts of the tumor.

Studies by Kristensen et al., Neena et al. and Yazdi[20–22] had reported prognostic significance of invasive front grading compared to lymph vascular space involvement, lymph node status, and grade of differentiation. According to Piikkó et al.,[23] multiparametric histopathological tumor front grade was significantly and independently associated with tumor-related death, irrespective of conventional Broder’s grade, and clinical stage of the tumors. On the contrary, a study by Weijers et al.[24] showed that neither of the histological grading systems has a strong predictive value and that none is superior to the other. Akinyamoju et al.[25] compared both Bryne’s and Broder’s grading system with clinicodemographic data of patients and reports that both systems provide similar results. However, the present authors suggest that Broder’s grading system is highly subjective and hence, when inter observer variability is encountered Bryne’s invasive front grading system can be used which is more of significance when compared to the conventional Broder’s grading system.

In this study, with respect to the relationship between the collagenous components in the stroma and the invading tumor cells, there have been some observable changes in different histological grades of OSCC. 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 tumor islands. This may be due to the deposition of collagen fibers which were in the form of thick bands and composed of closely packed fibrils, this feature being consistent with the reports by Montes and Junqueira.[18] They stated that the thick fibers were Type I collagen fibers and exhibited an intense birefringence of red, orange, and yellow color by polarizing microscopy and a weak birefringence of green when the fibers were thin fibrillar thus constituting Type III collagen.[18] Similarly, studies by Rich et al.[26] state that the hue component of collagen fibers (red, orange, yellow, and green) changes according to the thickness. George et al.[27] studied staining intensity of collagen, reticulin, acid mucins, fibrin, glycoproteins, sulfated mucins, and elastic fibers around the tumor islands and within the connective tissue of OSCC and reported highly significant changes in collagen and fibrin staining.

Carcinoma-associated fibroblasts are unique and have been studied in several types of human cancer, for example, breast, prostate, pancreatic, colon, and lung. They undergo dynamic changes in the accompanying tumor and results in desmoplasia and is associated with the recruitment of inflammatory cells and activation of angiogenic programs.[28] In this study, a few cases of well-differentiated carcinoma showed reddish-orange birefringence (thick fibers) and there was no significant difference between normal mucosa and well-differentiated OSCC cases, regarding the presence of thick collagen fibers. Whereas the presence of greenish-yellow fibers (thin fibers) in poorly differentiated cases was highly significant, with a P < 0.001. Similar results were obtained by Venigella on comparing the polarization colors with different grades of OSCC.[7] It is also consistent with the study by Sekiguchi et al.[29] who showed that carcinomas with scanty interstitial Type I collagen in biopsy specimens tended to have highly malignant characteristics. Similarly, Manjunatha et al.,[30] in their study, show that moderately to poorly differentiated OSCC cases demonstrate a gradual change in polarizing colors from yellowish-orange to greenish-yellow particularly, in the vicinity of invading tumor islands. Zhang et al.[31] have also proved that collagenous stromal changes in parakeratinized and orthokeratinized types of odontogenic keratocyst suggesting that the stroma of keratinizing cystic odontogenic tumor may play an important role in determining the neoplastic behavior of the lesion through epithelial–mesenchymal interaction.

Neoplasms undergoing malignant conversion show an increased architectural disorder at the invasive front of the neoplastic mass. Increased production of matrix remodeling enzymes and synthesis of many matrix components, most notably Type I collagen, occur at this site. MMPs (matrix metalloproteinases) produced by the cancer-associated fibroblasts as well as the inflammatory cells degrade the ECM structural components, both collagens as well as elastic, and thus helps in neoplastic progression. Indeed, cleavage of collagen Type I is required for endothelial cell invasion of the ECM and vessel formation.[30] The predominance of yellowish-green fibers/thin collagen fibers/Type III collagen in poorly differentiated carcinoma as seen from this study supports the above said changes produced by MMPs in advanced grades of OSCC. The statistical analysis for variation in the polarization colors of collagen from reddish-orange to greenish-yellow was observed to be significant for Bryne’s grading also.

Elastic fibers are major insoluble ECM assemblies that endow connective tissues with resilience. There is a lacuna in the current literature regarding changes in elastic fibers in the stroma of OSCC cases. In the normal mucosa, elastic fibers are seen in the form of long wavy fibers intermingled with few short and thick bundles of elastic fibers. The short and
thick bundles of fibers indicate that remodeling takes place within the stroma.

In this study, elastic fibers were present in all the cases of well differentiated and moderately differentiated/Grade 1 and Grade 2 cases of OSCCs. However, out of 15 cases of poorly differentiated OSCC, 4 cases showed minimal/no elastic fibers in the tumor-adjacent stroma. In the rest of the cases, the amount of elastic fibers were less than that of normal mucosa and consisted of relatively more of short/fragmented fibers.

Studies on lung carcinoma and breast carcinoma showed elastosis (increase in the amount of elastic fibers) in well-differentiated tumors and when present the prognosis was better. Hence, it is suggested that as elastic fibers can limit the tumor invasion, the presence of normal elastic fibers around the tumor cells can be counted as a prognostic indicator.\(^5\)\(^,\)\(^6\) Similarly, in the study by Agrawal et al.\(^6\) on OSCC, the lymph node metastasis was present in only (25%) of the total cases \(\left(P = 0.087\right)\), indicating that the presence of elastic fibers and tissue remodeling can act as a limiting factor in spread and metastasis of tumor. However, Agrawal et al.\(^6\) also state that cases of OSCC with dense lymphocytic infiltration did not show elastic fibers on VVG stain. In contrary to Agrawal et al., we have observed many elastic fibers showing fragmented pattern even in areas with dense lymphocytic infiltration. In a study by Piva et al.,\(^3\)\(^2\) it is suggested that intensity of the inflammatory infiltrate should not be used as a parameter in the prognostic assessment of OSCC as it exercises different functions in the various stages of carcinogenesis.

In this study, the morphological changes in the elastic fibers were taken for analysis rather than the amount and was found to be statistically significant according to both grading systems. Elastic fiber nature is shown to be more of frayed/fragmented/clumped in patients with pseudoxantheroma elasticum, Ehlers-Danlos syndrome and in aged persons,\(^3\)\(^3\) which shows a dysregulation in the synthesis-degradation of elastic fibers. Hence, the increase in the amount of fragmented elastic fibers in Grade 2 and Grade 3 cases of OSCCs clearly indicates a disturbance in the ECM surrounding the tumor cells probably by the influence of MMPs produced in tumor stroma.

**CONCLUSION**

To conclude, collagen and elastic fibers are showing significant changes in different grades of OSCC. The presence of greenish-yellow polarizing color of collagen fibers under polarizing microscope and the presence of fragmented/short elastic fibers have been shown as significant indicators of advanced grades of OSCC. These histochemical special stains being less expensive and less time consuming when compared to IHC can be routinely done on any biopsy specimen to understand the stromal changes and as an adjunct in determining the marginal clearance. The data shown with the collagen and elastic fibers staining pattern suggest that these could have potential prognostic relevance as well, however it needs to be validated in a large cohort of patient samples.

**Acknowledgment**

I acknowledge the Department of Oral Pathology, Sri Hasanamba Dental College, Hassan for the support.

**Financial support and sponsorship**

This study was supported by the Department of Oral Pathology, Sri Hasanamba Dental College, Hassan.

**Conflicts of interest**

There are no conflicts of interest.

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