Original Article

Evaluation of extracellular matrix changes among oral submucous fibrosis and oral squamous cell carcinoma patients of Malwa region of Punjab using special histochemical stains: An insight into cancerous transformation

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Abstract

Background: To assess extracellular matrix changes among oral submucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC) patients using special histochemical stains.

Materials and Methods: Twenty biopsy specimens of OSMF and 30 biopsy specimens of OSCC were included in the present study. Among 20 OSMF specimens, 10 were of early OSMF and the remaining 10 were of advanced OSMF. Out of 30 OSCC specimens, 10 cases each were of well-differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC. Three sections, each 4 μm thick, were obtained from all specimens. One section was stained with routine H&E staining, whereas the other section was stained with Masson's trichrome (MT) stain for collagen and Verhoeff–Van Gieson (VVG) for elastic fibres. Evaluation of all specimens was performed under the light microscope. The arrangement of collagen fibres and elastic fibres was compared between the OSMF group and OSCC group, in between different grades of OSMF and in between different grades of OSCC. The results were evaluated using SPSS software.

Results: Early OSMF cases were associated with fibrosis in the superficial lamina propria, whereas advanced OSMF had fibrosis involving deeper muscle fibres. In all early OSMF cases, elastic fibres were arranged in thin bundles, whereas in advanced OSMF cases, elastic fibres were in thick bundles. In well- and moderately differentiated OSCCs, the collagen fibres were arranged in thick bundles and in poorly-differentiated OSCCs, the collagen fibres appeared to be fragmented. The elastic fibres in well-differentiated OSCC and moderately-differentiated OSCC were thickly arranged, and poorly-differentiated OSCC showed thin fibres and 70% of cases showed the absence of elastic fibres.

Conclusion: Changes observed in both collagen and elastic fibres in the extracellular matrix (ECM) can be taken as a study model to further understand the progression of OSMF to OSCC using histochemical stains.

Keywords: Collagen, elastic fibres, oral submucous fibrosis, squamous cell carcinoma

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INTRODUCTION

Across the globe, oral cancer is one of the commonest occurring malignancies.[1,2]Among oral cavity cancer, the most routine histological form is oral squamous cell carcinoma (OSCC), which is responsible for the majority proportion of its cases. It is also the sixth most frequently encountered malignancy in the world; with a particularly higher prevalence rate in the South Asian region.[3,4] Pathogenesis of OSCC is multifactorial, and numerous precancerous disorders have been known to have been responsible for a significant proportion of OSCC cases. One such disorder is oral submucous fibrosis (OSMF). It is a chronic pathology, which results in scars and fibrosis and has significant premalignant potential. The buccal mucosa is one of the most frequent sites of its occurrence. As per the World Health Organization (WHO) data, globally, there are more than 5 million patients affected by OSMF. In the Indian subcontinent, female ponderance is usually seen among OSMF patients.[5,6] In OSMF stromal alterations are characterized by the deposition of both abnormal quality and quantity of collagen fibres, which are more resistant to decay. Also, hyaline degeneration, fragmentation and elastic degeneration are hallmark findings with the progression of the pathology.[7,8]

The role of the extracellular matrix (ECM) is the topic of extensive research over the past few decades. It is known to affect and modulate tumour behaviour. Also, inadequate synthesis/degradation of any component of ECM could alter cell functioning, thereby assisting cancerous progression. Production of matrix metalloproteinases (MMPs) tumour-associated fibroblasts and modulated immune cells are responsible for the destruction of ECM structural framework, that is, collagen along with elastic fibres, thereby assisting in a malignant spread. The exact role of collagen elastic fibres in the evolution of OSCC on the OSMF background is still unknown.[6-8] Literature quotes more than 25 collagen fibre types so far identified on the basis of their molecular structure. Among them, the most abundant type is type I collagen scattered with type three collagen in stromal connective tissue. Masson's trichrome stain (MT) demonstrates collagen quantitatively. Identification of abnormal arrangements of elastic fibres might of diagnostic importance. The use of special stains for assessing elastic fibres might lead to more definitive future studies. With the advancement of modern immunohistochemical techniques and markers, the utility of histochemical staining is getting undervalued. At the same time, these modern techniques are also expensive and time-consuming and hence, cannot be used for screening purposes in routine. Histochemical stains are cheaper, easy to carry out and aid in resolving various diagnostic quandaries. Verhoeff stain is a commonly employed stain for the assessment of elastic fibres.[8-10] Hence, the present study was conducted to assess the alterations in elastic and collagen fibres in OSMF and OSCC patients using special connective tissue stains; MT stain for collagen fibres and Verhoeff stain for elastic fibres.

MATERIALS AND METHODS

The approval from the ethics committee is obtained on (12th September, 2021). Twenty biopsy specimens of OSMF and 30 biopsy specimens of OSCC were included in the present study. Among 20 OSMF specimens, 10 were of early OSMF and the remaining 10 were of advanced OSMF. Out of 30 OSCC specimens, 10 cases each were of well-differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC. Three sections, each 4 μm thick, were obtained from all specimens. One section was stained with routine H&E staining, whereas the other section was stained with MT stain for collagen and Verhoeff–Van Gieson (VVG) for elastic fibres. All specimens were evaluated under the light microscope. In OSMF specimens, the extent of fibrosis and type of collagen bundle arrangement in the deeper portion of lamina propria were recorded. In OSMF specimens, elastic fibre arrangement was recorded. In both OSMF and OSCC specimens, elastic fibres and collagen fibres were graded into thick bundles, thin bundles and fragmented bundles. The arrangement of collagen fibres and elastic fibres was compared between the OSMF group and OSCC group, in between different grades of OSMF and in between different grades of OSCC. The results were evaluated using SPSS software followed by statistical analysis using the Chi-square test.

RESULTS

In the current study, 10 cases of early OSMF and 10 cases of advanced OSMF were enrolled. Of 10 cases of early OSMF, 40% (n = 4) cases showed fibrosis involving superficial lamina propria and 60% (n = 6) cases showed fibrosis extending up to deeper lamina propria. None of these cases showed the involvement of muscle fibres. Of 10 cases of advanced OSMF, 90% (n = 9) cases showed fibrosis extending deep into the submucosa involving the muscle fibres as shown in Table 1. Figure 1 shows the involvement of fibrosis up to muscle fibres. We observed that in all OSMF cases, elastic fibres were arranged in thin bundles, whereas in advanced OSMF cases, elastic fibres were arranged in thick bundles, which indicates that elastic

changes occur as the fibrosis progresses. Figure 2 shows thick bundles of elastic fibres. The Chi-square test was applied to evaluate the staining intensity of both stains and was found to be statistically significant as shown in Table 2. We also evaluated the collagen fibres and elastic fibres in 30 histologically confirmed cases of different grades of OSCC using MT stain and VVG stain. In well- (60%) and moderately (40%) differentiated OSCCs, the collagen fibres were arranged in thick bundles and poorly differentiated OSCCs, the collagen fibres appeared to be fragmented in about 80% of cases, which was statistically significant as shown in Table 3. Figure 3 shows the fragmentation of collagen bundles at the tumour invasive front. The elastic fibres in well-differentiated OSCC (40%) and moderately differentiated OSCC (30%) were thickly arranged, and in poorly-differentiated OSCC, 30% cases showed thin fibres and 70% cases showed the absence of elastic fibres as shown in Table 4. Figure 4 shows the fragmentation of elastic fibres at the tumour invasive front. While comparing the expression of collagen fibres between the OSMF group and OSCC group, it was seen that OSMF patients depicted higher arrangement into thick bundles in the deeper portion of lamina propria, whereas OSCC patients demonstrated a significantly higher proportion of thin bundle arrangement and fragmentation of collagen bundles as shown in Table 5. Elastic fibre arrangement was significantly more in the form of thin fibres and fragmentation in OSCC patients, whereas in OSMF patients, they were distributed equally as thick bundles and thin bundles as shown in Table 6.

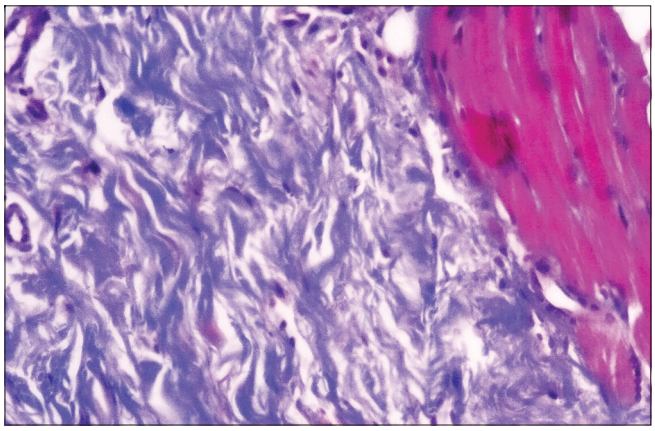

Figure 1: Involvement of fibrosis up to muscle fibres in OSMF cases as demonstrated by MT stain

DISCUSSION

Epithelial–mesenchymal transition (EMT) plays a significant and crucial role during morphogenesis. EMT is an area of high research in the current scenario because of the role it plays in carcinoma and fibrosis patients. Current researchers propose that EMT has an indispensable role in the occurrence and progression of OSCC. OSMF is a premalignant disorder affecting a significant proportion of the population of the Indian subcontinent. It manifests in the form of generalised fibrosis in the submucosal region. Its exact pathogenesis is multifactorial and is still debatable. In a simplified version, OSMF represents an altered version of wound healing in response to chronic, continuous injury caused by areca nut.[11,12]

In the present study of 10 cases of early OSMF, 40% (n = 4) cases showed fibrosis involving superficial lamina propria and 60% (n = 6) cases showed fibrosis

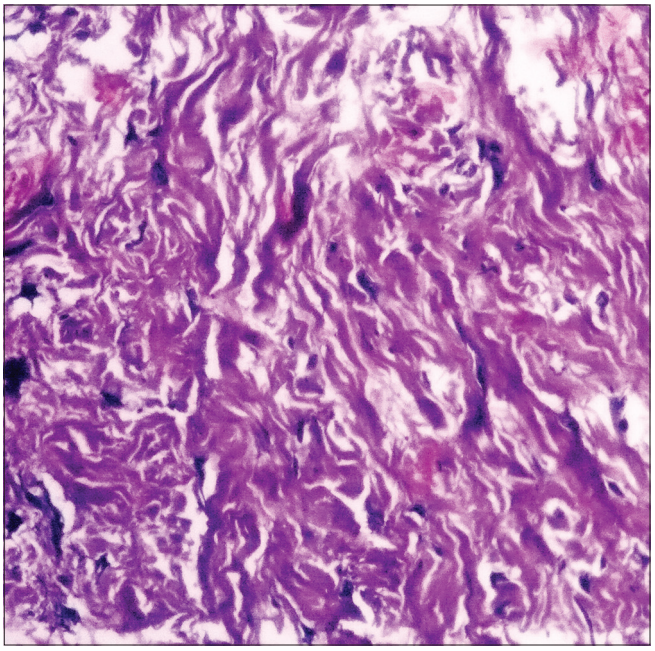

Figure 2: Thick bundles of elastic fibres in OSMF cases as demonstrated by VVG stain

Table 1: Comparison of fibrosis as evaluated by MT stain among OSMF patients

Extent of fibrosis	Early OSMF		Advanced OSMF	
	Number	Percentage	Number	Percentage
Fibrosis involving superficial lamina propria	4	40	0	0
Fibrosis involving deeper lamina propria	6	60	1	10
Fibrosis involving deeper muscle fibres	0	0	9	90
Total	10	100	10	100
P	0.019 (Significant)			

extending up to the deeper lamina propria. None of these cases showed the involvement of muscle fibres. Of 10 cases of advanced OSMF, 90% (n = 9) cases showed fibrosis extending deep into the submucosa involving the muscle fibres as shown in Table 1. We observed that in all early OSMF cases, elastic fibres were arranged in thin bundles, whereas in advanced OSMF cases, elastic fibres were in thick bundles, which indicates that elastic changes occur as the fibrosis progresses. The Chi-square test was applied to evaluate the staining intensity of both stains and was found to be statistically significant. MT stain was used because of the differential staining colour shown by muscle fibres and collagen, which helps in accessing the involvement of muscle fibres by fibrosis. Because OSMF shows fibroelastic changes in the lamina propria, we also tried to evaluate the pattern of elastic fibres using the VVG stain. We observed that in early OSMF cases, elastic fibres were arranged in thin bundles, whereas in advanced OSMF cases, elastic fibres were arranged in thick bundles, which indicates that elastic changes occur as the fibrosis progresses. The Chi-square test was applied to evaluate the staining intensity of both stains and was found to be statistically significant. It can be inferred that as the disease progresses, from early to advanced stage, there is the involvement of deeper submucosa and muscle fibres and changes in elastic fibres, which can be better appreciated using histochemical stains. Modak *et al*.,[5] in a previous study, correlated histopathological staging and analysed the polarisation colours and thickness of the collagen fibres in different stages of OSMF. The sample size was 40 subjects, of which 30 patients had OSMF, and 10 were in the control group. The correlation between clinical and functional staging was not significant, whereas the comparison of the functional staging with histopathological staging was more reliable than clinical staging as an indication of the severity of the disease.

Table 2: Comparison of elastic fibres arrangement as evaluated by VVG stain among OSMF patients

Arrangement of elastic fibres	Early OSMF		Advanced OSMF	
	Number	Percentage	Number	Percentage
Thin bundles	10	100	0	0
Thick bundles	0	0	10	100
Total	10	100	10	100
P	0.027 (Significant)			

We also evaluated the collagen fibres and elastic fibres in 30 histologically confirmed cases of different grades of OSCC using MT stain and VVG stain. In well- (60%) and moderately (40%) differentiated OSCCs, the collagen fibres were arranged in thick bundles and poorly differentiated OSCCs, the collagen fibres appeared to be fragmented in about 80% of cases, which was statistically significant. The elastic fibres in well-differentiated OSCC (40%) and moderately differentiated OSCC (30%) were thickly arranged and in poorly differentiated OSCC, 30% of cases showed thin fibres and 70% of cases showed the absence of elastic fibres. These fragmented collagen fibres and thin and absence of elastic fibres in poorly differentiated oral squamous cell carcinoma (POSCCs) could be due to dense inflammatory infiltration we observed in all these cases, which may release cytokines that cause ECM degradation and lysis of elastic fibres (Agrawal U *et al*., 2011).[12] In a similar study conducted by Dinesh *et al*.,[11] authors evaluated the morphological alterations demonstrated by elastic fibres among patients with epithelial dysplasia and OSCC. After performing a quantified analysis of elastic fibres among dysplasia and OSCC specimens, they observed a significant correlation. An alteration in the density and direction to the overlying epithelium along with dysplastic epithelial islands

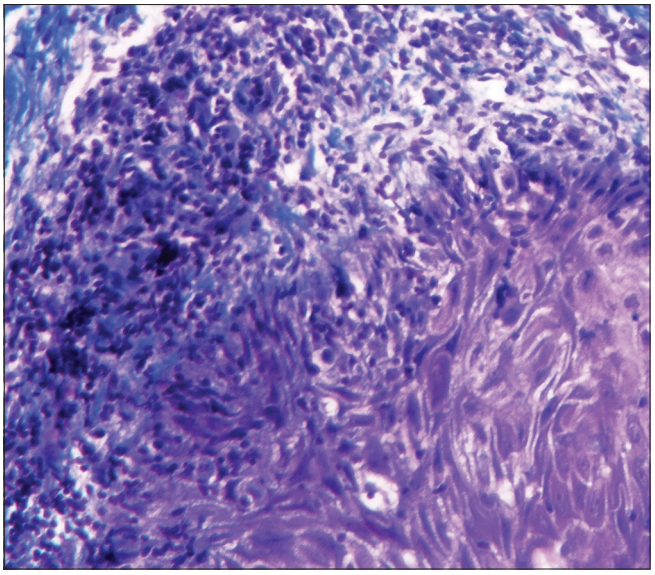

Figure 3: Fragmentation of collagen bundles in OSCC cases as demonstrated by MT stain

Table 3: Comparison of arrangement of collagen bundles as evaluated by MT stain among OSCC patients

Arrangement of collagen fibres	Well-differentiated OSCC		Moderately differentiated OSCC		Poorly differentiated OSCC	
	Number	Percentage	Number	Percentage	Number	Percentage
Thick bundles	6	60	4	40	0	0
Thin bundles	4	40	5	50	20	2
Fragmented	0	0	1	10	80	8
Total	10	100	10	100	10	100
P	0.023 (Significant)					

Table 4: Comparison of elastic fibres arrangement as evaluated by VVG stain among OSCC patients

Arrangement of elastic fibres	Well-differentiated OSCC		Moderately differentiated OSCC		Poorly differentiated OSCC	
	Number	Percentage	Number	Percentage	Number	Percentage
Thick bundles	4	40	3	30	0	0
Thin bundles	6	60	4	40	30	3
Fragmented	0	0	3	30	70	7
Total	10	100	10	100	10	100
P	0.007 (Significant)					

Table 5: Comparison of collagen fibres arrangement between OSMF patients (in the deeper portion of lamina propria) and OSCC patients

Arrangement of collagen fibres	OSMF		OSCC patients	
	Number	Percentage	Number	Percentage
Thick bundles	16	80	10	33.33
Thin bundles	4	20	11	36.67
Fragmented	0	0	9	30
Total	20	100	30	100
P	0.000 (Significant)			

Table 6: Comparison of elastic fibres arrangement between OSMF patients and OSCC patients

Arrangement of elastic fibres	OSMF		OSCC patients	
	Number	Percentage	Number	Percentage
Thick bundles	10	50	7	23.33
Thin bundles	10	50	13	43.33
Fragmented	0	0	10	33.33
Total	20	100	30	100
P	0.016 (Significant)			

was observed on progressing from well-differentiated to poorly differentiated OSCC. They concluded that the analysis of connective tissue stromal alterations might be utilised as an adjunct to histological grading.

In the present study, while comparing the expression of collagen fibres between the OSMF group and OSCC group, it was seen that OSMF patients depicted higher arrangement into thick bundles in the deeper portion of lamina propria, whereas OSCC patients demonstrated a significantly higher proportion of thin bundle arrangement and fragmentation of collagen bundles. Elastic fibre arrangement was significantly more in the form of thin fibres and fragmentation in OSCC patients, whereas in OSMF patients, they were distributed equally as thick bundles and thin bundles. Our results were in concordance with the results obtained by Gandhi and Prasad[8] who also reported similar findings. In their study, the authors also demonstrated significantly higher extracellular matrix alterations in OSCC patients in comparison to OSMF patients. There is a continuing investigation to assess the possible pathway by which epithelial cancer transforms from a disorder of connective tissue origin. From the data of recent researchers, it can be hypothesised that the process of malignant transformation and its further progression

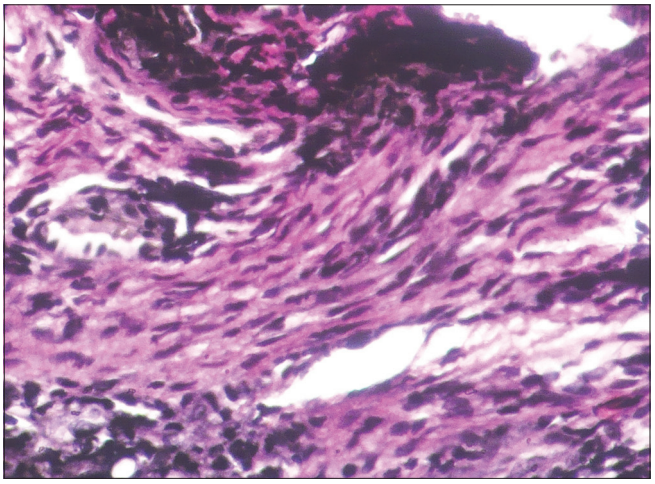

Figure 4: Fragmentation of elastic fibres in OSMF cases as demonstrated by VVG stain

is facilitated through interactions among epithelial and connective tissue components, chiefly collagen and elastic fibres.[13,14] Data from different animal research models also provide substantial evidence that fibrotic disorders have the malignant potential and there exist oncogenic factors in both the afflicted tissues. Referring to this with OSMF, it can be suggested that epithelial-mesenchymal interactions might result in significant alterations of epithelial phenotype, thereby increasing the possibilities of malignant transformation.[15,16] Zhang *et al*.[17] reemphasized that the irreversibility associated with fibrosis in relation to betal nut could be the outcome of resistance showed by cross-linked collagen to proteinases. Lehmann *et al*.,[18] suggested that collagen mRNA regulation by miRNAs could also be potentially important in bone metabolism. However; the exact role of collagen and elastic fibres in the malignant transformation of OSMF needs to be further elucidated.

CONCLUSION

Our results indicate that the changes observed in both collagen and elastic fibres in ECM can be taken as a study model to further understand the progression of OSMF to OSCC using histochemical stains. However; further studies are required to establish the efficiency of histochemical stains in evaluating these fibres.

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Conflicts of interest
There are no conflicts of interest.

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