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Original Article

Pathology Section

Architectural Analysis of Picrosirius Red Stained Collagen in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma using Polarization Microscopy

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ABSTRACT

Introduction: Collagen degradation is important both for carcinogenesis and in its progression. Research regarding the co-relation of collagen with Oral Epithelial Dysplasia (OED) and Oral Squamous Cell Carcinoma (OSCC) is less explored.

Aim: To elucidate the nature of collagen in Oral Epithelial Dysplasia (OED) and Oral Squamous Cell Carcinoma (OSCC) using Picrosirius Red Stain (PSR) under polarizing microscopy.

Materials and Methods: The study consisted of a total 40 samples which were divided into three groups. Group I included buccal mucosa as negative and irritation fibroma as positive control, group II consisted of OED and group III consisted of Oral Squamous Cell Carcinoma (OSCC). A histochemical analysis was conducted using PSR-polarization method by two independent observers.

Results: The control group shows predominantly reddish–orange birefringence. In OED with the advancement of grades, the colour changed from yellowish-orange colour to yellow-greenish with progressive increase in greenish hue. As OSCC regresses from well to poorly differentiated, the colour changed from reddish-orange to yellowish orange to greenish-yellow suggesting a transition from mature to immature collagen.

Conclusion: An observable gradual change in collagen of both OED and OSCC was noted as they were proceeding from benign to critical step. Thus, PSR is a useful tool for studying stromal changes as supporting collagen shows the transition in the form besides the alterations in epithelial cells.

Keywords: Birefringence, Greenish yellow, Reddish orange, Yellowish orange

INTRODUCTION

Oral Potentially Malignant Disorders (OPMD) is defined by WHO in 2005 as the risk of malignancy being present in a lesion or condition either at time of initial diagnosis or at a future date [1]. These disorders are histopathologically reported as Oral Epithelial Dysplasia (OED) that is graded as mild, moderate or severe. The most commonly used grading system for OED by the histopathologists is the WHO criteria given in 1978 [2]. The severe form of OED is likely to transform to Oral Squamous Cell Carcinoma (OSCC) which is composed of two discrete compartments i.e. the malignant epithelial cells and the stroma in which they are dispersed [3]. During the development of OSCC, a number of changes occur in the epithelium including the basal lamina breakdown. Once the basal lamina is degraded, neoplastic epithelial cell interacts with the stroma, particularly the collagen and this interplay is a crucial determinant of tumour progression [4].

The collagenous tissue, "basic skeleton" of the stroma undergoes extensive changes during the evolution and progression of carcinoma. Firstly, it can have antagonistic effects on tumour progression either by inhibiting the host immune response or by resisting the tumour spread via inducing an abundant collagenous stroma (walling off effect) [5]. Secondly, proteolysis or change in the collagen composition can facilitate the mobilization of neoplastic cells into the stroma, therefore aiding subsequent invasion and metastasis [6].

Histochemically, to detect collagen fibers traditional stains such as Van Gieson and trichrome stains which are the combinations of two or more anionic dyes are routinely used. Since, these methods lack precise selectivity, hence they are not ideal for collagen detection in light microscopy [7]. Another disadvantage of these methods is that they fail to reveal very thin collagen fiber which can further lead to underestimation of collagen content [7,8].

The potential problems encountered using these traditional stains was first resolved by Constantine and Mowry in 1968 wherein a combination of Picrosirius Red (PSR) and polarization microscope was used for selective demonstration of collagen [9]. PSR stain is a combination of two anionic dyes i.e. Sirius red F3BA (Direct Red 80) dissolved in a saturated picric acid solution. Sirius Red is a hydrophilic dye which has sulphonic acid groups. These groups react with basic groups present in the collagen molecule [10]. There is a parallel arrangement of the dye molecules with the long axes of collagen fiber. This parallel relationship between dye and collagen results in an enhanced birefringence. This birefringent property results in bright yellow to orange colour of collagen when viewed under polarized light [11]. Further, various studies have been conducted using this combination and found that polarization colours of PSR stained collagen are not only due to fiber thickness but the packing of collagen molecules also plays a key role suggesting this method can be a useful tool for the structural analysis of collagen [12,13].

Numerous investigators have utilized this combination for the detection and analysis of collagen in oral submucous fibrosis or other oral pathologic conditions like odontogenic keratocyst or ameloblastic fibroma, etc. [14-17]. Ganganna K et al., correlated changes in birefringence of collagen fibers with degrees of epithelial dysplasia in OSF and observed that there was a gradual change in the polarization colours of thick collagen fibers observed in mild, moderate to severe epithelial dysplasia seen in the epithelium of OSMF [18]. Although, studies have been conducted on OSCC using this method, but to the best of our knowledge, there are no studies which have compared the grades of OSCC with that of OED using this method [19-21]. Thus, the present study was an attempt to elucidate the nature of collagen in Oral Epithelial Dysplasia (OED) and Oral Squamous Cell Carcinoma (OSCC)

using Picrosirius Red Stain (PSR) under polarizing microscopy. The objective of the study was to examine the histochemical changes seen in collagen fibers in different grades of these lesions and to assess the relationship of this change with the nature of collagen with special reference to histopathological grading.

MATERIALS AND METHODS

For the present study, an approval from the ethical committee of a tertiary referee university was taken prior and the details of patients were kept confidential. The study included a total of 40 diagnosed cases which were retrieved from the Department of Oral Pathology and Microbiology of Sudha Rustagi College of Dental Sciences and Research, Faridabad, Haryana, India. The diagnosis of the cases was based on the clinical and microscopic findings of the incisional biopsy. These 40 cases were divided into three groups [Table/Fig-1].

From each paraffin block, two sections of 5-µm were obtained, one section was stained with Haematoxylin and Eosin (H&E) using standard protocol and other by the Picrosirius red stain. The procedure of PSR stain included the steps following deparaffinization and hydration in distilled water, the sections were then incubated in 0.1% (w/v) Direct Red 80/Sirius red (C.I- 365548-5G, Sigma-Aldrich, Switzerland) with saturated Picric acid solution (Qualigens, Mumbai, India) for 1 hour at room temperature. This was followed by rinsing with distilled water, stained with Mayer's haematoxylin (HI-media Labs. Mumbai, India) and differentiated in 1% HCl, alkalinization with tap water followed by the steps of dehydration and mounting.

Picrosirus red stained sections were examined under polarizing microscopy (Olympus BX41 TF) at a magnification of x100 for collagen fiber in the connective tissue and differences in the polarizing colours of the collagen fibers in different lesions were analysed. In OSCC, collagen fibers around tumour islands or cords were considered and areas showing dense inflammation or epithelial ulceration can have an impact on collagen arrangement thus were excluded. All the samples were analysed by two observers to check inter-observer variability and the data was computed.

RESULTS

The H&E stained slides were observed under normal light and it was noted that none of the groups showed excessive collagen fiber deposition except the positive control i.e., buccal mucosa irritation fibroma. Subsequently, PSR stained slides were examined under polarizing light for collagen fiber arrangement. Group I showed predominantly reddish-orange birefringence [Table/Fig-2,3] and group II exhibited a gradual change in the polarizing colours from yellowish-orange to slight increase in greenish tinge finally to intensified greenish hue on progressing from mild to severe OED [Table/Fig-2,4]. However, in Group III, a gradual change in the polarizing colours from reddish orange to yellowish orange to greenish yellow was seen from well differentiated to poorly differentiated OSCC [Table/Fig-2,5]. Inter-observer agreement was analysed using Kappa statistics and 91% agreement was found between the two observers for collagen birefringence.

GROUP	SPECIMEN	SAMPLE SIZE	TOTAL NUMBER
I –Control	Buccal mucosa- Negative control	5	10
	Buccal mucosa irritation fibroma- Positive control	5	
II - Grades of OED	Mild dysplasia	5	15
	Moderate dysplasia	5	
	Severe dysplasia	5	
III-Grades of OSCC	Well differentiated OSCC	5	15
	Moderate differentiated OSCC	5	
	Poorly differentiated OSCC	5	

[Table/Fig-1]: Total number of samples used in the present study
OED: Oral Epithelial Dysplasia; OSCC: Oral Squamous Cell Carcinoma

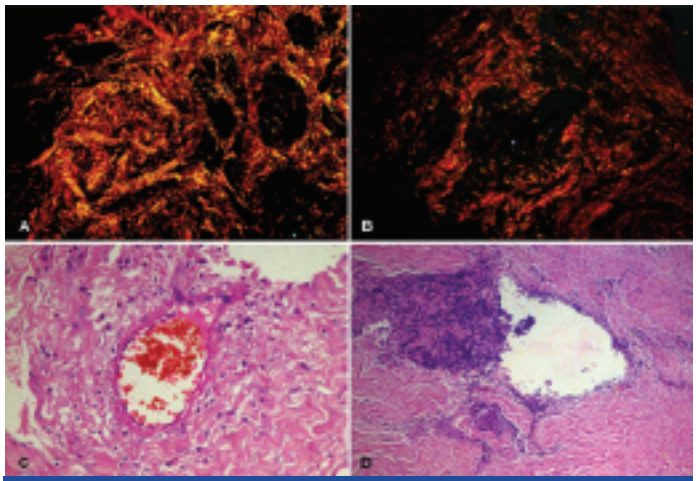
DISCUSSION

The development of OSCC is believed to occur in a stepwise fashion, beginning with OED progressing to carcinoma in situ and finally to OSCC. An essential step for OED to transform into OSCC is to invade the stroma by breakdown of basement membrane [22]. In general, the development of OSCC is intrinsically correlated with the surrounding stroma because during carcinogenesis, the tumour requires its stroma to grow beyond 1-2 mm size. The stroma acts as a mixed blessing, it provides the environment for nourishment, exchange of gas and waste material and also restricts the influx of inflammatory cells for the neoplastic cells. On the other hand, neoplastic cells in stroma may either induce desmoplasia or cause the lysis [20].

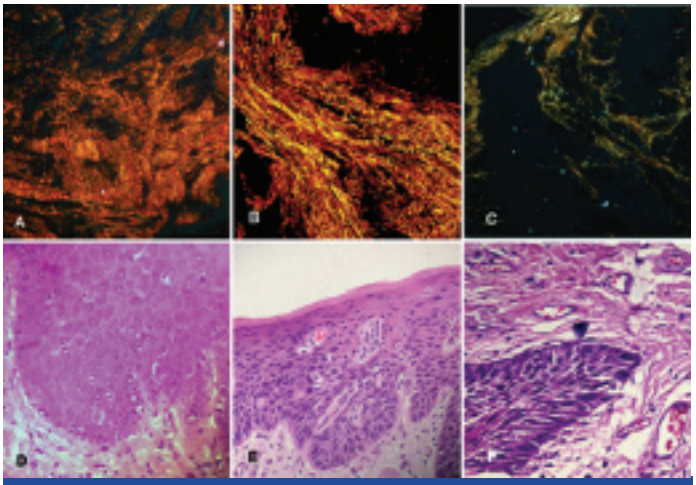
Thus, besides the malignant epithelial cells, the supporting stroma is equally important component for the existence and progression of OSCC. Collagen, one of the major elements of the stroma, is primarily affected in the stromal changes at the site of tumour cell

GROUP	SAMPLE	COLOUR OBSERVED by Observer 1 (x100)	COLOUR OBSERVED BY OBSERVER 2 (x100)
I. Control	Buccal mucosa- Negative control	Predominantly reddish-orange	Predominantly reddish
	Buccal mucosa irritation fibroma- Positive control	Predominantly reddish-orange	Predominantly reddish-orange
II. OED	Mild dysplasia	Yellowish orange with minimal areas of red birefringence	Yellowish orange
	Moderate dysplasia	Yellowish orange colour with slight greenish hue	Yellowish orange colour with slight greenish hue
	Severe dysplasia	Intensified Greenish hue	Greenish hue
III. OSCC	Well differentiated OSCC	Reddish orange birefringence	Reddish orange birefringence
	Moderately differentiated OSCC	Yellowish orange birefringence	Yellowish orange birefringence to slight greenish hue
	Poorly differentiated OSCC	Greenish yellow hue	Greenish-yellow hue

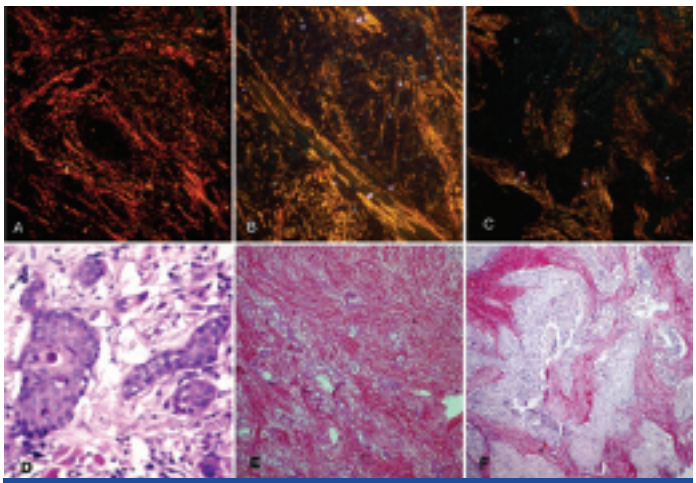
[Table/Fig-2]: Showing the polarizing colours observed (x100) in each of 40 samples
OED: Oral Epithelial Dysplasia; OSCC: Oral Squamous Cell Carcinoma



[Table/Fig-3]: a) Photomicrograph of histologically normal buccal mucosa as negative control showing predominantly reddish-orange birefringence. (Picrosirius red stain, X100)
b) Photomicrograph of buccal mucosa irritation fibroma as positive control showing reddish-orange colour. (Picrosirius red stain, X100)
c) Photomicrograph of histologically normal buccal mucosa used as negative control (H&E stain, X100)
d) Photomicrograph of buccal mucosa irritation fibroma used as positive control (H&E stain, X100)



[Table/Fig-4]: a) Photomicrograph of Mild dysplasia showing predominantly yellowish orange colour with minimal areas of red birefringence. (Picrosirius red stain, X 100)
b) Photomicrograph of Moderate dysplasia showing yellowish orange colour with greenish hue. (Picrosirius red stain, X 100)
c) Photomicrograph of Severe dysplasia showing intense greenish hue. (Picrosirius red stain, X100)
d) Photomicrograph of Mild dysplasia seen under a light microscope (H&E stain, X100)
e) Photomicrograph of Moderate dysplasia seen under a light microscope (H&E stain, X100)
f) Photomicrograph of Severe dysplasia seen under a light microscope (H&E stain, X100)



[Table/Fig-5]: a) Photomicrograph of Well differentiated OSCC showing reddish orange birefringence. (Picrosirius red stain, X100)
b) Photomicrograph of Moderately differentiated OSCC showing yellowish orange with sight greenish hue. (Picrosirius red stain, X100)
c) Photomicrograph of Poorly differentiated OSCC showing greenish yellow colour. (Picrosirius red stain, X100)
d) Photomicrograph of Well differentiated OSCC seen under a light microscope (H&E stain, X100)
e) Photomicrograph of Moderately differentiated OSCC seen under a light microscope (H&E stain, X100)
f) Photomicrograph of Poorly differentiated OSCC seen under a light microscope (H&E stain, X100)

invasion. Although numerous studies have been conducted on the relationship of stromal reaction with OSCC but research regarding the early stromal reaction in oral potentially malignant disorders is fragmentary [23]. Thus, the present study assessed the collagen fiber changes with progressive grades of OED and OSCC. In OED, beginning from mild with increasing grades of dysplasia, the birefringence alters from yellowish-orange to finally greenish colour passing through slight greenish hue and similar findings were mentioned by Yokoyama M [24]. In different grades of OSCC, there is a gradual change in birefringence ranges from reddish orange to yellowish orange and eventually to yellowish green. Such changes were in accordance with the study done by Aparna V et al., Kalele KK et al., and Manjunatha BS et al., [19-21]. However, another study done by Martins GB et al., suggested some changes in the collagen but did not mention colour gradation as the grades were progressive [25].

It is well-known that the PSR is considered to be one of the most widely used stain to visualize collagen fibers in histological tissues. For reasons like it's less tendency to fade unlike trichrome stain and has more selectivity thus making it ideal and superior for both staining and quantification of collagen. The reason behind its enormous application is that PSR enhances the birefringence properties of collagen which give rise to variety of colours [13].

Firstly, Junquiera et al., and Szendroi et al., suggested that the spectrum of colours may be due to the fiber thickness and they described that thin fibers usually show green to yellowish hue whereas thick fibers show orange to red polarization colour [26,27]. Later on, Dayan et al., proposed that packing of these collagen fibers also plays an important role in the pattern of polarization colours of PSR stained collagen. Tightly packed and well aligned collagen fibers showed polarization colours of longer wavelengths (reddish orange) [28]. Even, Trau H et al., said that PSR stained sections by polarizing microscopy can serve as a procedure for differentiating procollagens, intermediates and other non-tightly packed collagen fibers from normal tightly packed fiber [29]. Sharf Y et al., conducted a study using nuclear resonance technique which revealed a colour profile of orange to red which corresponded to the well packed fibers and the green to greenish yellow to poorly packed fibers [30]. Recently, Rich L and Whittaker P demonstrated that PSR with polarization microscopy is the best method to analyse structure of collagen [13]. Thus, it can be stated that primarily the compactness of collagen can be studied by the change in polarization colour.

In the present study, the colour detected in early stages of OSCC (i.e. in well differentiated grade) was reddish-orange with advancing grade the colour changed to yellowish-green. A similar progressive change was noted in the OED with varying grades. Hence, the present study indicated that the change in the polarizing colour of collagen fiber which coincided with the change found in the stages of both OED and OSCC. However, this peculiar change in colour with respect to the type of collagen fibers as the lesion is invading and advancing exist but the exact mechanism behind such process is still unclear.

It is a well-known fact, that the hallmark of carcinoma is the neoplastic cell migration and invasion. During the transformation from dysplasia to carcinoma, hypoxia arises which induces genetic instability and accelerate angiogenesis thus making the stroma edematous and unstable. As carcinoma progresses, neoplastic cell transform collagen mainly by the production of Carcinoma-Associated Fibroblasts (CAFs) and increase collagenolytic enzyme activity. This altered fibroblast phenotype contribute to the production of altered collagen. Also, by increased formation of collagenases, the invading neopalstic cell is able to dissolve the collagen eventually leading to disarranged stroma [31]. Several studies have already proven that during tumourigenesis different types of collagen, in particular collagen type I (thick) is synthesized which when stained with PSR gives reddish orange hue [27]. The severe grades of both OEDD and SCC showed yellowish-greenish hue suggesting the presence of type III (thin) collagen fibers. Thus, our findings confirm previous results that collagen type I is decreased and replaced by type III during the transformation of initial to higher grades, both in OED and in OSCC. Therefore, suggesting that as the cancer progresses, the surrounding stroma co-evolves into the active state through continuous tumour-stroma interactions [6,20,24,25].

So far all the PSR stain related studies have used linear polarized light, though, there are some limitations using this technique. The first disadvantage of using linear polarized light is that PSR-stained fibers will appear dark if they are aligned parallel to the transmission axis of either of the two linearly polarizing filters. This can be overcome by using rotating microscope stage which will change the orientation of the tissue section with respect to the transmission

axes. But some collagen fibers are frequently crimped or wavy and so will appear dark irrespective of rotated microscope stage. Thus, the total collagen content especially in tissue containing large amounts of wavy fibers may be underestimated. Here, we should emphasize that the fiber hue does not permit identification of collagen fiber type as some have suggested. Secondly, type III fibers are usually thinner than type I fibers but the green colour does not necessarily signify type III and can also represent either an immature type I or sectioning artifact smeared of thick type I fiber. Thirdly, materials such as keratin and fibrin are weakly birefringent which is almost similar to that of the thinnest collagen fibers and thus complicating the analysis [11].

CONCLUSION

In the present study, observable collagenous changes were seen in progressive grades of OED and OSCC. We have demonstrated that the combination of PSR with polarization microscopy is a powerful tool for its structural analysis and the colour changes observed in collagen fibers reflect its gradual shift from thicker towards thinner fibers in stroma during tumour progression. Like any other technique even PSR with polarization microscopy has some limitations. Thus, it should be supplemented with gold standard i.e., H&E and molecular markers on a larger sample including the tumour invading front to extrapolate this knowledge.

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