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Research Article

Histochemical Analysis of Collagen Reorganization at the Tumor-Stromal Interface in Oral Squamous Cell Carcinoma- A Polarizing Microscopic Study

Alrani D, Niranjan KC*, Acharya S and Hallikeri K

Department of oral Pathology, SDM College of Dental Science and Hospital, India

***Corresponding author:** Niranjan KC, Department of Oral Pathology, SDM College of Dental Science and Hospital, Dharwad, Karnataka, India

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Abstract

Stromal changes are the key factors for provision of nutrition and growth to any tumor and also they act as a barrier for spread of the tumor. There are limited histochemical studies in the literature on methods to detect, quantify and analyze the collagen in the invasive tumor front (ITF) of oral squamous cell carcinoma (OSCC).

Aim: 1) To analyze the nature of collagen (thickness, hue, density, birefringence) with respect to cohesive and discohesive tumor front of oral squamous cell carcinoma. 2) To correlate the nature of collagen at the different grades (Broder's and Bryne's grading system) of oral squamous cell carcinoma. 3) Clinicopathological correlation with the nature of collagen at the invasive front of oral squamous cell carcinoma.

Materials and Methods: Tissue sections of 30 OSCC cases with ITF were stained with hematoxylin and picrosirius red stains for evaluation of the nature of collagen under polarizing microscope.

Results: Tumor with cohesive front will have thick collagen fiber which is predominantly organized red- yellow in color, well packed and shows strong birefringence ($p < 0.005$). A gradual change in the nature of collagen fiber was observed in the discohesive tumor front where the collagen fiber were thin disorganized, yellow-orange to green-yellow in color loosely packed with weak birefringence ($p < 0.005$).

Conclusion: Cohesive tumor front with organized collagen fibers resist the tumor against invasion and metastasis, preventing it to increase in size and thus associated with initial stage of tumor (I&II) whereas in discohesive tumor front the fibers may enhance the movement of tumor cells towards invasion and metastasis.

Keywords: Collagen; Picrosirius Red (PSR); Oral Squamous Cell Carcinoma (OSCC); Invasive Tumor Front (ITF); Cohesive tumor front; Discohesive tumor front

Introduction

OSCC is composed of two discrete components, the malignant epithelial cells and the stroma in which they are dispersed [1]. One of the major aspects of tumor cell invasion and metastasis is the interaction between cancer cells and extracellular matrix component [2]. The stroma is composed of ground substance composed of proteoglycans, glycoproteins and water, the fibrous component including interstitial collagen and elastic fibers [3]. Extracellular matrix components are the key factors for nutrition and growth to any tumor and also they act as barrier for spread of the tumor [4]. Alteration in ECM may play a role in recurrence and in facilitation the invasion of tumor cells.

Collagen which constitutes 34% of the total extracellular matrix (ECM) proteins forms the integral part of connective tissue stroma and plays a vital role in maintaining structural integrity and in determining tissue function. The collagenous tissue in the stroma gives strength to the tumor by giving a skeleton to the tumor. Increase in collagen content of the extracellular matrix increases the mechanical stiffness and transport resistance of the tumors [3]. A recent study by Li, et al. in 2013 mentions that collagen fiber plays an important role in ECM destruction and remodeling [2], so the study of interstitial collagen has been the mainstay of investigative histological procedures in understanding the pathogenesis of the lesion.

Collagen has natural birefringence which is attributed to the arrangement of its fibers which is enhanced by special stains like van gieson, masson's trichrome and picrosirius red. Picrosirius red stain is considered a highly specific and selective stain for collagen fibers due to its ability in differentiating between different types of collagen fibers in various pathological conditions.

There are limited histochemical studies in the literature on methods to detect, quantify and analyze the collagen in the tumor

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Table 1: Categorical distribution of Clinicopathological features of Oral Squamous cell carcinoma patients.

Parameter	Category	No of cases	Percentage
Gender	Male	24	83%
	Female	5	17%
Age	<45	14	50%
	>45	15	50%
Habit	T(tobacco)	18	63%
	Smoking	5	17%
	Combination	6	21%
Frequency of habit	0 year	5	17%
	0-9 year	5	17%
	10-19 year	9	30%
	>20 year	10	36%
Size of tumor	< 4cm	17	59%
	>4cm	12	41%
Site	BM,RMT,BGS	22	76%
	T	6	20%
	GIN,ALV	1	4%
Type	Exophytic	23	80%
	Endophytic	6	20%
Stage	I + II	17	57%
	III + IV	12	43%
Broder's Grading	Well	20	67%
	Mod, mod poor	9	33%
IFG grading	4-8	2	10%
	9-12	11	37%
	13-16	16	53%
Lymph node status	Negative (0)	20	70%
	Positive (1)	9	30%
Pericapsular invasion	Negative (0)	21	73%
	Positive (1)	8	27%
Surgical Margins	Negative (0)	25	87%
	Positive (1)	4	13%
Pattern of invasion	Cohesive	10	37%
	Discohesive	19	63%

Abbreviations: BM= Buccal Mucosa; RMT= Retromolar Trigone; BGS= Bucco-Gingival Sulcus; T= Tongue; ALV=Alveolus; GIN= Gingiva; Well= Well Differentiated Carcinoma; MOD= Moderately Differentiated Carcinoma; Mod-Poor= Moderately to Poorly Differentiated Carcinoma

invasive front of oral squamous cell carcinoma (OSCC).

Aims and objectives

1. To analyze the nature of collagen (thickness, hue, density, birefringence) with respect to cohesive and discohesive tumor front of oral squamous cell carcinoma.

2. To correlate the nature of collagen at the different grades (Broder's and Bryne's grading system) of oral squamous cell carcinoma.

3. Clinicopathological correlation with the nature of collagen at the invasive front of oral squamous cell carcinoma.

Materials and Methods

Twenty nine histologically diagnosed cases of OSCC and two controls of normal mucosa were retrieved from the archives of Department of Oral Pathology, S.D.M College of Dental Sciences and Hospital, Dharwad. All the sections were subjected to haematoxylin and eosin and also picrosirius red (PSR) staining, Sirius Red F3B (C.I.35780). All the patients were subjected to radical surgery, accompanied by removal of lymph nodes conducted in S.D.M College of Dental Sciences and Hospital from year 2009 to 2015.

Majority of the OSCC cases showed discohesive tumor front 19 (63%) which is represented by high degree of tumor cell dissociation followed by cohesive tumor front 11 (37%) (Table 1), clinicopathological features of Oral Squamous cell carcinoma patients.

Inclusion criteria: All the patients of OSCC who were subjected to radical surgery and accompanied by removal of lymph nodes.

Exclusion criteria: Incisional biopsies only, patients with lack of clinical details and areas of necrosis were not included in the study.

Analysis of collagen

All the PSR stained sections of OSCC were analyzed under Polarizing Microscope to observe and note the nature of collagen i.e. thickness, organization, hue, density, and birefringence irrespective the tumor is cohesive or discohesive at the ITF by 3 blind observer. The nature of collagen fibers was analyzed under the corresponding grades of carcinoma for both Broder's and Bryne's grading system. Data of each case was tabulated and were subjected for statistical analysis. Pearson's correlation test was employed for comparison between various parameters in the different groups.

Results

In this study we found that there is positive correlation between the nature of collagen fiber in relation to patterns of invasive front. Tumor with cohesive front showed thick collagen fibers which are predominantly organized red- yellow in color, well packed and shows strong birefringence. A gradual change in the nature of collagen

Table 2: Nature of collagen fiber in relation to patterns of invasive front.

Parameter	Categories	Cohesive n=10	Discohesive n=19	p-value
Thickness	Thick	11(100%)	4(21%)	0.000
	Thin	0(0%)	15(79%)	
Organization	Organized	10(91%)	0(0%)	0.000
	Disorganized	1(9%)	19(100%)	
Colors	Red –orange	11(100%)	3(16%)	0.000
	Yellow-orange	0(0%)	3(16%)	
	Green-yellow	0(0%)	13(68%)	
Density	Well packed	10(91%)	2(12%)	0.000
	Loosely packed	1(9)	17(88%)	
Birefringence	Strong	11(100%)	5(26%)	0.000
	Weak	0(0%)	14(74%)	

*=p-value is highly significant (< 0.01).

Table 3: Clinicopathologiocal correlation with nature of collagen fibre.

Clinicopathological Parameter	Parameter	Category	< 4cm	>4cm	p-value
SIZE OF TUMOR	Thickness	Thick	8(47%)	6(50%)	0.876
		Thin	9(53%)	6(50%)	
	Organization	Organized	6(35%)	3(25%)	0.555
		Disorganized	11(65%)	9(75%)	
	Colors	Red-orange	8(47%)	5(42%)	0.883
		Orange-yellow	2(12%)	1(8%)	
		Yellow-green	7(41%)	6(50%)	
	Density	Well packed	9(53%)	2(17%)	0.047*
		Loosely packed	8(47%)	10(83%)	
	Birefringence	Strong	10(59%)	5(42%)	0.362
		Weak	7(41%)	7(58%)	
TNM STAGE	**Parameter**	**Category**	**I , II**	**III , IV**	**p-value**
	Thickness	Thick	9(53%)	6(46%)	0.713
		Thin	8(47%)	6(54%)	
	Organization	Organized	7(41%)	3(23%)	0.297
		Disorganized	10(59%)	9(77%)	
	Colors	Red-orange	8(47%)	6(46%)	0.921
		Orange-yellow	2(12%)	1(8%)	
		Yellow-green	7(41%)	5(46%)	
	Density	Well packed	10(59%)	1(15%)	0.016*
		Loosely packed	7(41%)	11(85%)	
	Birefringence	Strong	12(71%)	3(31%)	0.030*
		Weak	5(29%)	9(69%)	
METASTASIS	**Parameter**	**Category**	**Absent**	**Present**	**p-value**
	Thickness	Thick	11(55%)	4(44%)	0.599
		Thin	9(45%)	5(56%)	
	Organization	Organized	8(40%)	2(22%)	0.351
		Disorganized	12(60%)	7(78%)	
	Colors	Red-orange	9(45%)	5(56%)	0.837
		Orange-yellow	2(10%)	1(11%)	
		Yellow-green	9(45%)	3(44%)	
	Density	Well packed	11(55%)	1(11%)	0.026*
		Loosely packed	9(45%)	8(89%)	
	Birefringence	Strong	11(55%)	4(44%)	0.599
		Weak	9(45%)	5(55%)	

*=p-value is significant (< 0.05).

fiber was observed in the discohesive tumor front where the collagen fiber were thin disorganized, yellow-orange to green-yellow in color loosely packed with weak birefringence (p< 0.01) (Table 2).

Density of collagen fiber

When compared between the density of collagen fiber with the size of the tumor the result was statistically significant (p< 0.05). More that 50% of the lesions having size below 4 cm, collagen fiber were observed to be well packed and in more than 80% of lesions with size above 4 cm shows collagen fiber which were loosely packed (Table 3).

Density of collagen fiber was also seen to be statistical significant (p< 0.05) with TNM stage and metastasis of lymph node. The dense, well packed collagen fiber in the picrosirius stained section of more than 50% of cases of OSCC were noticed to be in stage I and II of TNM with absence of metastasis to lymph node whereas more than 80% of cases where collagen fiber were loosely packed were in stage III and IV of TNM with evident metastasis. Of all the parameter, density of packing has shown maximum correlation with Clinicopathological feature (Table 3).

Table 4: Correlation between the histological grading and nature of collagen fiber.

Category	Parameter	Categories	Well	Mod, Mod-poor		p-value
Broder's grading	Thickness	Thick	10(47%)	5(55%)		0.782
		Thin	10(53%)	4(45%)		
	Organization	Organized	8(36%)	2(22%)		0.351
		Disorganized	12(64%)	7(88%)		
	Colors	Red-orange	11(52%)	3(33%)		0.537
		Yellow-orange	2(11%)	1(11%)		
		Green-yellow	7(37%)	5(56%)		
	Density	Well packed	9(42%)	3(33%)		0.657
		Loosely packed	11(58%)	6(67%		
	Birefringence	Strong	11(58%)	3(33%)		0.225
		Weak	8(42%)	6(67%)		
Modified Invasive front grading		**Categories**	**4-8**	**9-12**	**13-16**	
	Thickness	Thick	1(50%)	5(45%)	8(50%)	0.972
		Thin	1(50%)	6(55%)	8(50%)	
	Organization	Organized	1(50%)	4(36%)	4(25%)	0.686
		Disorganized	1(50%)	7(64%)	12(75%)	
	Colors	Red-orange	1(50%)	6(54%)	6(37%)	0.224
		Yellow-orange	1(50%)	0	2(13%)	
		Green-yellow	0	5(46%)	8(50%)	
	Density	Well packed	1(50%)	4(36%)	6(37%)	0.934
		Loosely packed	1(50%)	7(64%)	10(63%)	
	Birefringence	Strong	1(50%)	8(72%)	6(37%)	0.198
		Weak	1(50%)	3(28%)	10(63%)	

Abbreviations: Well= Well Differentiated Carcinoma; MOD= Moderately Differentiated Carcinoma; Mod-Poor= Moderately to Poorly Differentiated Carcinoma

Clinicopathological consideration

None of the other Clinicopathological parameter shows correlation with the advancing tumor front and nature of collagen fiber except age, size of tumor, TNM staging and metastasis of lymph node.

Histological grading system

Results were not statistically significant with Broder's and Bryne's grading system of OSCC but observation suggested that significant correlation was present. Majority of cases (>50%) which were graded as poor to moderate – differentiated according to Broder's and Bryne's grading system shows collagen fiber at the advancing tumor

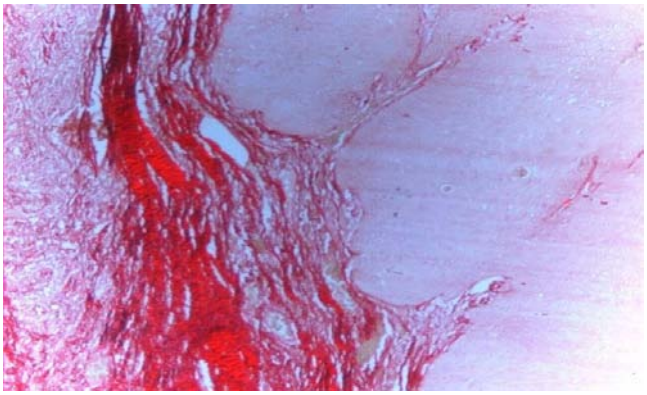

Figure 1A: Photomicrograph of Cohesive tumor front (Picro Sirius red stain without polars, 10X).

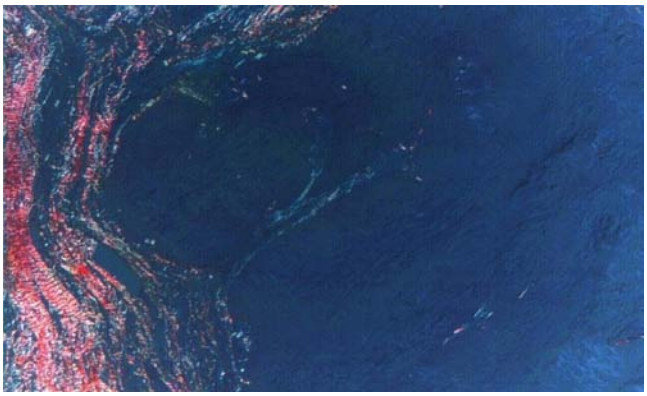

Figure 1B: Photomicrograph of Cohesive tumor front (Picro Sirius red stain with polars, 10X).

front as disorganized, loosely packed, greenish yellow in color with weak birefringence (Table 4).

Discussion

One of the most prevalent keratinocyte based cancers is squamous cell carcinoma. The highly invasive and metastatic nature of OSCC makes it one of the most frustrating cancers to treat [5]. However, to be invasive, a tumor cell must be able penetrate and move through the stroma [6]. The process of tumor dissemination involves specific interactions with tumor cell-surface adhesion receptors and multiple adhesive components of the extracellular matrix (ECM).

Tumor stroma plays a critical role in carcinogenesis; to grow beyond a minimal size of 1-2 mm, the tumor requires stroma [4]. The stroma is composed of ground substance composed of proteoglycans, glycoproteins and water, and the fibrous component including interstitial collagen and elastic fibers [1]. Which provides the vascular supply, nourishment, oxygen supply, and also limits the influx of inflammatory cells, thereby acting as a barrier for immunological rejection. Fibrous component of stroma has shown to be associated with OSCC and hence stromal changes which indicate the propensity of tumor cells to infiltrate and metastasize are now being studied as one of the prognostic indicators [1].

Van Gieson and trichrome stains may not be ideal for detection of collagen fibers as both these methods fail to reveal thin collagen fibers, stain's tendency to fade and these disadvantages lead to under estimation of collagen content. This perplexing issue incited Puchtler and colleagues to seek a better method and they found that Sirius red dissolved in a saturated picric acid solution (picrosirius red) consistently stained thin collagen fibers, did not fade, and was appropriate for use with polarized light microscopy [7].

Combination of sirius red and picric acid was first considered to be a special stain for connective tissue in 1964, especially for differentiating the subtype of collagen. It works on the principle that sulphonic group of sirius red reacts with the basic groups in collagen molecules. 126 sirius dye molecules bind to purified collagen types I, II and III. The enhanced birefringence of collagen is due to the attachment of the elongated dye molecules parallel to the long axis of the collagen. The orientation of the fibers to polars and collagen birefringence provides brightness to the collagen. Thickness, density of packing and spatial arrangement determines the polarization birefringence of the collagen. Picrosirius red stain thus helps in better understanding of the collagen function and pathology [8-11]. In addition the picrosirius red stained sections when observed under a polarizing microscope show birefringence due to the anisotropic properties of collagen [12,13].

As proposed in different studies that different patterns of invasion (representing different grades of tumor cell dissociation) are associated with the prognostic outcome in cancer [14]. An attempt has been made in this study to assess the nature of collagen at the invasive front of OSCC using picrosirius red and polarizing microscope.

Previous literature confirmed that Invasive front grading in contrast to Broder's grading is of high prognostic value, because of the following reasons:

1. Tumors often are more poorly differentiated in invasive than in superficial parts [15].

2. Blood group H antigen is often lost in invasive tumor margins of OSCC and this lost is associated with poor prognosis [16].

3. Increased expression of proliferation associated structure in the invading tumor front [17].

4. Melanoma cells from the deep tumor parts have a higher DNA content than more superficial cells [18].

5. Increased expression of Ki-67, L-myc, c-myc, N,myc, c-erbB-2 oncoprotein in most invasive lung cancer [19,20].

6. Increased labeling of bromodeoxyuridine at the site of invasion [21].

All of these studies show that the key to a better understanding of the invasive and metastatic behavior of cancer cells may reside within the invasive margins of different tumors so in the present study nature of collagen fiber was analyzed with the modified invasive front grading.

As proposed by Bryne, et al. [22], pattern of invasion is divided into four grades,

Grade I: Pushing, well delineated infiltrating borders,

Grade II: Infiltrating, solid, cords, bands and/ or strands,

Grade III: Small groups or cords of infiltrating cells (n >15),

Grade IV: Marked and wide spread cellular dissociation in small groups and/ or in single cells (n< 15).

The cases were divided into 2 groups

a) The majority 19 (63%) of the tumor showed discohesive tumor front represented by grade II, III, IV (66%)

b) Followed by cohesive tumor front 11(37%) represented grade I.

In the present study with respect to the relationship between the collagenous component in the stroma and the invading tumor front, there have been some observable changes with different advancing tumor front i.e. cohesive and discohesive.

In cohesive tumor front densely packed distinct collagen, shows reddish orange color which was mainly concentrated around tumor islands probably to prevent the tumor invasion. This may be due to deposition of type 1 collagen fiber which were in the form of thick bands and composed of densely packed, well organized with strong birefringence (Figure 1A, 1B), while in discohesive tumor front collagen fiber were thin, loosely packed, disorganized, with weak birefringence, probably due to majority of Type III collagen fiber (Figure 2A, 2B, 3 and 4). This feature being consistent with the concept of Jungeria, et al. [23] and Montes, et al. [24]. They stated that the thick fibers were type I collagen fiber and exhibit an intense birefringence of red, orange and yellow color by polarizing microscope and a weak birefringence of green when fiber were thin fibrillar thus consisting type III collagen. Observation is further supported by study by Stenback, et al. [25], the presence of a delicate meshwork (reticular) of type III collagen at the invading front of tumor islands in increasing gradations of skin cancer. Study on "stromal reaction during tumor progression in oral mucosa" by Megumi Yokoyama revealed that type I collagen which was stained red by PSR, were decreased with advanced dysplastic grading [26].

Thus the color changes observed in the present study clearly indicate some alteration in the stromal tissue around the tumor island of advancing front, which in turn may be due to carcinogenic agents that are involved in tumorigenesis. The above results are further supported by Brekken, et al. [27] who stated that the tumor progression is influenced by extracellular matrix. This finding is further supported by study on mechanics of capsule formation which reveals that a more robust extracellular matrix and capsule results in slower growth of tumor [28].

Further, nuclear resonance studies on the physical aggregation of the collagen fiber by Sharf, et al. [29] have also revealed a color profile of orange to red while corresponded to the well packed fiber and green to greenish yellow to poorly packed fibers. This collagen may be from the tumor cell in origin, thus benefiting the tumor by reducing the access to the host lymphocytes.

Alternatively, the collagen could be of stromal cell origin, thus benefiting the host by walling off the invading tumor.

Results in the study also revealed that 9 cases (53%), tumor having size less than 4 cm shows densely packed collagen fiber and 10 (83%) tumor having size greater than 4 cm shows loosely packed collagen fiber around the tumor island of advancing front. Hence tumors

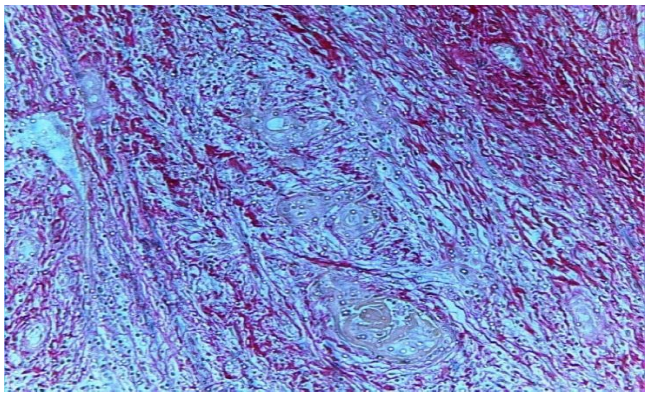

Figure 2A: Photomicrograph of Discohesive tumor front (Picro Sirius red stain without polars, 10X).

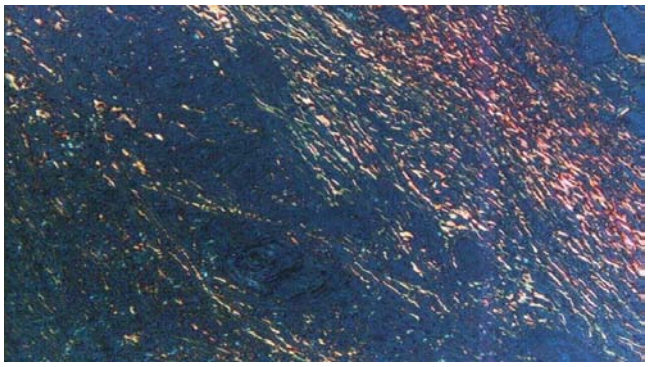

Figure 2B: Photomicrograph of Discohesive tumor front (Picro Sirius red stain with polars, 10X).

with excessive collagen in the stroma may respond in this manner as seen in study on breast cancer have also shown that an increase in collagen content of extracellular matrix increase the mechanical stiffness and transport resistance of tumor [30]. As also study on myocardial infarction concluded that the collagen degradation and loss after myocardial infarction is associated with infarct expansion and followed by functional decline [31].

Present study also revealed that majority of cases which were graded as TNM stage I and II 9 (56%) with absence of lymph node metastasis 10 (53%) shows densely packed collagen fiber whereas tumor in stage III and IV, 11 (85%) with evident metastasis 8 (89%), collagen fiber were loosely packed. Alon, et al. [32] in their study on stromal differences in salivary gland tumors found that 50% of collagen fiber in polymorphous low grade adenocarcinoma and adenoid cystic carcinoma were greenish yellow, whereas in pleomorphic adenoma, only 13% of them were greenish yellow. In similar manner study of SCC of skin and lower lip revealed that high grade of tumor cell dissociation, represented by a spray like pattern of invasion (PI), was significantly associated with a high frequency of metastatic as well as recurrent disease [33,34]. It was also reported that non-cohesive (spray like) pattern of invasion was significantly associated with the invasive with lymphovascular space involvement and large tumor size.

Contrary to our result the nature of collagen fiber was not significant with the Broder's grading system as mentioned in the

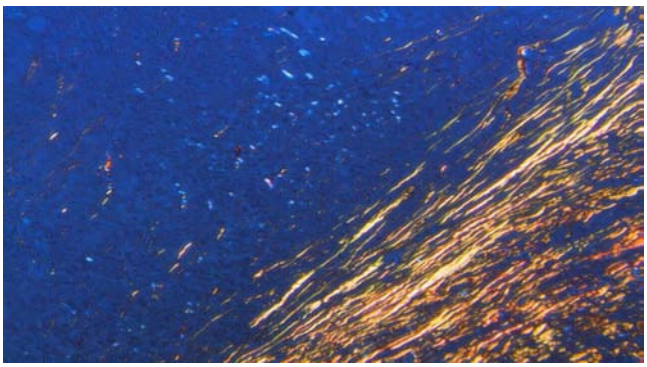

Figure 3: Photomicrograph of Discohesive tumor front showing thin collagen fibers with yellow orange color birefringence (Picro Sirius red stain 10X).

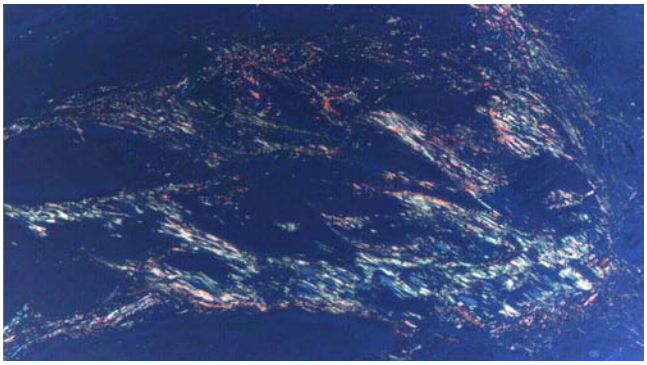

Figure 4: Photomicrograph of Discohesive tumor front showing thin, disorganized, yellow orange color birefringence, loosely packed collagen fibers. (Picro Sirius red stain 4X).

study done by Venigella [3] and Kalele [4], that collagen fiber in well differentiated carcinoma revealed polarizing colors of reddish orange around the tumor island, which gradually changes into yellow-orange in moderately differentiated and greenish yellow with weak birefringence in poorly differentiated carcinoma, but association was observed with the invasive front grading by Bryne, et al. [35]. Previous literatures have also confirmed that Invasive front grading in contrast to the conventional Broder's grading is of high prognostic value. Irrespective of difference scoring system the common findings of all study was that a discohesive tumor front was associated with metastasis, large tumor size, advanced tumor stage (TNM stage III and IV) increased recurrence and decrease survival.

Conclusion

Based on the above study it is concluded that picrosirius red stain with the use of polarizing microscope is most suitable stain to visualize collagen fibers and application of stain is a relatively simple tool to study the changes in extracellular matrix in particular the structural integrity of collagen fibers at the different invasive front of OSCC.

In the present study an observable change in the collagen with the different pattern of invasion was evinced with the pattern of invasion. Adjacent to the cohesive tumor front represented by pushing borders of invasion is positive correlated with thick bands of collagen which were well organized, and resist the tumor against invasion and metastasis, preventing it to increase in size and thus associated with initial stage of tumor (I&II). In discohesive tumor front the fibers are thin, disorganized which may enhance the movement of tumor cells towards invasion and metastasis.

Determination of collagen fiber nature in different patterns of invasion of oral squamous cell can help for targeting the stroma for various treatment strategies. Further research with larger sample size is with advanced techniques of using immunohistochemistry and collagen gene identification, Second harmonic generation (SHG) microscopy, Confocal laser microscopy are probably more specific and sensitive for collagen detection in this direction.

Drawbacks and Limitations

Though picrosirius red stains very thin collagen fibers in comparison to other collagen stains, Factors like pH, concentration of stain and the duration of staining will attribute to the variation in results.

Sample of the stains can be deteriorated when kept in for more than four years. Under this condition the solution loses its specificity and besides staining collagen, it also stains muscle and epithelium [11].

It is not advisable to use the staining technique on tissue preserved in formalin for many number of days. Hence, researchers must aim at ultra structural features of connective tissue in different stages of OSCC in future.

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