

RESEARCH ARTICLE

Editorial Process: Submission:12/15/2017 Acceptance:02/19/2018

Evaluation of Collagen in Leukoplakia, Oral Submucous Fibrosis and Oral Squamous Cell Carcinomas Using Polarizing Microscopy and Immunohistochemistry

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Abstract

Objectives: Picrosirius red and MMP are capable of degrading extracellular matrix proteins, expressed in lesions such as squamous cell carcinomas. The present study was undertaken with an aim to analyze and compare changes in collagen using Picrosirius red staining under polarizing microscopy and immunohistochemical staining using anti MMP-13 in samples of oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma. **Materials and Methods:** A total of 70 slides were prepared and divided into 3 groups. Group I comprised 10 slides of normal gingival tissue, Group II 40 slides of potentially malignant disorders and Group III 20 slides of well differentiated oral squamous cell carcinoma. Half the slides for each group were stained with Picrosirius red stain and the remainder with antibodies to MMP-13. **Results:** In Group II, MMP-13 connective tissue expression was greater in OSMF as compared to leukoplakia. Group III showed elevated expression among 70% of cases. Picrosirius red staining in Group II cases, showed higher staining Yellow-Orange and Green-Yellow mature fibers in OSMF than leukoplakia cases while in Group III, 50% OSCC cases showed Green-yellow stained immature thin fibers. **Conclusion:** In future, therapeutic measures targeted against MMP-13 may inhibit collagenolysis to some extent and delay spread of tumors. An easy and reliable method to determine the state of the stroma in such cases may be Picrosirius red staining with polarizing microscopy.

Keywords: Immunohistochemistry-MMP-13- picrosirius red stain- polarizing microscopy- oral squamous cell carcinoma

Asian Pac J Cancer Prev, **19** (4), 1075-1080

Introduction

The use of tobacco in different forms is highly prevalent in the Asian subcontinent. Tobacco contains many carcinogens like nicotine, N-nitrosamines, and many others which induce generation of free radicals and reactive oxygen species, that affect the essential constituents of cell membrane and might be involved in carcinogenesis. The oral cavity witnesses the development of a variety of Red and White lesions associated with tobacco use. These lesions often precede the development of frank cancerous lesions (Lodi et al., 2006).

Oral potentially malignant lesions comprises of a multitude of histologically diverse lesions with variable but overall increased risk for development of invasive oral squamous cell carcinoma (OSCC). Leukoplakia is the most common premalignant or “potentially malignant” lesion of the oral mucosa. The reported prevalence ranges from 0.2 to 5%, with remarkable regional differences:

India (0.2-4.9%), Sweden (3.6%), Germany (1.6%), and Holland (1.4%) (Marrija, 2000).

Oral Submucous fibrosis (OSMF) is a chronic, progressive, scarring disease that predominantly affects the people of South –East Asian origin. The disease was first described by Schwartz (1952) and he ascribed the term “atrophica idiopathica (tropica) mucosae oris”. Later, in 1953, Joshi from Bombay redesignated the condition as oral submucous fibrosis, implying predominantly its histological nature (Rajendran, 1994).

Despite the attainments already achieved concerning OSCC diagnosis and therapy, mortality and morbidity rates are still exceedingly high, challenging the available methods of prognosis assessment and encouraging the search for new and better markers. Recent advancements in Cancer Genetics and the role of molecular markers in pathogenesis of cancer are particularly notable.

One of the major aspects of tumor cell invasion and metastasis is the interaction between cancer cells and the

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surrounding extracellular matrix (ECM). This interaction involves all the components of the extracellular matrix, of which type I collagen is the most abundant and is synthesized predominantly by fibroblasts.

Collagen is involved in tumor progression in two very different ways. The desmoplastic response to a tumor results in excess deposition of collagen around the tumor. Conversely, collagen degradation and decreased synthesis allow invasion of tumor cells through the stroma. Degradation of the extracellular matrix is dependent on specific interactions between tumor and host cells (Fenhalls et al., 1998).

Traditionally, stains like Van Gieson and other trichome stains were used to demonstrate collagen fibers in tissue sections. But they lacked the precise selectivity and failed to reveal very thin collagen fibers. Sirius Red is an elongated dye molecule which reacts with collagen with high specificity and promotes an enhancement of the normal birefringence of collagen under polarizing microscopy (Constantine and Mowry, 1968).

Matrix metalloproteinase's (MMPs) are zinc dependent endopeptidases that are capable of degrading extracellular matrix proteins. The activity of MMPs is seen not only during normal organogenesis and wound healing but also in pathological conditions like inflammatory diseases and tumor invasion (Mehanna et al., 2009).

Myofibroblasts (MF) has been reported and plays a key role in ECM synthesis, re-organization and tissue contractions during both physiologic and pathologic processes like wound healing and tumourigenesis (Desmoulier et al., 2004).

It may be postulated that an excess of MF in malignancies may secrete excess MMP, which breakup ECM thus allowing for easier and faster dissemination of epithelial cells into the matrix. Moreover, the MF, also secrete numerous growth factors and cytokines that stimulate epithelial cell proliferation (Desmoulier et al., 2004).

Hence MMPs appear to be essential for tumor invasion and metastatic spread, and it may be hypothesized that positive identification and quantification of MMP may be useful in the prognosis of malignancies.

Therefore the present study was undertaken with an aim to analyze and compare the changes in collagen using Picrosirius red staining under polarizing microscope and immune histochemical staining using Anti MMP-13 in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma.

Materials and Methods

The present study was conducted in the Department of Oral Pathology and Microbiology after the clearance from the ethical committee of the institute. Formalin fixed paraffin embedded blocks of previously diagnosed cases as leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma were taken from the archives of the department. Normal blocks of oral gingival tissues were taken as control.

A total of 70 slides were prepared from these blocks taken from the archives of the department and were divided in to 3 groups. Group I consisted of 10 slides

which were prepared from normal gingival tissue which was excised during implant exposure (Stage II), and these served as negative control. Group II consisted of 40 slides out of which 20 were clinical and histopathologically diagnosed as oral leukoplakia and epithelial dysplasia respectively and 20 were clinically and histopathologically diagnosed as oral submucous fibrosis. Group III consisted of 20 slides were histopathologically diagnosed as well differentiated oral squamous cell carcinoma.

Further, half the slides for each group were stained with Picrosirius Red Stain. The remaining halves from each group were stained with immune histochemical stains for Antibody to MMP-13. Further they were observed under Polarizing microscope, where 10 fields from each sample were observed under oil immersion for different polarizing colors.

The slides that were prepared were evaluated by one senior oral pathologist and one resident oral pathologist of the department. Each slide was observed under polarizing microscope and was evaluated for the degree of the staining for MMP-13 and scores were given as follow (Culhaci et al., 2004):

- 0 : No staining of the tumor cells or stromal cells
- 1+ : Weak (<50%) positive staining of the tumor cells or weak staining of stromal cells
- 2+ : Moderate (>50%) positive staining of the tumor cells and or moderate staining of stromal cells
- 3+ : Extreme staining of the tumor cells and or strong staining of stromal cells

The data collected was archived and tabulated and statistical evaluation was done using SPSS (v16) software.

Results

Table 1 shows the comparison of MMP-13 immuno-histochemical expression in epithelium among all the three groups.

Table 2 exhibits the comparison of MMP-13 immuno-histochemical expression in connective tissue among all the three groups.

Figure 1 depicts photomicrograph of epithelial/collagen tissue fibres with Anti- MMP-13 expression in Leukoplakia, OSMF and OSCC.

Table 3 shows the comparison of Polarizing colors of picrosirius red staining among all the three groups.

Figure 2 depicts photomicrograph of collagen tissue fibres stained with Picrosirius red stain seen under Polarizing microscope in Leukoplakia and OSCC.

Table 4, Table 5, Table 6 materializes the co-relation between immuno-histochemical expression of MMP-13

Table 1. Comparison of MMP-13 Immuno-histochemical Expression In Epithelium Among All Three Groups

Scores		Groups			
		I	II	III	
		Normal	Leukoplakia	OSMF	OSCC
0	Negative	4 (80%)	0 (0%)	2 (20%)	0 (0%)
1+		1 (20%)	1 (10%)	4 (40%)	2 (20%)
2+	Moderate	0 (0%)	6 (60%)	2 (20%)	3 (30%)
3+	Extensive	0 (0%)	3 (30%)	2 (20%)	5 (50%)

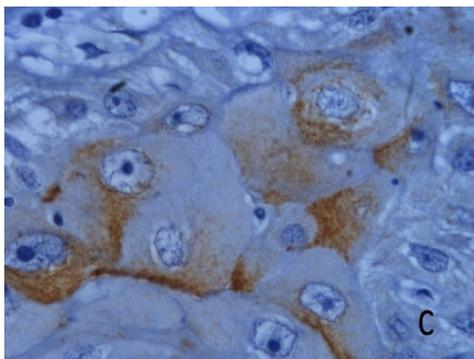
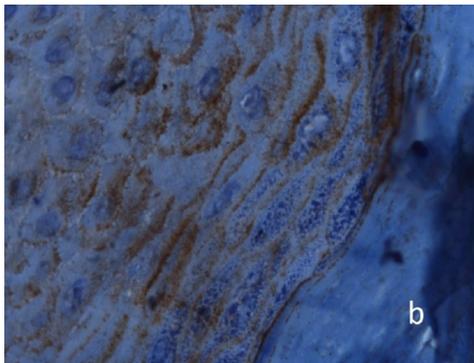
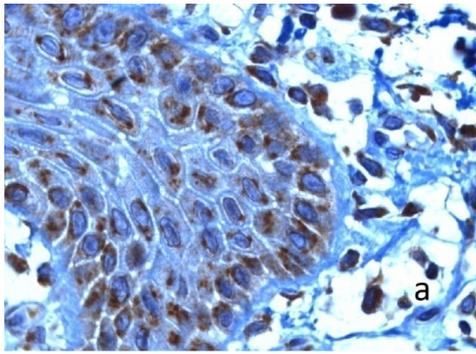


Figure 1. Photomicrograph of Epithelium/Collagen Fibres (Anti MMP-13 Expression) a) Leukoplakia b) Oral Submucous Fibrosis c) Oral Squamous Cell Carcinoma

and picosirius polarizing staining.

Discussion

Oral cancer is a significant health problem in the world. Most oral cancer patients are diagnosed at a late stage, when treatment is less successful and treatment associated morbidity is more severe. A number of new diagnostic aids to conventional oral examination have

Table 2. Comparison of MMP-13 Immuno-histochemical Expression in Connective Tissue Among All Three Groups

Scores		Groups			
		I	II		III
		Normal	Leukoplakia	OSMF	OSCC
0	Negative	5 (100%)	1 (10%)	0 (0%)	1 (10%)
1+		0 (0%)	6 (60%)	4 (40%)	2 (20%)
2+	Moderate	0 (0%)	2 (20%)	4 (40%)	3 (30%)
3+	Extensive	0 (0%)	1 (10%)	2 (20%)	4 (40%)

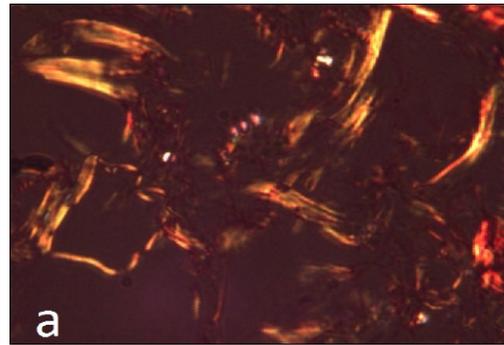


Figure 2. Photomicrograph Showing Collagen Fibres (Stained with Picro-Sirius Red Stain) in Under Polarizing Microscope a) Oral Squamous Cell Carcinoma b) Leukoplakia

recently been introduced to assist in the early detection of oral neoplasia. A Workshop coordinated by WHO collaborating centre for oral cancer and pre-cancer at London in May 2005 did not favor subdividing pre-cancer into lesions and conditions and the consensus view was to refer to all clinical presentations that carry risk of cancer under the term potentially malignant disorders to reflect their widespread anatomic distribution (Warnakulasuriya et al., 2007).

Numerous studies have stressed on the importance of the stroma in the spread of Oral cancer. Of the many steps of invasion and metastasis, the breakdown of matrix components and the migration of cells through the degraded matrix are important determinants. The increase in collagen fiber bundles in Oral Submucous Fibrosis results in diminished vascularity which causes atrophy of the overlying epithelium, making it more susceptible to carcinogenic agents. Stains for collagen are useful in when they are highly selective. An ideal collagen stain should not stain anything besides collagen, with the remainder of the tissue visualized by the counter stain. It is well known that collagen viewed by polarized light is birefringent. Staining with Picosirius red F3BA

Table 3. Comparison of Polarizing Colors of Picosirius Red Staining Among All Three Groups

Staining Colors Observed	Groups			
	I	II		III
	Normal	Leukoplakia	OSMF	OSCC
Red-Yellow	1 (20%)	3 (30%)	4 (40%)	3 (30%)
Yellow-Orange	4 (80%)	6 (60%)	4 (40%)	2 (20%)
Green-Yellow	0 (0%)	1 (10%)	2 (20%)	5 (50%)

Table 4. Co-relation Between Immuno- Histochemical Expression of MMP-13 and Picrosirius Polarizing Staining in Group I

	Picro-Sirius Staining Data				MMP-13			
	S No	Red Yellow	Yellow Orange	Green Yellow	3+	2+	1+	0
Normal	1		P					P
	2		P					P
	3		P					P
	4		P					P
	5	P						P
Total		1	4	0	0	0	5	

Table 5. Co-relation Between Immuno- Histochemical Expression of MMP-13 and Picrosirius Polarizing Staining in Group II

	Picro-Sirius Staining Data				MMP-13			
	S No	Red Yellow	Yellow Orange	Green Yellow	3+	2+	1+	0
OSMF	1			P	P			
	2	P				P		
	3		P				P	
	4	P					P	
	5	P				P		
	6		P			P		
	7		P				P	
	8		P				P	
	9				P	P		
	10	P						P
Total		4	4	2	2	4	4	
Leukoplakia	1		P			P		
	2		P			P		
	3	P					P	
	4	P						P
	5				P	P		
	6		P				P	
	7		P				P	
	8		P				P	
	9		P				P	
	10	P					P	
Total		3	6	1	1	2	7	

produces a remarkable degree of birefringence of all the collagen fibers which appear bright yellow when viewed by polarized light. This property permits even very small fibers to be seen easily. This intensity of the birefringence after Sirius red exceeds greatly that seen either in unstained sections or after any of the other procedures tested here (Constantine et al., 1968).

Sirius red does not impart birefringence to structures that lack it normally. This helps distinguish collagen from other substances that are also stained by Sirius red. Moreover, it also helps to differentiate between thick fibers and thin fibers, which is otherwise very difficult under the light microscope (Venigella and Charu, 2010).

The use of Picrosirius stain along with Polarizing microscopy has been especially helpful in these studies.

Usually, normal thin collagen fibers show green to greenish yellow polarization, whereas thick fibers show yellowish-orange through orange to red polarization. It has also been stated that in both thin and thick fibers, green to greenish yellow colors suggest that the collagen is poorly packed and orange red color originates from tightly packed fibers. It was suggested by the authors that change in polarization colors of collagen fibers may be indicative of neoplastic transformation (Gangana et al., 2012).

It has been reported that 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. Whereas, moderately differentiated and poorly

Table 6. Co-relation Between Immuno-histochemical Expression of MMP-13 and Picrosirius Polarizing Staining in Group III

	S No	Picro-Sirius Staining Data			MMP-13			
		Red Yellow	Yellow Orange	Green Yellow	3+	2+	1+	0
OSCC	1			P	P			
	2	P						P
	3	P					P	
	4			P	P			
	5		P			P		
	6			P	P			
	7		P			P		
	8			P		P		
	9	P						P
	10			P	P			
Total		3	2	5	4	3	3	

differentiated squamous cell carcinoma in the same study showed a gradual change in the polarizing color from yellowish orange to greenish yellow, particularly in the immediate vicinity of the invading tumor islands (Venigella and Charu, 2010).

The transformation of lesions from a pre-neoplastic to cancerous state is associated with an increase in collagenolytic activity. Cancer cells produce collagenases as collagen type I and III are the most abundant component in the extracellular matrix of dermal and oral submucous connective tissue. By increased formation of collagenases, the invading tumor cells are capable of dissolving collagen in connective tissue obstructing its course, and migrate through the degraded matrix (Gangana et al., 2012).

MMPs are a family of zinc-dependent neutral endopeptidases collectively capable of degrading all components of the extracellular matrix. The MMP family contains at least 24 structurally related members, which are divided into collagenases, gelatinases and stromelysin like MMPs. Members of the collagenase subgroup of MMPs i.e. collagenase 1 (MMP-1), collagenase 2 (MMP-8), and collagenase 3 (MMP-13), are the principal neutral proteinases capable of degrading native fibrillar collagens in the extracellular space. They all cleave type I, II and III collagens at a specific site, generating $\frac{3}{4}$ N-terminal and $\frac{1}{4}$ C-terminal fragments, which then denature in physiological temperature and are degraded by the other MMPs, e.g., gelatinases [2,3 of nina Johansson]. MMP-13 is a member of the collagenase family, which degrades fibrillar collagens of types I, II, III, IV, X and XIV, tenascin, fibronectin, aggrecan, vesicant, and fibrillin-I. MMP-13 also cleaves native type I collagen in the N-terminal non-helical telopeptide [4 of nina Johansson]. It also plays a key role in the activation cascade, both activating and being activated by several MMPs. Elevated MMP-13 levels have been reported in a number of malignancies, and has also been associated with tumor behavior and prognosis (Johansson, 1999).

In our study, we attempted to study the expression of MMP-13 in different Oral potentially malignant disorders, and compare the findings with those seen in Oral Squamous cell carcinoma. Moreover, we also studied the

stroma in the same study group by Picrosirius staining, as this has been reported to describe the stroma better than routine H and E staining. Further, we attempted to see whether there was any co-relation between the levels of expression of MMP-13 and the color of collagen seen under polarizing microscopy.

Our study showed a significantly increased expression of MMP-13 in the connective tissue of Oral Squamous cell carcinoma cases as compared to the expression in potentially malignant disorders (Leukoplakia and Oral submucous fibrosis). Further, an increased expression was also noted in the pre-neoplastic group when compared with normal tissue, which did not show any expression at all. An intra-group comparison of the distribution of MMP-13 between Leukoplakia and Oral submucous fibrosis showed increased expression in Submucous fibrosis. Our findings are contrary to the widespread view that a reduction in collagenolytic activity is responsible for the pathogenesis of Oral Submucous Fibrosis. It is possible that the cases studied by us were in an advanced stage, or that there may be other unknown factors acting.

Moreover, our study showed half of the cases of Oral Squamous cell carcinoma showing extensive expression of MMP-13 in the epithelium which was significantly more than that in the premalignant group. While most cases (60%) of Leukoplakia showed moderate expression of MMP-13 in the epithelium, most of the cases of Oral Submucous Fibrosis (60%) showed negative/minimal expression, correlating well with the fact that oral epithelium undergoes atrophy in this lesion.

Picrosirius staining under fluorescent microscopy showed predominance of red-yellow and yellow-orange staining (40% each) in Oral submucous fibrosis while more than half of the cases of Leukoplakia and 80% of normal tissue showed yellow-orange staining. However, a significant finding was that half of the cases of Oral squamous cell carcinoma showed Green-yellow birefringence. This finding suggests that Oral malignancy is indeed associated with a breakdown of matrix.

There have been numerous studies in literature which have studied the collagen fibers in submucous fibrosis and

in Oral squamous cell carcinomas. These studies were based on the supposition that tightly packed collagen fiber bundles would act to inhibit the progression of cancer cells. Numerous other studies have focused on the expression of MMP-13 in these lesions as it is a principal agent in degradation of Type I collagen. These studies were based on the supposition that a breakdown in the extracellular matrix would allow for easy spread of the neoplastic cells. An analysis of studies of these natures suggested to us that MMP-13 expression, which is known to be an indicator of progression of neoplastic lesions, should also have a correlation to the type of collagen present in the connective tissue stroma of the lesions. It would only be logical to assume that an increased expression of MMP-13 should be associated with thin or loosely packed collagen fibers (Johansson, 1999).

In our study, an increased expression of MMP-13 in the connective tissue stroma of oral Squamous cell carcinoma correlated well with the demonstration of greenish-yellow birefringence with picosirius red stain. Also, the predominance of yellow-orange birefringence in the connective tissue of pre-malignancies correlated with minimal / no expression of MMP-13. This stands to logic as the connective tissue in normal tissue (80%) also showed yellow-orange birefringence. The findings of our study seem to suggest that the connective tissue undergoes significant alterations as lesions progress from pre-malignancy to malignancy. Furthermore, our study also shows that the use of Picosirius stain with polarizing microscopy may be a more economical, rapid and easy to perform procedure to evaluate the connective tissue changes in lesions, as compared to immune-histochemical study of MMP-13 which is more technique sensitive, takes more time and more expensive (Veli-Matti, 2003).

In conclusion, the findings of our study seem to suggest that the connective tissue undergoes significant alterations as lesions progress from pre-malignancy to malignancy. Furthermore, our study also shows that the use of Picosirius stain with polarizing microscopy may be a more economical, rapid and easy to perform procedure to evaluate the connective tissue changes in lesions, as compared to immune-histochemical study of MMP-13 which is more technique sensitive, takes more time and more expensive. Thus Picosirius stain can be used independently or as an adjunct with MMP-13 for evaluation of connective tissue.

While cancer therapy is directed on one end towards the malignant cells per se, numerous studies are directed towards associated developments. It is probable to assume that in future, therapeutic measures targeted against MMP-13 may inhibit collagenolysis to an extent and delay the spread of tumor. An easy method to determine the state of the stoma in these cases may be the use of Picosirius Red stain with Polarizing microscopy.

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