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Evaluation of Mast cell count in different Grades of Oral Submucous Fibrosis: an Invitro study

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Abstract

Introduction: Oral SubmucousFibrosis [OSMF] is a premalignant condition commonly seen in South East Asia. It is a chronic, progressive scarring disease leading to distinct clinical features that can be attributed to areca nut chewing.

Aim: To determine the expression of Mast cell count in different grades of OSMF

Materials and Methods: Mast cell expression was determined with 1% Toluidine blue stain for all the 48 cases.

Results: Mast cell count was found to be increased in Grade I and II but not in Grade III. On statistical analysis the results were found to be insignificant statistically

Conclusion: Mast cells count were found to be increased in early grades, but decrease in their numbers seen in advance grade. Statistically the results were found to be insignificant. Further studies with larger sample size is required to establish the exact correlation between mast cell count and the severity of OSMF.

Keywords: Oral submucous Fibrosis, Mast cell, Angiogenesis.

Introduction

Oral Submucous Fibrosis [OSMF] is a premalignant condition commonly seen in South East Asia. It is a chronic, progressive disease leading to distinct clinical features that can be attributed primarily to areca nut chewing[1, 2]. The prevalence of OSMF is about 0.5% with a range of 0.2-2% in different regions of India. Epithelial dysplasia has been observed in 10-15% and carcinoma in at least 5% of cases submitted for biopsy[2].

Histologically, excessive fibrosis in the oral mucosa seems to be the main pathology, secondarily leading to atrophic changes in the epithelium[2]. OSMF can be grouped into four histological grades of increasing severity: very early, early, moderately advanced and advanced[3].

The presence of mast cells and their role in OSMF have been discussed by many authors. Some studies have found increased mast cells in early grades of OSMF while others have not found any significant relation between mast cell count and OSMF grade[3]. In normal mucosa mast cells ranged between 0- 5/high power field(hpf) while in OSMF the number varied between 2-9 cells /hpf in various grades.[3]

Mast cells are activated by a number of stimuli. The functional activity of mast cells is represented by mast cell degranulation. When activated by immunological or non – immunological stimuli, degranulation of mast cells release various preformed mediators.[2] Mast cells secrete various preformed mediators like histamine, heparin, serotonin, tryptase, chymase, lipid derived mediators like leukotrienes - LTB4, LTC4, LTD4, LTE4, pro inflammatory cytokines like Tumour necrosis factor alpha (TNF-α), Interleukin (IL-1), mutagenic cytokines– IL-3, IL-5 and immunomodulatory cytokines like IL-4, IL-5 and other mediators like platelet activating factor (PAF), macrophage inflammatory protein (MIF-1α), prostaglandin and arachidonic acid metabolites. [4,5] These mediators degrade connective tissue matrix to provide space for new blood vessels.[2] Mast cell promotes angiogenesis by secreting angiogenic factors that degrade extracellular matrix.[2] These factors are histamine, basic fibroblast growth factor (b- FGF), heparin, tryptase. Mast cell- derived heparin and TGF- β have chemotactic activity for endothelial cells and also stimulate the growth of fibrotic tissue.[2] The release of mast cell granules may initiate a change in the connective tissue ground substance.[3]

Though there are several studies on the Mast cell expression in different grades of OSMF, but none of them gives clear picture. Mast cells play an important role in the pathogenesis of OSMF. This study is an honest attempt to evaluate their expression in different grades of OSMF and hence to better understand their role in pathogenesis of OSMF.

Materials and Methods

Once the ethical approval was taken, cases from the archives of our department which were already histologically confirmed as OSMF were considered for the study

A total of 48 cases were selected according to the inclusion and exclusion criteria. Clinical details were obtained from the request forms and histopathological slides were retrieved from the archives of the department.

Inclusion Criteria

1. Cases histologically diagnosed as OSMF.

Exclusion Criteria

1. Any coexisting premalignancy, other than OSMF
2. Any coexisting malignancy
3. Any coexisting acute or chronic specific infections, other than pulp and periapical diseases, gingivitis and periodontitis
4. Known cases of Systemic sclerosis

Histological Grading of OSMF

Haematoxylin and Eosin stained sections of 48 cases of OSMF were retrieved from the archives of the department. The diagnosis was reconfirmed and the cases were histologically graded using the criteria given in Table 1.

Table 1: Histological grading of oral submucous fibrosis (Pindborg and Sirsaat, 1966 and Khanna and Andrade, 1995)[6]

Grades	Stages	Features
Grade I	Very Early	1. Finely fibrillar collagen. 2. Marked oedema. 3. Fibroblasts: plump and young. 4. Blood vessel (BV): normal 5. Inflammatory cells: neutrophils, occasional eosinophils.
	Early	1. Juxta-epithelial hyalinzation. 2. Collagen: Thickened, separate bundles. 3. BV: Dilated and congested. 4. Inflammatory cells: Mostly lymphocytes, eosinophils and occasional plasma cells.
Grade II	Moderately Advanced	1. Collagen- moderately hyalinized, thickened collagen bundles. 2. Adult Fibrocytes- Spindle shaped. 3. BV: Constricted. 4. Inflammatory cells: Lymphocytes and plasma cells.
Grade III	Advanced	1. Collagen completely hyalinized and no separate bundles. 2. Fibroblasts – reduced to absent. 3. Inflammatory cells – reduced.

After histological grading, the final sample included 17 cases of grade I, 25 cases of grade II and 06 cases of grade III OSMF. For simplicity, in grade I cases, both very early and early stages were evaluated together

Mast Cell Count in Different Grades of OSMF

A. Staining with Toluidine blue

Mast cell count was studied by 1% Toluidine Blue staining. Formalin-fixed, paraffin embedded tissue blocks of OSMF cases were sectioned to a thickness of 4 µm. The slides were stained with toluidine blue as follows:

1. **Preparation of Stock Solution:** 1% toluidine blue stock solution was prepared by mixing

- Toluidine Blue powder 1.0 gm
- 70 % alcohol: 100.0 ml

2. **Preparation of Working solution:** The working solution was prepared fresh, by mixing

- Toluidine blue, Stock 5.0 ml
- 1% Sodium chloride (45.0 ml Sodium Chloride: 0.5 gm in distilled water: 50.0 ml)

3. **Staining technique**

a) Dewaxing: Sections were treated with two changes of xylene for 30 minutes.

b) Rehydration: The sections were rehydrated through 100 %, 90 % and 70 % alcohol for 1 minute each, followed by rinsing in distilled water for 15 seconds each.

c) Staining: The sections were then treated with 1%Toluidine blue solution for 2 minutes. They were then washed with three changes of distilled water.

d) Dehydration: The sections were then quickly dehydrated through 70 %, 90 % & 100 % alcohol with 5 dips each. The sections were subsequently drained, blotted and placed in xylene for 20 minutes.

e) Mounting: Stained sections were mounted using DPX mountant.

B. Evaluation of Toluidine Blue staining:

All the stained slides were examined using binocular bright field microscope (Olympus CX 21 I) and on ocular grid of 400 squares. Mast cells were identified by their **purple metachromatic granules and sky blue nuclei**. Mast cells (both intact and degranulated) were counted in each case. Mast cells were counted in 10 randomly selected high power fields under X10 objective. MCC was

calculated in each case by two observers independently and a mean MCC was obtained.

Statistical Analysis

I. Statistical Plan

A normality test was conducted using **Shapiro Wilk** test to check for the normal distribution of the data. Subsequently, it was found that the data was not normally distributed and hence relevant non-parametric tests were used for further analyses.

II. Descriptive Statistics

The demographic characteristics of the study cases were expressed in terms of number and percentage.

III. Inferential Statistics

Kruskal Wallis test was used to check any statistical significant difference among the three study groups for the mast cell count and TGF- β distribution, and corresponding 95% confidence interval (CI) was estimated from this model with level of significance of P<0.05

Results

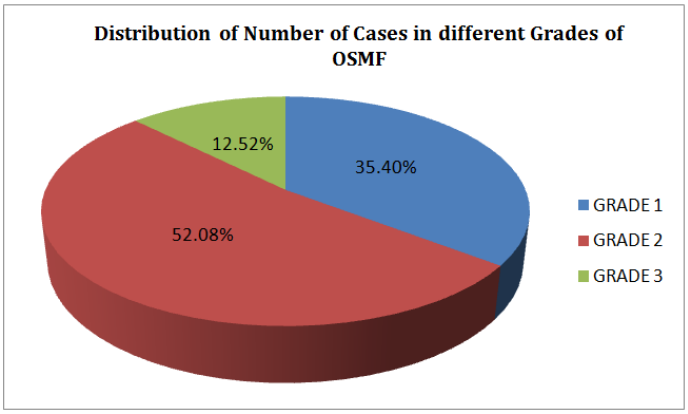
Demographic Characteristics

Number Of Cases: The total number of cases (n) was 48. The distribution of cases under each grade of OSMF is given in Table 2 and Graph 1. The most common histological grade was grade II, followed by grade I and grade III was the least common.

Table 2: Distributionof number of cases in different grades of OSMF

	N (Number)	% (Percentage)
Grade I	17	35.40
Grade II	25	52.08
Grade III	6	12.52

Graph 1: Distribution of number of cases in different grades of OSMF.



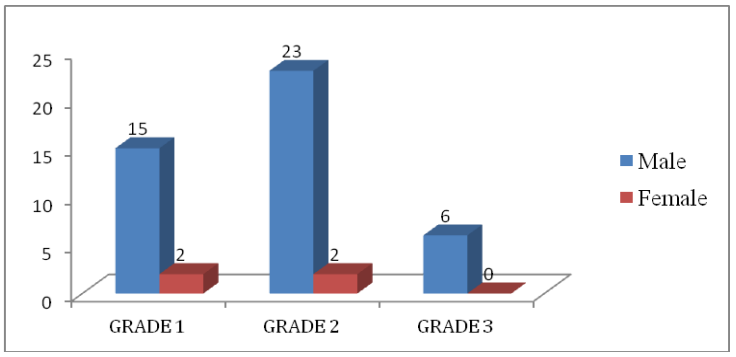
Gender

Among the 48 cases, 44 were males and 4 were females, thereby giving a male to female ratio of (11:1). Gender-wise distribution of cases under each grade is given in Table 3 and Graph 2.

Table 3: Gender wise Distribution among the study cases in different grades of OSMF

Grade	Male	Female
Grade I	**15**	**2**
Grade II	**23**	**2**
Grade III	**6**	**0**

Graph 2: Gender wise Distribution of number of cases in different grades of OSMF



Mean Age

The youngest in the study was 21 years and the oldest was 70 years old. The mean age in each grade of OSMF is given in Table 4. There was no significant difference in age between the grades.

Table 4: Age-wise Distribution among the study cases in different grades of OSMF

Grades	Youngest	Oldest	Mean Age
Grade I	22 years	54 years	34.53 +/-10.05
Grade II	21 years	70 years	36.72 +/- 13.54
Grade III	22 years	42 years	34.17 / - 7.65

Mast Cell Counts In Different Grades of OSMF

Mast cells were found in all cases of OSMF. They were seen to be distributed throughout the connective tissue, including muscle and adipose tissue. In majority of the cases, they were concentrated just below the epithelium and around the blood vessels. **(Photographs 14 through 17)**

Mast cells were counted in 10 random fields for every case. The mean mast cell count was calculated and tabulated. The lowest mean MCC was 1.0 and the highest was 20.2. In grade I OSMF cases, the lowest was 1.3 and highest counts were between 17 and 19. In grade II cases, the lowest was 1 and the highest counts were between 19 and 21. The lowest MCC in grade II was 2.2 and the highest count was not more than 8.0.

A Kruskal Wallis test was conducted to examine whether there was any statistical difference among the three study group with respect to mast cell count **(Table 5)**. The result revealed that there was no statistically significant difference (p = 0.14) found among all the three grades of OSMF i.e Grade I OSMF [mean = 9.04 +/- 4.64 (95% CI = 6.65 – 11.43)]; Grade II OSMF [mean = 8.58 +/- 4.83 (95 % CI = 6.58 +/- 10.57)]; Grade III OSMF [mean = 5.04 +/- 1.99 (95 % CI = 2.96 -7.13)].

Table 5: Comparison of mast cell counts in different grades of OSMF

GRADES	MAST CELL COUNTS								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	P-Value
					Lower Bound	Upper Bound			
Grade I OSMF	17	9.04	4.64	1.13	6.65	11.43	2.05	17.2	0.14
Grade II OSMF	25	8.58	4.83	0.97	6.58	10.57	1.2	19.4	
Grade III OSMF	6	5.04	1.99	0.81	2.96	7.13	3.05	7.95	

Hence, when the mean of mast cell count was compared in all the three grades of OSMF, it was found that mast cell count was increased in Grade I and II when compared to Grade III. In Grade I and Grade II, there was a wide range of MCC compared to grade III.

Summarizing The Results

MCC were found to be higher in grades I and II, when compared to grade III OSMF. However, the difference was not statistically significant.

List of Figures

Toluidine Blue Staining For Mast Cells

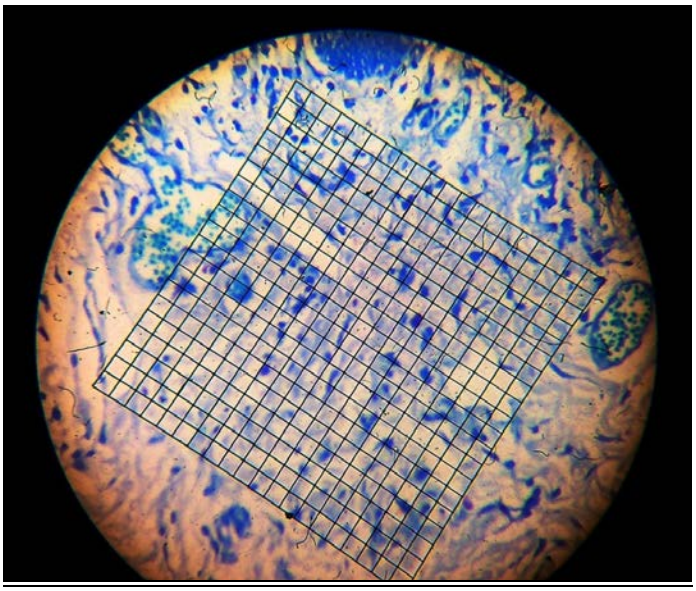


Fig. 1: Toluidine blue stain.Mast cells counting using ocular grid (X40).

Grade I

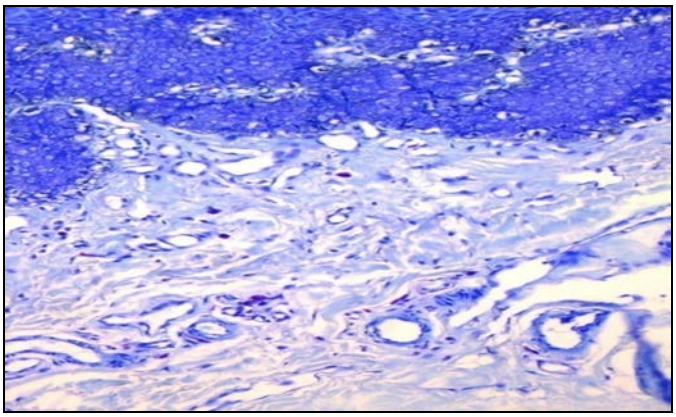


Fig.2: Toluidine Blue stain. Grade I OSMF. Mast cell concentrated perivascularly. This case also exhibited mild epithelial dysplasia.(X40).

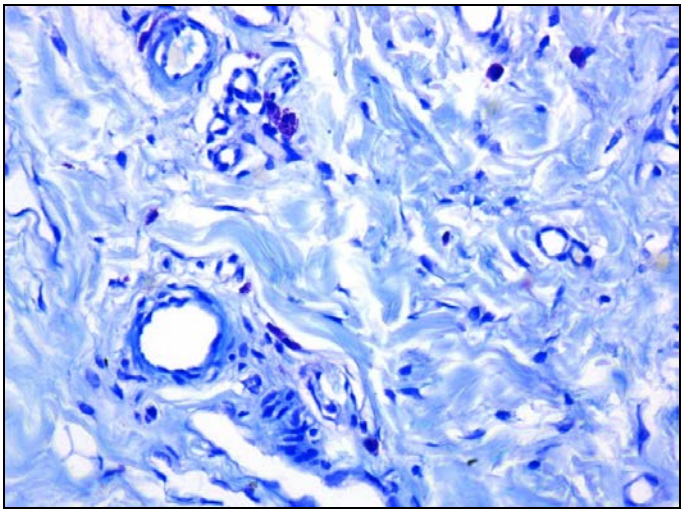


Fig. 3: Toluidine blue staining for demonstration of Mast cells (X100). The mast cell granules metachromatically stain purple.

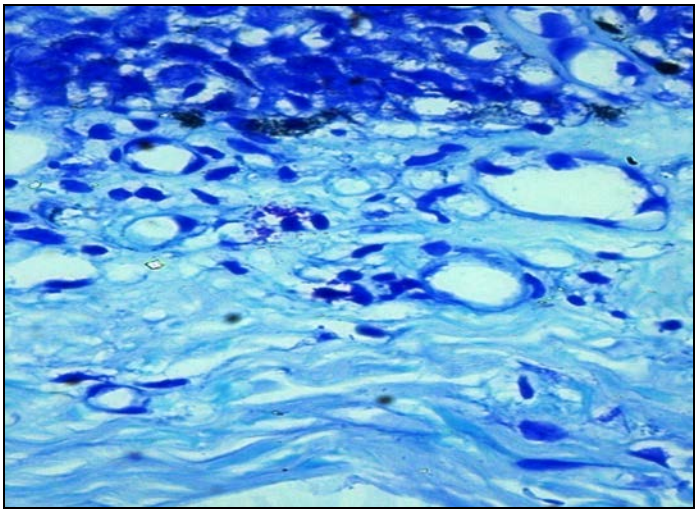


Fig. 3:Toluidine blue staining for demonstration of Mast cells (X200)

Discussion

OSMF is a precancerous condition, predominantly affecting south East Asians[7]. Epidemiological studies in

India have shown a female predominance of OSMF by some authors. Conversely, few authors have reported a male predominance.[8] Our findings were in agreement with the latter and showed an 11:1 male to female ratio. This could be explained by the fact that in the city, areca nut and betel quid chewing habit is more prevalent among males, whereas in the rural areas, betel quid chewing is still prevalent among females.

Age predilection of OSMF reported in literature varies widely. More et al [9] state that OSMF can be commonly seen in individuals between 16 and 35 years of age. In our study, majority of the cases were between 30 and 40 years of age. The occurrence of OSMF was seen at a younger age group in case of Grade I when compared to Grade II and Grade III. Grade II was also seen at a younger age group than Grade III.

The most important feature is mucosal rigidity due to fibrosis of juxtaepithelial connective tissue, which later involves deeper portions, resulting in a progressive inability to open the mouth. [8] Various aetiologic agents such as chillies, nutritional deficiency, chewing of areca nut, genetic susceptibility, altered salivary constituents, autoimmunity and collagen disorders may be involved in the pathogenesis of this condition. However, the most accepted aetiologic agent is arecanut chewing.[10]

Mast Cells in OSMF

Mast cell was first described by Paul Ehrlich in 1877 as "Mastzellan" – a well fed cell[4]. They are mobile and bone marrow derived.[5] They are similar to basophils. Basophils leave the bone marrow after they are mature, whereas, mast cells exit the bone marrow in an immature form and mature at the tissue site.[11] They are concentrated in submucosa of areas which communicate with the exterior such as skin, lung, gastrointestinal tract (including the mouth), conjunctiva and nose.[11] They are also found in the dental pulp.[5] They are distributed in perivascular and perineural regions. They have a lifespan of weeks to months.[5]

Light microscopy of mast cells

Under light microscopy, they stain similar to fibroblasts. They are easily identifiable using a selective stain – 1% toluidine blue. They are large spherical or elliptical cells. The most characteristic feature is the presence of 80 – 300 granules. With toluidine blue, the mast cell granules stain metachromatically and appear purple and the nucleus stains sky blue.[4,5] Based on light microscopic appearance with toluidine blue, three types of mast cells have been identified[7].

1. **Cells deeper in connective tissue:** (except that in close vicinity to blood vessels): these are round / oval in and dark purple in colour. They have well-defined borders. The nucleus is covered by granules and barely visible. These are called Intact cells.
2. **Cells in superficial connective tissue and near blood vessels**: The cells appear flattened / irregular with granular cytoplasm.They are known as **spreading cells** with cell borders not defined and nucleus only partially appreciable. These cells are found in lichen planus below the epithelium.
3. **Cells within the infiltrate:** These are **degranulated cells** and appear paler as staining changes from metachromatic violet to light pink (because of less granules), nucleus blue in colour & well defined.

Ultrastructure of mast cell [5]

In oral mucosa and skin, in mast cells are complex with amorphous region located next to crystalline region. Variation is seen in crystalline region and differs with the three types of mast cells described above.

Mast cell markers

Mast cells have been studied using anti mast cell tryptase antibody.[2] Khatri MJ et al [12] used c-kit (CD 117) to evaluate mast cells in OSMF and compared it with MCC

using 1% toluidine blue. They found a significantly higher mast cell count using c-kit in normal and advanced cases and hence concluded that using c-kit is a more reliable method for identification of mast cells.

Mast cell functions

The mast granules are rich in histamine, heparin, leukotrienes (B4, C4, D4, E4), TNF-α, IL-1, I, IL-3, IL-5, IL-10, chondroitin sulphate, proteoglycan and numerous enzymes including collagenaseand tryptase.[4,5,11] On activation of mast cells, degranulation occurs releasing these mediators and they cause a variety of effects depending the anatomic location and the response required. They produce variety of lipid and protein alpha mediators with pro inflammatory activities including chemotactins, cell activating and cell growth factors.[7] Mast cells are not uniform in all locations, the granule content of mucosal and connective tissue mast cells are different from each other.[5,11] The mucosal mast cells are T-cell dependent.[11]

They play a role in remodelling of extracellular matrix (ECM) during wound healing.[5] Mast cells have been studied in various conditions like wound healing, chronic inflammation, keloid, pulmonary fibrosis and angiogenesis.[4] They have been thought to play a role in progression of rheumatic arthritis and progression of fibrotic diseases.[5] They are also found in neurofibromas.[1] Mast cells have also been studied in various oral diseases such as periapical lesions, reactive lesions (pyogenic granuloma in particular), orofacialgranulomatosis, odontogenic cysts, gingivitis, OSMF, OL, OLP, and OSCC [2,5,11]

Mast cells have been found to be increased in OSMF when compared to normal mucosa by many researchers [2,3,4,5,11,12]. Savita JK et al [3], in their review cite previous authors that the MCC in normal mucosa is 0-5 cells/high power field (HPF) and in OSMF it is increased to 2-9 cells/HPF. Ankle MR et al [8] reported a MCC of 25.5 cells /sq.mm in normal oral mucosa as against 48.25cells /sq.mm. in OSMF. In our study, we found a wide range of MCC in different areas in each section. When MCC was correlated with the grade of OSMF, there was no significant difference. However, MCC was higher in grade I and II when compared to grade III OSMF. Many other researchers have also obtained a similar result.[11,12] The plausible reasons are:

- In grade I and II of OSMF, mast cell degranulation occurs continuously and their contents are depleted. These flattened cells, though they are present may not be easily visible in light microscopy, even with Toluidine blue. This postulation stems from Khatri MJ's study[19], where they found more mast cells in advanced cases of OSMF on immunohistochemical staining with c-kit.
- The lifespan of mast cells is weeks to months. It takes a long time for progression of OSMF, and by this time, all the mast cells recruited in early phases may have undergone programmed cell death.
- With increase in severity of OSMF, complete fibrosis of connective tissue take place. This dense fibrosis and compressed blood vessels may impede the migration of inflammatory cells (including mast cells) in advanced cases.

Sabrinath B et al [2] compared and correlated mast cell density (MCD) and microvascular density(MVD) in normal oral mucosa and different grades of OSMF. They found that MCD and MVD were higher in OSMF when compared to normal. As MCD increased, MVD increased and this was most prominent in moderately advanced OSMF. Thus they concluded that mast cells are important players in angiogenesis seen during early stages of OSMF.

Conclusion

This study shows Mast cell count is increased in Grade I and Grade II of OSMF. In Grade III cases of OSMF Mast cell count was seen decreased. Though statistically significant relation was not found, but increased Mast cell count in early cases cannot be ignored. Therefore, for better understanding of the disease process, further study is required with larger study sample to understand the pathogenesis of the disease process and finally in the management of the disease

Abbreviation

Oral Submucous Fibrosis (OSMF)

Mast Cell Count (MCC)

High power field (hpf)

Leukotrienes – LT

Interleukin – IL

Extracellular Matrix (ECM)

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