Matrix metalloproteinase-1 expression in oral submucous fibrosis: An immunohistochemical study

Gauri Mishra, K Ranganathan[1]

Department of Oral and Maxillofacial Pathology, Rama Dental College, Hospital and Research Centre, Kanpur, Uttar Pradesh, [1]Ragas Dental College, Hospital and Research Centre, Chennai, Tamilnadu, India

ABSTRACT

Context: Oral submucous fibrosis (OSF) is a form of pathological fibrosis affecting the oral mucosa. There is compelling evidence to implicate the habitual chewing of areca nut with the development of OSF. Because collagens are the major structural components of connective tissues, including oral submucosa, the composition of collagen within each tissue needs to be precisely regulated to maintain tissue integrity. Arecoline stimulates fibroblasts to increase the production of collagen by 150%.

Aim: As the role of collagenase is implicated in cleaving the collagen under physical conditions, this study was carried out to evaluate the role of collagenase-1 (matrix metalloproteinase [MMP]-1) in a pathologic condition like OSF.

Settings and Design: A total of 40 patients were included in the study, comprising of 30 OSF as Group 1 and 10 normal buccal mucosa tissue as Group 2.

Materials and Methods: Both the groups were stained for MMP-1 by the immunohistochemical method using the streptavidin HRP–biotin labeling technique. MMP-1 expression intensity in the epithelium and connective tissue was decreased in Group 1 when compared to Group 2.

Statistical Analysis Used: Chi-square test of association was used to determine the difference in the expression of MMP-1 between OSF and normal buccal mucosa and among different histological gradings of OSF.

Results: The results were statistically significant. However, there was no statistically significant difference between the expression of MMP-1 among different histological grades of OSF in Group 1.

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Matrix metalloproteinases (MMPs) are a family of neutral proteases that are produced by a variety of cells during both physiologic conditions, like normal development and wound healing, and a wide variety of pathological processes.[1,2] Currently, 28 human MMPs have been identified, and these enzymes have been classified according to their substrate specificity and structural similarities. The major subgroups are interstitial collagenases, gelatinases, stromelysin and memebrane-bound MMPs.[3] They are produced by several cell types, including fibroblasts, macrophages, neutrophils and some epithelial cells. Their secretion is induced by certain stimuli, including growth factors, cytokines and physical stress.[4] Most MMPs are synthesized as propeptides that require proteolytic cleavage for activation.[5] Collagenase, a member of the MMP family, is secreted as a latent precursor (procollagenase) and is activated by chemicals such as free radicals produced during the oxidative burst of leukocytes and proteinases.[4] Collagenase is the principal human enzyme that cleaves native fibrillar collagen.[6] Collagenase-1 (MMP-1) is produced by a wide variety of normal cells, e.g. stromal fibroblasts, macrophages, endothelial cells and epithelial cells, as well as by numerous tumors, suggesting a broad-base role for this collagenase in biology.[3]

Oral submucous fibrosis (OSF) is a form of pathological fibrosis affecting the oral mucosa and contiguous areas of the upper aerodigestive tract, with a relentless course of progression. Once initiated, the disease is ever-progressive even after cessation of the putative causative factor of areca nut chewing.[7] There is compelling evidence to implicate the habitual chewing of areca nut with the development of OSF. The areca nut component of betel quid plays a major role in the pathogenesis of OSF. Arecoline, an active alkaloid found in betel nuts, stimulates the fibroblasts to increase the production of collagen by 150%.[8] Flavanoid, catechin and tannin in betel nuts cause collagen fibers to cross-link, making them less susceptible to collagenase. This results in increased fibrosis due to both increased collagen production and decreased breakdown.[9]

Address for correspondence:
Dr. Gauri Mishra
E-mail: gaurimishra12@gmail.com

Because the role of collagenase is implicated in cleaving the collagen under physical conditions, this study was carried out to evaluate the role of collagenase-1 in a pathologic condition like OSF.

MATERIALS AND METHODS

Patients and tissues

Thirty cases of OSF (Group 1) and 10 of normal (Group 2) constituted the study material. The study was conducted in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, to record the clinical details of patients with OSF and to study the expression of MMP-1 by immunohistochemistry (IHC) in paraffin-embedded tissues. Clinically, the Group 1 patients showed presence of fibrous bands in the labial and/or buccal mucosa, loss of elasticity of the buccal/labial mucosa, difficulty to open the mouth and had a habit of chewing areca nut in some form. Ten patients with clinically normal buccal mucosa, reporting to the out patient department of oral and maxillofacial surgery for removal of the impacted third molar, constituted Group 2. They had no habit of smoking, alcohol consumption or chewing areca nut, were apparently healthy with no systemic disorders and were not on any medications. Detailed case history including age, sex, occupation, past medical and dental history, history of habits, drugs and trauma were recorded. General examination and intraoral examination were performed. Biopsies were taken after obtaining patient consent from the representative areas.

Staining

The tissue taken was immediately transferred to 10% buffered formalin. After adequate fixation, paraffin blocks were made. From the blocks, 5-μ-thick sections were cut and used for routine hematoxylin and eosin staining to confirm the clinical diagnosis. IHC staining was carried out. The primary antibody used was anti-MMP-1 mouse monoclonal antibody, clone: 41-1E5; isotype: IgG2a (Calbiochem- Merck International EMD Biosciences, San Diego, California, USA, Cat. No. IM35L). Secondary antibody used was from IHC Select Immunoperoxidase Secondary Detection System (Chemicon International, 28820 Single Oak Drive, Temecula 92590, USA, Cat. No. DAB150). The breast carcinoma specimen tissues were fixed, processed, embedded, sectioned and stained in a similar manner and used as positive control. One positive control tissue slide was included for each batch of staining.

Analysis

The mounted slides were viewed under a light microscope. The positively stained MMP-1 cells take up brown color within the cytoplasm. The intensity was evaluated and graded using the staining intensity of the positive control as negative (-), mild (+), moderate (++) and intense (+++) staining.

Data entry was performed with the aid of Statistical Package for Social Sciences (SPSS 10.5 version). Descriptive analysis was presented for all the variables. Chi-square test of association was used to find out the difference in expression of MMP-1 between OSF and normal buccal mucosa and among different histological grading of OSF. A *P*-value <0.05 was considered significant. Kappa analysis was performed for measurement of agreement with a second evaluator to rule out intraobserver bias.

RESULTS

Subjects

A total of 40 patients were included in this study, comprising of two groups: Group 1 was the study group of comprising of 30 histologically confirmed OSF patients while Group 2 was made up of 10 normal patients.

Group 1 consisted of 28 (93.3%) males and two (6.7%) females, with a male:female ratio of 19:1. In Group 2, there were six (60%) males and four (40%) females, with a male:female ratio of 3:2 [Table 1]. Group 1 had the youngest patient, aged 23 years, while the eldest was 57 years old. Most of the patients in Group 1 fell in the age group of 25–44 years, with the mean age of 35.43 + 9.13 years. In Group 2, the youngest was 29 years and the oldest was 49 years, with a mean age of 33.80+6.11years [Table 2].

All the study subjects of Group 1 gave a history of consuming areca nut either as commercially available pan masala, gutkha, hans, mava or raw areca nut as 19 (63.3%), 3 (10%), 3 (10%), 2 (6.7%) and 3 (10%), respectively. In Group 2, none of the subjects had habit of chewing raw areca nut or areca nut products. All the 30 study subjects of Group 1 were histologically graded as early stage, intermediate stage and advanced stage, with 10 (33.3%) subjects in each stage [Figures 1-4].

Table 1: Gender distribution between Group 1 and Group 2

Group	Gender		*P*-value
	Male, n (%)	Female, n (%)	
Group 1 (n=30)	28 (93.3)	2 (6.7)	0.01
Group 2 (n=10)	6 (60)	4 (40)	

Gender distribution between Group 1 and Group 2

Table 2: Age distribution between Group 1 and Group 2

Group	<25 years, n (%)	25–34 years, n (%)	35–44 years, n (%)	45–54 years, n (%)	≥ 55 years, n (%)	*P*-value
Group 1 (n=30)	2 (6.7)	12 (40)	12 (40)	2 (6.7)	2 (6.7)	0.445
Group 2 (n=10)	-	7 (70)	2 (20)	1 (10)	-	

MMP-1 connective tissue stain intensity between Group 1 and Group 2

Staining results

When the intensity of MMP-1 expression in the epithelium was compared between Group 1 and Group 2, it was found that in the former, of the 30 samples, 15 (50%) and 12 (40%) showed mild (+) staining and moderate (++) staining intensity, respectively. Epithelial expression was negative (-) in three (10%) tissue samples. While in Group 2, of the 10 samples, eight (80%) samples showed mild (+) epithelial staining and two (20%) showed moderate (++) staining intensity. The difference in epithelial staining was statistically significant between Group 1 and Group 2 (P=0.007) [Graph 1]. However, on comparing connective tissue staining, of the 30 samples of Group 1, 13 (43.3%) tissue samples showed mild (+) expression and 13 (43.3%) showed moderate (++) staining intensity. Negative (-) expression was seen in four (13.4%) samples. In Group 2, of the 10 samples, mild (+) connective tissue expression was seen in eight (80%) tissue samples and moderate (++) staining intensity seen in one (10%) sample. No (-) expression was seen in one (10%) sample. The difference in connective tissue staining was statistically significant between Group 1 and Group 2 (P=0.011) [Graph 2].

MMP-1 expression of the epithelium and connective tissue was evaluated among different histological grades of OSF in Group 1. In early stage, of the 10 samples, three (30%) showed mild (+) and six (60%) showed moderate (++) epithelial and connective tissue staining intensity while negative (-) expression was seen in one (10%) tissue sample. Of the 10 samples of intermediate stage, six (60%) samples showed mild (+) and three (30%) showed moderate (++) epithelial staining, while mild (+) and moderate (++) connective tissue staining was observed in five (50%) and four (40%) samples, respectively. However, epithelial and connective tissue expression was negative (-) in one (10%) tissue sample. In the samples of advanced stage (10), the epithelial staining was assessed as mild (+), moderate (++) and negative (-) in six (60%), three (30%) and one (10%) tissue sample, respectively, as compared to five (50%), three (30%) and two (20%) tissue samples showing mild (+), moderate (++) and negative (-) connective tissue staining. The result was not statistically significant (P=0.38) [Graphs 3 and 4].

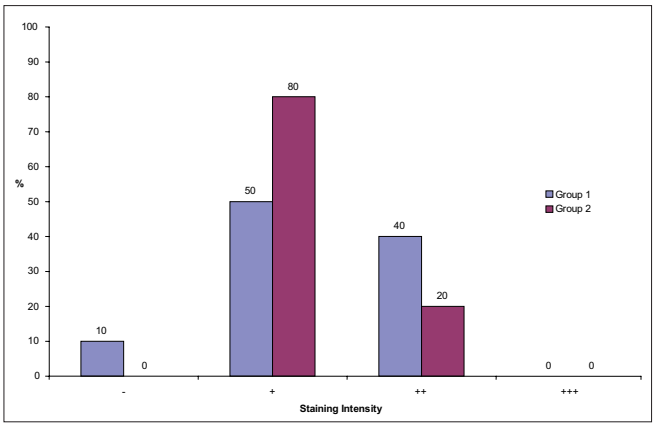
DISCUSSION

The term OSF was derived from oral (meaning mouth), submucosal (meaning below the mucosa of the mouth) and fibrosis (meaning hardening and scarring). OSF is a form of pathological fibrosis affecting the oral mucosa and contiguous areas of the upper aerodigestive tract, having a relentless course of progression, and it is not amenable to reversal at any stage of the disease process.[10] There is compelling evidence to implicate the habitual chewing of areca nut with the development of OSF. It occurs predominantly in the Indian subcontinent and South Asian countries, where the habit is more prevalent. The WHO definition for an oral precancerous condition, "a generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer," accords well with the characteristics of OSF. The malignant conversion rate of the precancerous condition ranges from 8 to 10%.[11] It is therefore important to identify the marker that could help us in diagnosing OSF in early stage so as to treat it aggressively and prevent the malignant conversion.

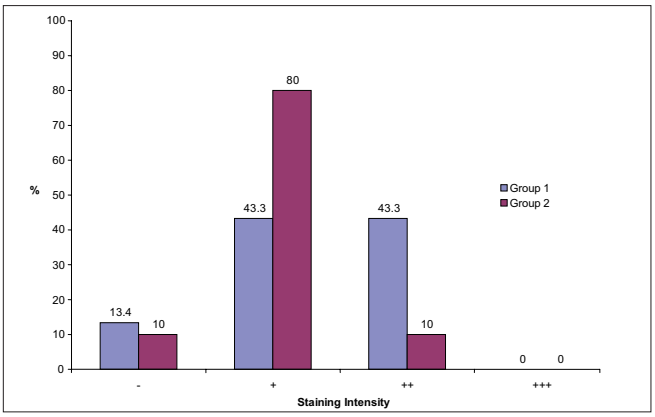
In 1960s and 1970s, only sporadic cases of OSF were seen, more often in older women. The prevalence of OSF has been increasing in India, with an estimated eight-fold increase in the last 15 years.[12] The rate varies from 0.2 to 2.3% in males and from 1.2 to 4.57% in females in Indian communities.[13] During the last two decades, with the advent of "pan masala," the practice of chewing habit has increased along with the prevalence among males. The present study showed a high preponderance of OSF in males, with a male:female ratio of 19:1. This was in accordance with the findings of other authors like Sinor *et al.*, Shah and Sharma, Ranganathan *et al.* and Ariyawardana *et al.*, who found the male:female ratio to be 29:1, 1.7:1, 9.9:1 and 4.6:1, respectively.[7,12,14,15]

The mean age at which OSF is seen now is much lower than what was seen two decades ago. The present study showed the mean age of the patients with OSF as 35.43+9.13 years. Other studies conducted have observed the mean age of OSF to be 29, 30.42+10.9, 32.4+10.4, 43.9+14.02 and 38.8+10.6 years.[7,12,14-16] In this study, the youngest patient with OSF was 23 years old. Quicker onset and higher male prevalence has been reported for OSF. Sinor *et al.* and Shah and Sharma have reported that males are affected more than females in the younger age groups of 11–20 and 15–24 years, respectively.[12,14] The study by Ranganathan *et al.* had the youngest OSF patient of 16 years.[7] This shows the increasing trend of OSF among the younger age group probably due to the easy availability of commercial products like pan masala. Lower age of onset of OSF means greater chances of malignant transformation among these patients.

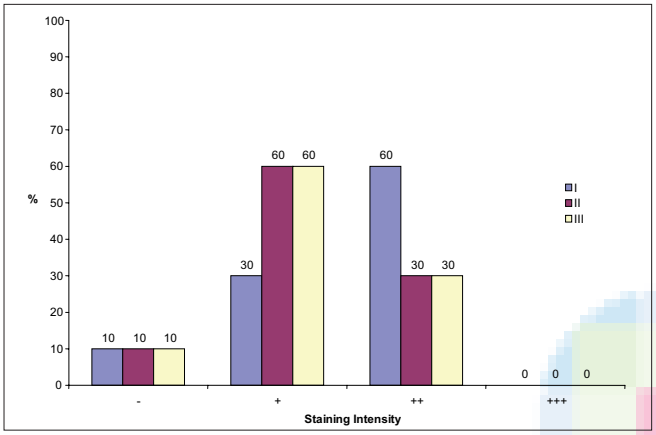
Various evidences implicate the habitual chewing of areca nut with the development of OSF. Worldwide, estimates of oral submucous fibrosis indicate that 2.5 million people are affected, with most cases concentrated on the Indian subcontinent, especially in southern India.[9] Ranganathan *et al.*, in their hospital-based study, have observed an increase in the use of areca nut, particularly pan masala, in the younger age groups in India, leading to an increasing incidence of OSF.[7] Various other factors such as genetic, autoimmune and nutritional factors are also thought to be the causative agents of OSF.[12] Several authors have confirmed a strong association between areca nut use and OSF and the increasing use of pan masala in place of the conventionally used areca nut.[7,12,14,15,17] In this study, all the subjects with OSF had the habit of chewing areca nut in various forms. Of all the subjects in the study group, 10% of



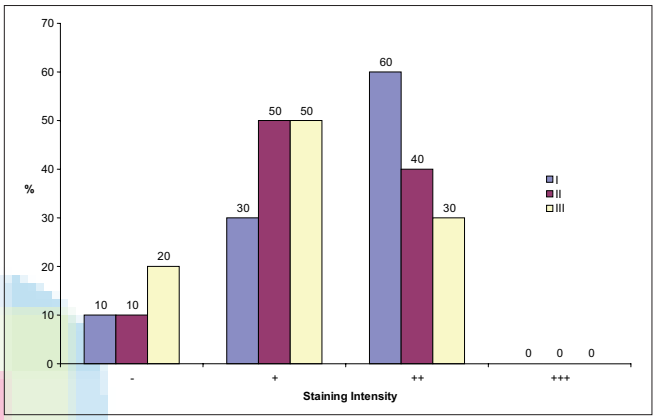
Graph 1: MMP-1 epithelial stain intensity between Group 1 and Group 2



Graph 2: MMP-1 connective tissue stain intensity between Group 1 and Group 2



Graph 3: MMP-1 epithelial stain intensity in various histological grades of Group 1



Graph 4: MMP-1 connective tissue stain intensity in various histological grades of Group 1

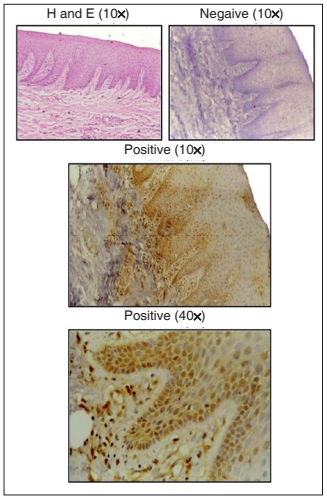


Figure 1: Staining pattern of normal buccal mucosa

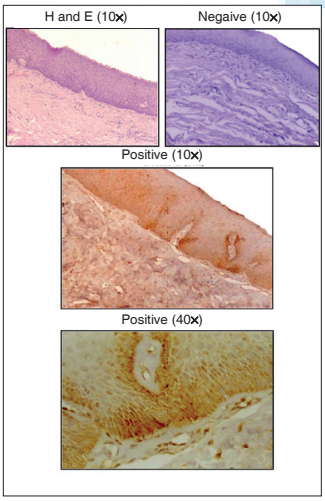


Figure 2: Staining pattern of early-stage oral submucous fibrosis

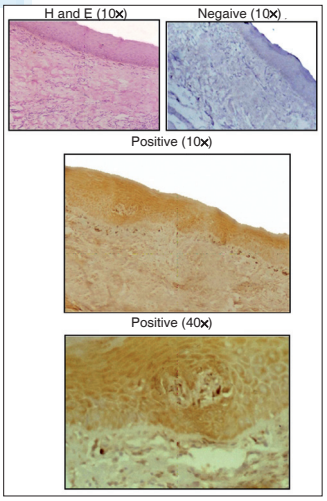


Figure 3: Staining pattern of intermediate-stage oral submucous fibrosis

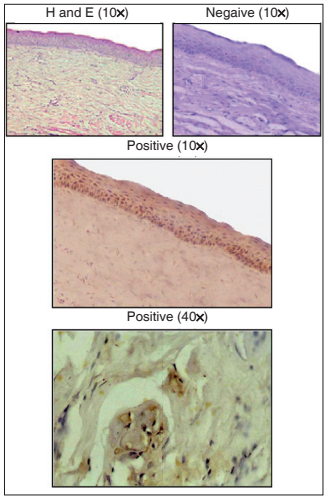


Figure 4: Staining pattern of advanced-stage oral submucous fibrosis

the patients consumed areca nut in the conventional form while others used some form of commercially available products. 63.3% of the study subjects chewed pan parag, 10% of the study subjects consumed gutkha, 10% chewed arecanut with hans, 6.7% consumed mava while 10% had the habit of chewing raw areca nut.

OSF is characterized by juxtaepithelial inflammatory reaction

followed by fibroelastic change in the lamina propria and associated epithelial atrophy. The important histological characteristic of OSF is the deposition of collagen in the oral submucosa.[18] Pindborg and Sirsat studied in detail about the histopathology of OSF and classified it histologically as very early, early, moderately advanced and advanced.[19] This classification was modified by Utsunomiya *et al*., who divided OSF into three stages as early, intermediate and advanced.[20] Based on this classification, in this study, 33.3% of the subjects were graded as early stage, 33.3% of the subjects as intermediate stage and 33.3% of the subjects were graded as advanced stage.

There is compelling evidence to suggest that OSF occurs due to disturbance in the homeostatic equilibrium between synthesis and degradation of the extracellular matrix (ECM), wherein collagen forms a major component, and can thus be considered as a collagen-metabolic disorder.[18] The excessive collagen deposition associated with connective tissue disorders, including fibrosis and drug induced tissue overgrowth, may be explained by several mechanisms. First, there may be accelerated production of collagen by fibroblasts that exceeds the rate of degradation. Second, increased collagen deposition might result from increased formation of type IV collagen that is resistant to fibroblast collagenases.[21] The present study was undertaken to evaluate the expression of MMP-1 in different histological grades of OSF and to compare the MMP-1 expression in OSF with that of the normal buccal mucosa using IHC. It was in view of altered degradation of collagen in OSF.

Huang and Shieh have demonstrated that collagen content in the advanced group with oral submucous fibrosis was higher than that of the normal mucosa group.[22] Van Wyk *et al*. concluded that there is excessive increase of collagen in OSF.[23] Tsai *et al*. have demonstrated marked deficiency of collagen and fibronectin phagocytosis of OSF fibroblasts.[24] In this study, the expression of MMP-1 in the epithelium of OSF was 90% and of the normal mucosa was 100%, with a statistical significance of P=0.007. This indicates decrease in the expression of MMP-1 in the epithelium in OSF compared to normal. Sutinen *et al*. have demonstrated a low expression of MMP-1 in oral epithelial dysplasia.[25]

Shieh and Yang have demonstrated that collagenase activity in OSF was much lower than that of the normal buccal mucosa.[26] Chang *et al*. have also reported decreased activity of collagenase in OSF.[21] In this study, MMP-1 expression in the connective tissue of OSF was 86.6% and that of the normal buccal mucosa was 90%. Our results confirm previous researcher's findings and provide further evidence that there is decrease in the expression of MMP-1 in OSF compared to normal. The normal buccal mucosa in the present study group, showed 100% diffuse positivity for MMP-1 in the cytoplasm of the epithelial cells, with 80% showing mild staining and 20% showing moderate staining intensity, with the buccal layer taking up most of the stain. Within the connective tissue, 80% of the stromal cells showed mild staining intensity, 10% showed moderate staining and 10% showed no stain. On evaluation of MMP-1 cytoplasmic epithelial stain intensity in different grades of OSF, it was found that in early OSF, negative, mild and moderate staining were seen in 10%, 30% and 60% of the samples, respectively, while both in intermediate and advanced OSF cases, negative, mild and moderate staining were seen in 10%, 60% and 30% of the samples, respectively. On evaluation of connective tissue intensity for MMP-1 in early OSF, negative, mild and moderate staining were seen in 10%, 30% and 60% of the samples, respectively; in intermediate OSF, 10% showed no stain, 50% showed mild stain and 40% showed moderate stain; in advanced OSF, 20% showed no stain, 50% showed mild stain and 30% showed moderate stain. The results from present study show altered pattern of staining intensity between different grades of OSF; however, the results were not statistically significant.

The results of the present study clearly demonstrate decreased expression of MMP-1 in OSF when compared with normal buccal mucosa. However, the staining pattern among different stages of OSF was not statistically significant. This could be attributed to the small sample size in each grade.

SUMMARY AND CONCLUSION

A total of 40 patients were included in the current study, comprising of 30 OSF cases taken, as Group 1 and 10 normal buccal mucosa tissues, taken as Group 2. Group 1 had a male:female ratio of 19:1, with a mean age of 35.43+9.13 years while in Group 2 the male:female ratio was 1.5:1, which was seen with 33.80+6.11 years as the mean age. All tissues were from the buccal mucosa. Both the groups were stained for MMP-1 by the IHC method using the streptavidin HRP–biotin labeling technique. The intensity of the cytoplasmic stain was evaluated manually using the staining intensity of the positive control (breast carcinoma) as the benchmark and graded on a four-point scale: (-) negative staining, (+) mild staining, (++) moderate staining and (+++) intense staining. MMP-1 expression intensity in the epithelium and connective tissue was decreased in Group 1 when compared to Group 2. The results were statistically significant. There was no statistically significant difference between the expressions of MMP-1 among different histological grades of OSF in Group 1.

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