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Original Article

Epithelial Dysplasia and Silver binding nucleolar organizer region proteins (AgNORs) in Oral Submucous Fibrosis.

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ABSTRACT

Background and Objectives: Oral submucous fibrosis is an insidious chronic disease of multifactorial etiology. The chewing of areca nut is thought to be an important etiological factor. It was suggested that substantial amount of copper is released from areca nut products which induces lysyl oxidase activity thus up regulating collagen synthesis by fibroblasts facilitating its cross linkage and thereby inhibiting collagen degradation. Nucleolar organizer regions proteins (NORs) are associated with proliferative activity and prognostic marker in several precancerous conditions and cancers. Different grades of OSMF were studied to find out its correlation with AgNORs counts and grade of dysplasia in OSMF. Material and Methods : Biopsy was taken from the buccal mucosa of OSMF patients. 3 - 4µ sections were cut from paraffin embedded sections. H & E stain, AgNORs stain &AgNORs counting was done. Results : The data analysis revealed that Mean AgNORs (p value 0.0001, 0.039, 0.046, 0.0001 in grade I, grade II, grade III, grade IV respectively) counts were increased as grade advances. Conclusion :. There is a positive correlation of AgNORs counts with clinical and histological grading, and AgNORs could truly serve as prognostic marker as a measure of dysplasia in OSMF.

1. Introduction

Oral mucous membrane is a unique tissue which is continuously exposed to various kinds of stressors, such as heat, cold, microorganisms, chemicals and mechanical irritations in the process of food intake. In response to these stressors, both epithelial and connective tissue layers of the oral mucosa also exhibit acute and chronic reactive changes.

Oral cavity is lined by a keratinized or non-keratinized stratified squamous epithelium. Several lines of evidence including clinical, experimental, and morphological data support the concept that squamous cell carcinoma of the upper aero digestive tract arises from noninvasive lesions of the squamous mucosa. This includes "premalignant lesions" and "premalignant conditions"[1].

The latest WHOmonograph on the head and neck Tumors (2005) uses the term 'Epithelial precursor lesions or conditions".1 from all premalignant conditions the incidence of OSMF is very high in India and its neighboring countries [2]. Uncertainty towards the present treatment modalities has challenged the clinicians and the research workers alike[2].

According toJ.J.Pindborg (1966) 3OSMF is defined "as an insidious chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although, occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by fibro elastic change of the lamina propria, with the stiffness of the oral mucosa trismus and inability to eat"[3].

The precise etiopathogenesis of OSMF is yet not known. Various factors that have been proposed include chronic irritation of the oral mucosa due to pan, tobacco, gutkhas, smoking, alkaloids in areca nuts, excessive consumption of chilies and spices, nutritional deficiencies genetic susceptibility, immune medicated damage and excess copper consumption[4-7]. OSMF is most commonly associated with the habitual chewing of areca nut (betel nut)[8,9].Areca nut is a mild stimulant, causing a hot sensation in the body, heightened alertness and sweating, although the effects vary from person to person. The areca nut contains tannin, gallic

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acid, a fixed oil gum, a little terpineol, lignin, various saline substances and three main alkaloids: Arecoline, Arecaine and Guracine which have vasocontricting properties[8-11].

Nucleolar organizer regions (NORs) are loops of ribosomal DNA that transcribe to ribosomal RNA and thus ultimately the protein. NORs have been utilized by cytogenetics for evaluation of certain genetic disorders notably trisomes. Mammals have several chromosomes possessing NORs, these areas are equivalent to sites which hybridize with RNA and each NOR has 40 transcription units. In man and chimpanzees NORs are located on five acrosentric chromosomes viz. 13,14,15,21 and 22. [12-14]NOR staining represents actively transcribing NORs and thus rRNA transcription and maintaining configuration of rRNA. The frequency of NORs per nucleus may partly reflect cell ploidy or proves useful as replicatory markers. Increased expressions of NORs sites would therefore be expected in actively proliferating cells[15,16].Studies have shown Ag-NORs to be useful to reflect the biologic behavior in oral squamous carcinoma[17].They have also been used in oral submucous fibrosis and hold promises for predicting the biologic behavior of oral submucous fibrosis, as can be correlated to clinical histological grading[18].

In our study we have done AgNORsstaining and tried to predict and assess the biologic behavior of lesion by correlating clinical and histological grades with AgNORs count.

2.Material and Methods

A total 30 patients of Oral Submucous Fibrosis were collected who attended the Dept. of Oral Maxillofacial pathology and microbiology and, K.M.Shah Dental College and Hospital, Vadodara. The age group of these patients varied from 15 70 years. The selection of these patients was based on the following criteria[19].

1) Positive history of areca nut chewing.

2) Burning sensation of mouth on eating normal spicy food.

3) History of gradual restricted mouth opening.

4) Blanched mottled and pearly appearing buccal mucosa.

5) Stiffening of buccal mucosa with or without palpable fibrous bands.

Other conditions causing restriction in mouth opening were carefully excluded. All the patients had not received any kind of treatment for Oral Submucous Fibrosis and none of them were suffering from any systemic diseases.

Biopsy specimens were obtained with 6mm punch from the buccal mucosa and kept in 10% formalin for fixation and processed. 3- 4 µ sections were cut from paraffin embedded blocks and 4 slides were made from each block, 2 slides for H & E stainingwere used for histological grading and 2 slides for AgNORs stain.

1) Hematoxylin and Eosin stain [20]

The specimens stained with H & E stain and observed under 10X objective (Microscope LABOMED Vision 2000).

It was used for,

1. Histological classification of OSMF. So that it could be correlated with AgNORs counts.

2. Identification of silver nitrate stained specimens.

Histological Evaluation and Histological Grading was done based on criteria used according to Khanna JN and Andrade NN 1995[21].

Epithelial dysplasia if observed was evaluated by two or more of features described by Paul M. Speight 2007.22

2) Silver nitrate staining:

The silver nitrate staining for AgNORs was performed according to modified method of Ploton et al (1988)[20].

In each case, AgNORs were evaluated under 100X oil immersion objective (Microscope LABOMED Vision 2000) and cedar wood oil. One hundred nuclei from epithelia randomly selected and observed for each case and AgNOR dots were counted. Randomness was accomplished by moving in a sequenced fashion where adjacent field did not overlap. The number of individual discernible and separated black dot or blebs in each nucleus was recorded and average for each case was computed.

3.Results

Age and gender distribution of the study subjects, Correlation between histological grading and mean mouth opening (Cms), Correlation between clinical staging and epithelial findings, Correlation between clinical staging and epithelial dysplasia, Correlation between clinical staging and corresponding mean AgNORs count in OSMF patients, Correlation between epithelial dysplasia and mean AgNORs count in OSMF patients are tabulated in table a g. Figure 1- 4 represents sections from various cases, representing histopathological characteristics. Figures 5 7 represents sections from various cases, representing AgNOR dots.

CHART 1 (Table a)

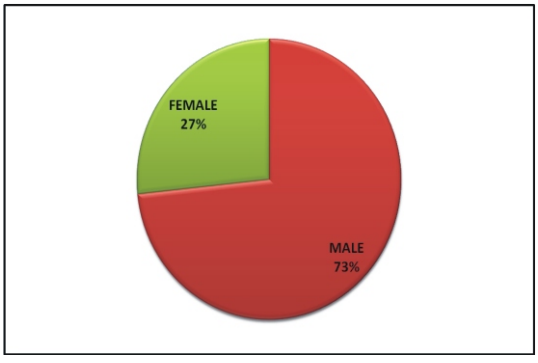


CHART 2(Table b)

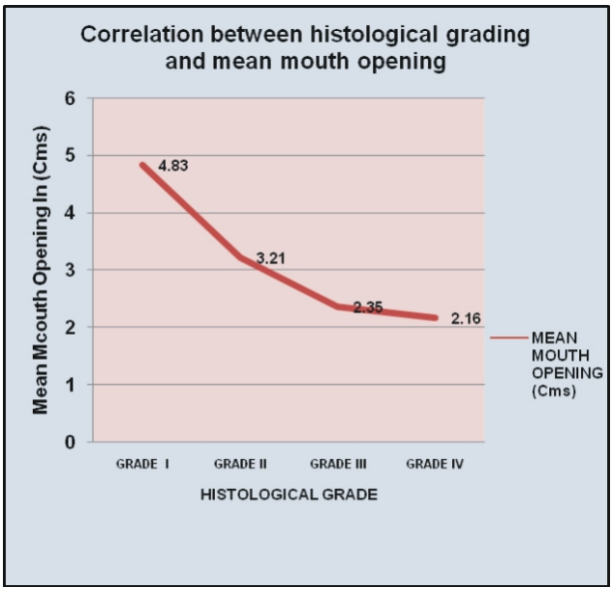


CHART 3(Table c)

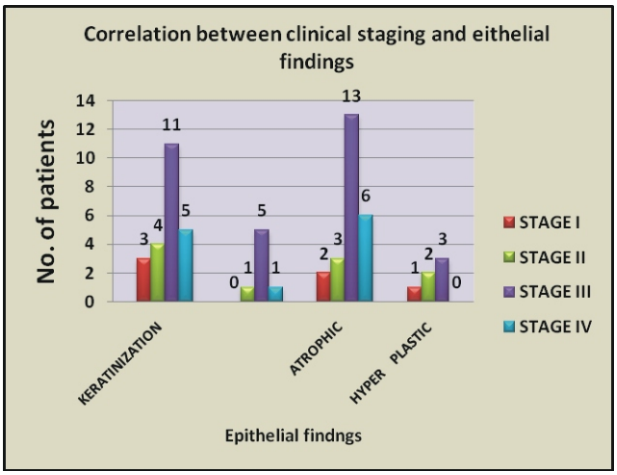


CHART 4(Table d)

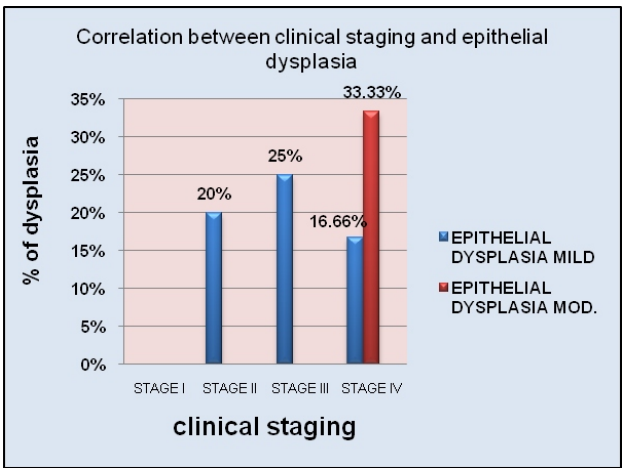


CHART 5(Table e)

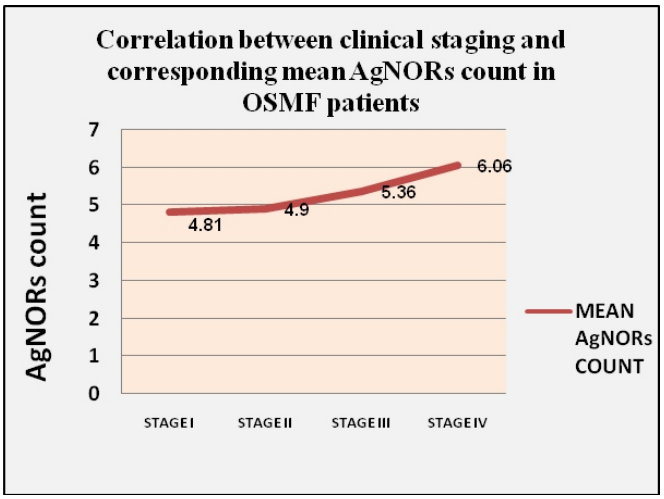


CHART 6(Table f)

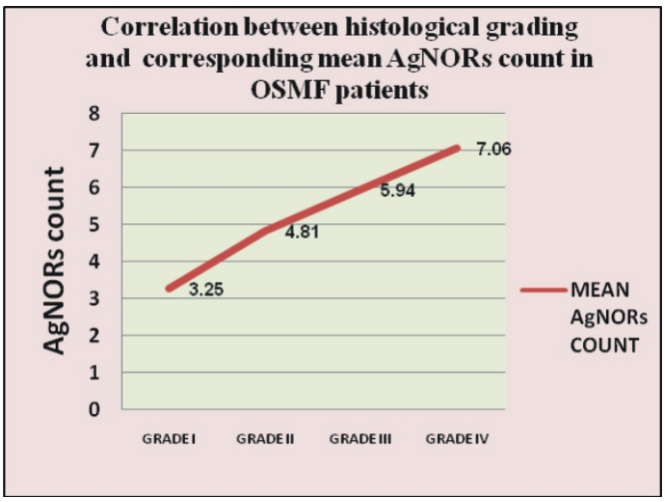


CHART 6(Table g)

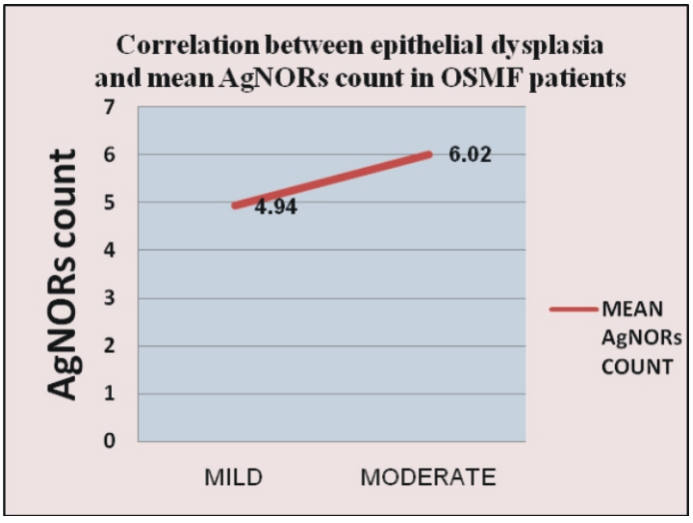


Figure 1 : Photomicrograph of oral submucous fibrosis showing atrophic epithelium, subepithelial band of chronic inflammatory cells, dense connective tissue. (10X, H & E stain)

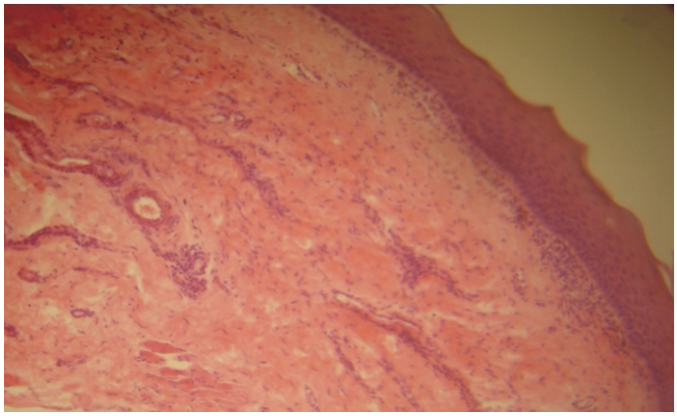


Figure 2 : Photomicrograph of oral submucous fibrosis showing hyperplastic epithelium, sub epithelial chronic inflammatory cells and dilated blood vessels in connective tissue. (10X H & E stain)

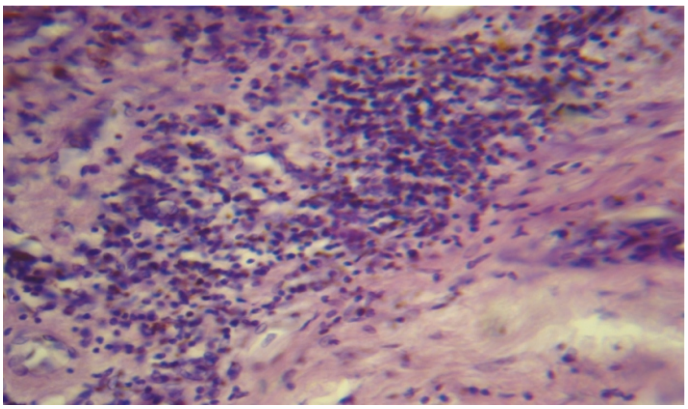


Figure 3 : Photomicrograph of oral submucous fibrosis showing focal collection of chronic inflammatory (40X, H & E stain)

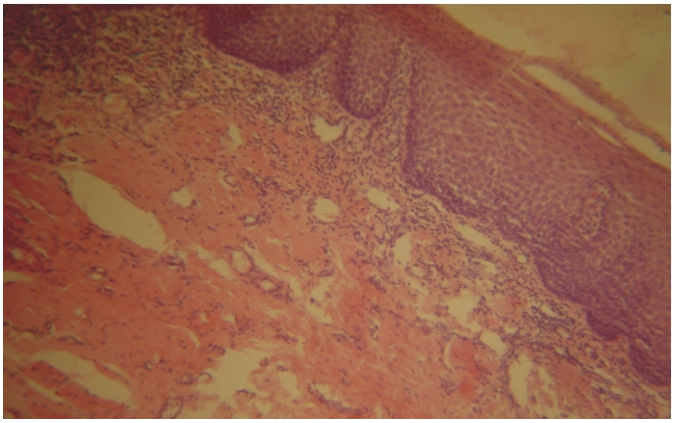


Figure 4 : Photomicrograph of oral submucous fibrosis showing dilated blood vessels, hyperplastic epithelium and diffuse chronic inflammatory cells. (10X, H & E stain)

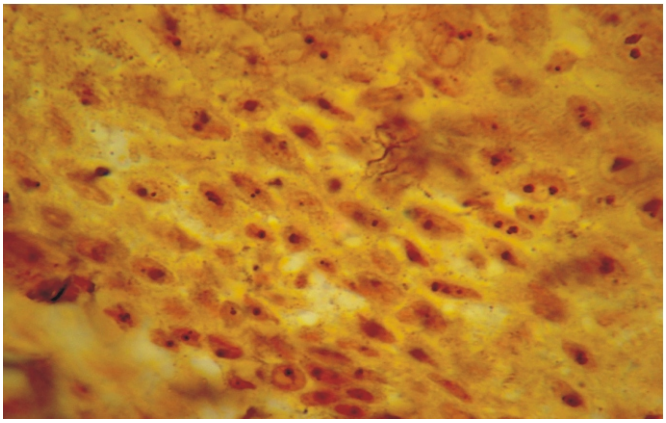


Figure 5 : Photomicrograph of oral submucous fibrosis showing 2 -3 AgNOR dots in epithelial cells. (AgNOR stain, 100X oil immersion

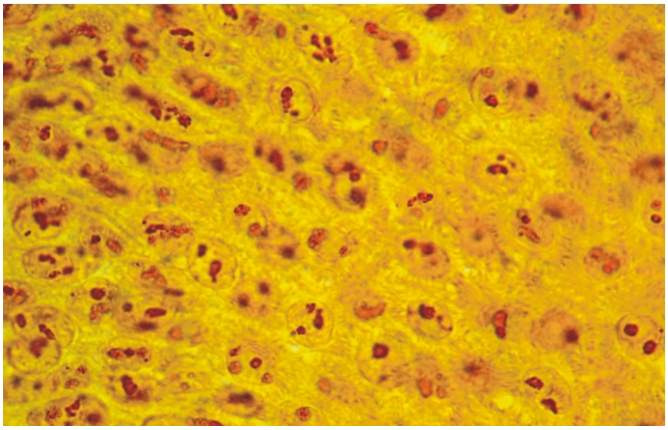


Figure 6 : Photomicrograph of oral submucous fibrosis showing 4 - 5 AgNOR dots in epithelial cells. (AgNOR stain, 100X oil immersion)

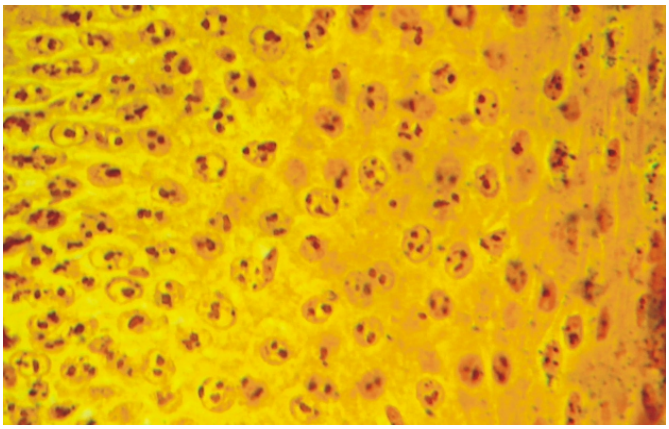
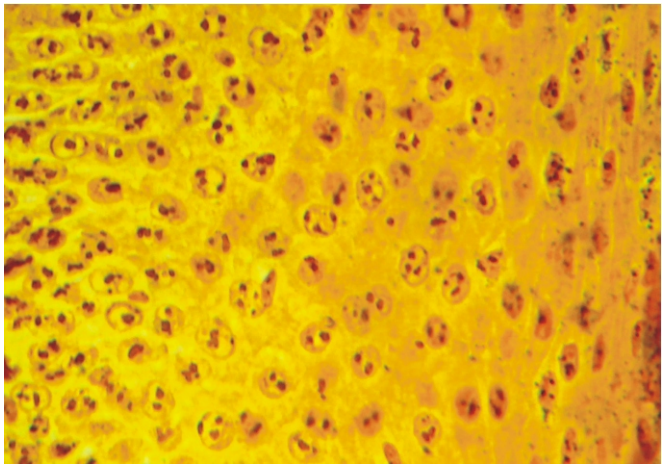


Figure 7 : Photomicrograph of oral submucous fibrosis showing 5 - 6 AgNOR dots in epithelial cells. (AgNOR stain, 100X oil immersion



TABLES :Table a : Distribution of OSMF patients according to age and sex(CHART 1)

GENDER	TOTAL	AGE GROUP					
		0 -9	10 - 19	20 - 29	30 - 39	40 - 49	50 - 59
MALE	22	0 (0%)	1 (45.45%)	6 (27.27%)	8(36.36%)	3 (13.63%)	4 (18.18%)
FEMALE	8	0 (0%)	0 (0%)	1 (12.5%)	1 (12.5%)	3 (37.5%)	3 (37.5%)
TOTAL	30	0 (0%)	1 (3.33%)	7 (23.33%)	9 (30%)	6 (20%)	7 (23.33%)

Mean age : Males 36.27yrs 9.63
Females - 43.29 yrs11. 11

Table b :Correlation between histological grade and mean mouth opening (CHART 2)

Histological Grade	Mean Mouth Opening (cms)
GRADE I	4.83
GRADE II	3.21
GRADE III	2.35
GRADE IV	2.16

Table C : Correlation between clinical staging and epithelial findings (CHART 3)

CLINICAL STAGING	TOTAL	KERATINIZATION		ATROPHIC	HYPER PLASTIC
		PARA	ORTHO		
STAGE I	3	3 (100%)	0 (0%)	2 (66.66%)	1 (33.33%)
STAGE II	5	4 (80%)	1(20%)	3 (60%)	2 (40%)
STAGE III	16	11 (68.75%)	5 (31.25%)	13(81.25%)	3 (18.75%)
STAGE IV	6	5 (83.33%)	1 (16.66%)	6 (100%)	0 (0%)
TOTAL	30	23 (76.66%)	7(23.33%)	24 (80%)	6(20%)

Table D : Correlation between clinical staging and epithelial dysplasia. (CHART 4)

CLINICAL STAGING	TOTAL	EPITHELIAL DYSPLASIA					
		MILD		MOD.		SEVERE	
STAGE I	3	0	(0%)	0	(0%)	0	(0%)
STAGE II	5	1	(20%)	0	(0%)	0	(0%)
STAGE III	16	4	(25%)	0	(0%)	0	(0%)
STAGE IV	6	1	(16.66%)	2	(33.33%)	0	(0%)
TOTAL	30	6	(20%)	2	(6.66%)	0	(0%)

Table E : Correlation between clinical staging and corresponding mean AgNORs count in OSMF patients (CHART 5)

CLINICAL STAGING	MEAN AgNORs COUNT	P value (t test)
STAGE I	4.81	0.000 (S)
STAGE II	4.9	0.027(S)
STAGE III	5.36	0.001 (S)
STAGE IV	6.06	0.045 (S)

S- Significant

Table F : Correlation between histological grading and corresponding mean AgNORs count in OSMF patients (CHART 6)

HISTOLOGICAL GRADING	MEAN AgNORs COUNT	P value (t test)
GRADE I	3.25	0.000 (S)
GRADE II	4.81	0.039 (S)
GRADE III	5.94	0.046 (S)
GRADE IV	7.06	0.001(S)

S- Significant

Table G : Correlation between epithelial dysplasia and mean AgNORs count in OSMF patients. (CHART 7)

EPITHELIAL DYSPLASIA	MEAN AgNORs COUNT	P value(t test)
MILD	4.94	0.0001(S)
MODERATE	6.02	

S- Significant

4.Discussion

OSMF, a crippling disease of the oral mucosa, evokes the dental professionals in different parts of the world. Its occurrence in various parts of India, South Africa and among Indian emigrants has been reported in dental literature. The rate at which oral precancerous and cancerous lesions are spreading like an epidemic is alarming. The prevalence of oral precancerous lesions is much higher than that of oral cancer and these lesions provide useful clinical markers for oral cancer. Immunological and biochemical alterations in the sera of such patients can help not only in early diagnosis, appropriate treatment but also as indicators of prognosis, as the disease progresses[23]. The present study is undertaken to correlate clinical stage and histological grade with AgNORs count and tried to predict the biological behavior of the disease in 30 cases of OSMF. Of the 30 cases of OSMF studied, 73.33% cases were males and 26.77% were females. A literature survey shows wide variation in age sex distribution of OSMF. Some of the epidemiological survey shows female predominance in the occurrence of this entity, Murti et al [24], Laskaris et al [25] and Caniff et al [26]. A male predominance in OSMF cases were shown by Sinor et al [27], Tinky Bose and Anita Balan [28]., Kirankumaretal [29], Pindborg JJ and SirsatM[30].

R.Maher et al noticed that mouth opening is progressively reduced as the disease become severe and more intra oral sites were involved. A number of disease related factors such as, stiffness of the oral mucosa, submucosal bands, submucosal collagen deposits extending deep to muscle, intra oral location and extend of diseases, and pain and exacerbation of ulcers may contribute to the difficulty in mouth opening[30].This findings also confirmed by Haider SM et al (2000)31.In our study we also found a progressively decreased in mouth opening as the severity of disease is increased.

In our study we found keratinization in all the cases where as in Pindborg JJ and Sirsat SM [3], it was found in 64% of all the cases.

Oral submucous fibrosis shows characteristic histopathologicalfetures consisting of an atrophic epithelium with juxtaepithelial hyalinization and collagen of varying density. Pindborg in his study of 53 biopsies found atrophy of the epithelium in 71.4% of the OSMF patients.32 81.25% of all clinical stage III and all the clinical stage IV cases in our study showed epithelial atrophy.

In our study, we found epithelial dysplasia in 26.66% of cases which is slightly higher than Pindborg JJ and Sirsat SM [3], who found dysplasia in 11.2% of OSMF patients, andmuch lower than R. Maher et al [30]who found dysplasia in 48.6% of OSMF patients. In agreement to these Murti et al [33] who found epithelial dysplasia in OSMF vary from 7 26%. In our study, mild dysplasia was found in 20% cases of OSMF which is slightly lower than R Maher et al[30] who found mild dysplasia in 28.3% cases of OSMF, and much lower thanKiran Kumar et al [29], who found 42% of cases of OSMF. In the present study, moderate dysplasia was in 6.66% cases of OSMF which is slightly lower than R Maher et al [30]who found moderate dysplasia in 10.8% cases of OSMF and much lower than Kiran Kumar et al [29], who found moderate dysplasia in 52% cases of OSMF. We did not find severe dysplasia in our study in contrast to thisKiran Kumar et al [29]found 2% cases with severe dysplasia in OSMF and R. Maher et al[30]who found 9.2% cases with severe dysplasia in OSMF. We did not have any case of OSMF with features of malignat transformation.

Silver staining is a useful tool for the study of both the structure of nucleolus and of the variation of the nucleolar activity Howell WM et al, [34]. Quantitative and qualitative changes in AgNORs may provide useful information about nucleolar activity in hyperplastic and neoplastic conditions Allision et al, [35].Malignant tumors have a significantly higher mitotic rate than their benign counterparts Bryan et al, [36]. The relationship between AgNORs count and cellular proliferation has been studied using immunohistochemistry. These studies used antisera to Ki 67, PCNA, and bromodeoxyuridine in order to assess the degree of proliferative activity. In all the cases they demonstrated that greater the proliferation activity, higher the AgNORs counts[31]. The number of NORs in the nuclei may reflect the state of activation of nuclei or even malignancy [37]. The use of AgNORs technique in tumor histopathology has been made possible primarily due to the work of Crocker 1987 and Egan 1988.

The result of the present study shows clearly indicate significant increases in the mean AgNORs count from lower grade to higher grade of OSMF, which is Similar to the findings of R.Rajenderan et al and SM Nair [18].

Higher AgNORs count was also found in tissue showing dysplasic changes. These findings are similar to the findings of Rajendran R & Nair SM [18].

5.Conclusion

In our study we noted, OSMF was more prevalent in males (73%) than females (27%) (M:F = 3: 1) and the mean age male (36.27yrs) was significantly less compared to females (43.29yrs), (p 0.009). Mouth opening is decreased as histological grade of OSMF advances, (p - 0.001). Keratinization was seen in all (100%) cases of OSMF. Atrophic epithelium is significantly (p - 0.039) Increased as clinical stage advances. Significantly increased mAgNORs count in OSMF as the clinical stage and histological grade advances. (p< 0.05). Significantly increased mAgNORs count in moderate dysplasia than mild dysplasia, (p 0.001).

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