

ORIGINAL ARTICLE

Estimation of argyrophilic nucleolar organizer regions in different grades of oral submucous fibrosis

KL Girish, KL Kumaraswamy¹, Usha Balan², Maji Jose³

Department of Oral Pathology, Sree Mookambika Institute of Dental Sciences, Kulasekharam, Tamil Nadu, ¹Department of Oral Pathology, Farooqia Dental College, Mysore, ²Department of Oral Pathology, KMCT Dental College, Calicut, Kerala, ³Department of Oral Pathology, Yenepoya Dental College, Mangalore, Karnataka, India

Address for correspondence:

Dr. KL Girish,
Department of Oral Pathology and Microbiology,
Sree Mookambika Institute of Dental Sciences,
V.P.M. Hospital Complex, Kulasekharam,
Kanyakumari District - 629 161, Tamil Nadu, India.
E-mail: klgowda@yahoo.com

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ABSTRACT

Context: Assessment of potential for malignant transformation of oral submucous fibrosis (OSF) through clinical or light microscopic examination of hematoxylin and eosin stained tissue sections is not totally satisfactory. The search is for such a tissue marker that will differentiate those cases of OSF, which carry a higher risk for malignant transformation. During the past few years, numerous workers have validated the usefulness of enumerating argyrophilic nucleolar organizer regions (AgNORs) in predicting the malignant potential of lesions. The present study was carried out to validate the diagnostic potential of this marker. **Objectives of the Study:** Quantitative and qualitative assessment of AgNORs in different grades of OSF and to compare the count of AgNORs in different grades of OSF and normal mucosa. **Materials and Methods:** AgNORs were investigated in tissue specimens from 90 patients diagnosed with different histopathological grades of OSF. AgNORs were identified in tissue specimens stained with silver nitrate, using light microscope. AgNORs were counted as small, large and total count to analyze them both quantitatively and qualitatively. **Statistical Analysis Used:** Results were subjected to statistical analysis for obtaining significance value (*P* value) by unpaired Student's *t*-test. **Results:** The mean total count of AgNORs was 2.464 ± 0.101 , 4.358 ± 0.108 , 3.704 ± 0.106 and 3.279 ± 0.161 in normal mucosa, Grades I, II and III of OSF, respectively. A qualitative difference was observed in the presentation of AgNORs in different grades of OSF. Mean value of small nucleolar organizer regions (NORs) decreased while the mean of large NORs increased as the grade of OSF increased. **Conclusions:** The study findings suggest that AgNORs are increased in OSF and they can serve as a reliable tool adjunct to histopathological diagnosis. Their ease of demonstration and high specificity to cellular proliferation make them the best available histopathological marker in the arsenal of an oral pathologist.

Key words: Argyrophilic nucleolar organizer regions, oral submucous fibrosis, silver staining

INTRODUCTION

Oral submucous fibrosis (OSF) is a peculiar, chronic progressive, insidious, irreversible, crippling disease of the oral cavity characterized by fibrotic change and severe burning sensation with restricted opening of the mouth.^[1-4] It

was first described by Schwartz^[5] which he called "atrophia idiopathica mucosae oris." Later in 1953, Joshi coined the term submucous fibrosis.^[6] It largely affects people of South-East Asia and immigrant population from this area in other parts

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of the world. OSF is a lesion found among people who chew betel nut with or without tobacco and other ingredients.^[7]

Carcinomas of the oral cavity are highly prevalent in India. Oral carcinomas are often preceded by numerous precancerous lesions or conditions, the treatment of which could prevent the progression to malignancy. OSF is one such potentially malignant disorder, with a high risk for cancer development.^[8,9]

During the past few years, numerous workers have validated partly or wholly the usefulness of enumeration of nucleolar organizer regions (NORs) in the nuclei for predicting the malignant potential of lesions. NORs are loops of ribosomal DNA found in nucleolus that can transcribe for ribosomal RNA. NORs are associated with non-histone nucleoproteins which can be identified by silver staining (argyrophilic nucleolar organizer regions [AgNORs]). AgNORs have been correlated to the proliferative activity of lesions and hence may be prognosis related.^[10] The prediction of prognosis using a histological parameter if possible can exert favorable influences clinically.

MATERIALS AND METHODS

The present study included 90 cases of clinically and histopathologically confirmed cases of OSF. Selected cases were further grouped into Grades I, II and III OSF based on clinical and histopathological features. Ten sections of normal buccal mucosa were used as the control group.

Grading of OSF posed a difficult task, as there are many grading systems given by various authors that vary. Hence, our own grading system was followed and only those cases in which the clinical staging correlated with histological grading were selected [Tables 1 and 2].

Table 1: Clinical staging

Stages	Signs and symptoms
I	Mild blanching, burning sensation while taking spicy food and hot fluids, normal mouth opening
II	Obvious blanching, increased burning sensation without stimuli, palpable bands, reduced mouth opening
III	Severe burning sensation, tongue protrusion affected, ulcerative lesions on cheek, thick palpable bands, very little mouth opening

Table 2: Histological grading

Grades	Histopathological features
I	Epithelium shows hyperkeratosis, intra-cellular edema, little basal cell hyperplasia, rete-ridges present
II	Epithelium undergoing atrophy, rete-ridges less prominent, connective tissue showing thickened collagen bundles, less cellularity, fibrosed blood vessels with moderate amount of hyalinization
III	Marked atrophy of epithelium, absence of rete-ridges, connective tissue showing abundant hyalinization, cellularity absent in connective tissue

The tissues obtained were fixed in 10% neutral buffered formalin. From each block, two sections were cut. The sections were standardized by maintaining the thickness at 3 μ . One set of sections were stained by Harris hematoxylin and eosin for histological diagnosis and the other set was stained by the improved method of silver staining technique for nucleolar organizing regions; with a slight modification of the incubation temperature (at 40°C for 20 min). The optimal staining results were arrived at after a pilot study carried out in the department.

Mode of study

All stained sections were examined under a binocular research light microscope. Two observers recorded the findings of the same slides to eliminate the inter-observer differences. Observers were given different code numbers and to avoid errors in interpretation, clinical history was not given before examination. Photomicrographs of sections showing AgNORs were taken initially and the enumeration of AgNORs was standardized. The observers recorded the counting of the same slides at intervals to remove the intra-observer bias.

Enumeration of argyrophilic nucleolar organizer regions

In each of the sections, 100 individual cells were examined from the representative areas of the epithelium. The methodical and systematic quantification of AgNORs was carried out using an oil immersion objective ($\times 100$). AgNORs were counted in each nucleus and those, which appeared as blebs, intra-nucleolar clusters were categorized as large NORs and those which appeared as discrete small dots as small NORs. The summation of both showed the total number of AgNORs in each cell. The AgNORs were counted as per the standardized procedure recommended by Crocker *et al.* and photomicrographs were obtained using a photomicroscope.

RESULTS

Ten cases with normal buccal mucosa that constituted the control group and 90 cases of OSF were considered. Based on clinical and histopathological correlation, the 90 cases of OSF were further divided into three groups representing Grade I, Grade II and Grade III. Staining of AgNORs was carried out^[11] and enumeration of AgNORs was done.^[12] The results obtained were tabulated [Table 3].

DISCUSSION

The occurrence of OSF is not a rare condition in the South Indian population.^[2,13-16] The Indian habit of repeatedly insulting the oral mucosa with very spicy, pungent foods and irritant like supari (areca nut), pan (betel leaves), tobacco (chewed and smoked) over a period of years, nutritional deficiencies,

Table 3: Mean, range and SD of small, large and total AgNORs in normal buccal mucosa and Grade I, Grade II and Grade III OSF

Groups	n	Mean	SD	Range
SN				
Normal	10	1.662	0.050	1.58-1.74
Grade I	30	3.184	0.054	3.10-3.27
Grade II	30	2.418	0.053	2.33-2.50
Grade III	30	1.396	0.061	1.30-1.50
LN				
Normal	10	0.802	0.050	0.72-0.88
Grade I	30	1.174	0.054	1.09-1.26
Grade II	30	1.286	0.053	1.20-1.37
Grade III	30	1.883	0.119	1.58-2.02
Total				
Normal	10	2.464	0.101	2.30-2.62
Grade I	30	4.358	0.108	4.19-4.53
Grade II	30	3.704	0.106	3.53-3.87
Grade III	30	3.279	0.161	2.97-3.52

n: Number of cases, SN: Small nucleolar organizer regions, LN: Large nucleolar organizer regions, SD: Standard deviation, OSF: Oral submucous fibrosis, AgNORs: Argyrophilic Nucleolar organizer regions

genetic abnormalities and autoimmunity have all been incriminated by various authors as causative agents.^[1,2,7,8,17-22]

All the cases of OSF in our study gave a history of betel nut chewing; either in the form of packed pan masala or locally made products involving betel nut, betel leaf, lime and tobacco. When compared to the duration of the habit with severity of signs and symptoms, it showed a positive correlation. The inability to open the mouth and the number of fibrotic bands were more in those patients with a history of long duration of chewing habits.

It is felt that the buccal mucosa and lips are commonly affected at an early stage besides palate and faucial pillars.^[23] The oral mucosa is usually involved symmetrically.^[24] In our study, also the buccal mucosa was found to be affected more severely. The reason for this severity could be due to their habit of keeping the quid in the buccal sulcus for longer duration.

Considerable evidence has accumulated to suggest that OSF is a potentially malignant disorder; probably the atrophied epithelium is conditioned for the positive action of the carcinogens. Though various authors like Pindborg *et al.*,^[8] Wahi *et al.*^[25] have reported the association of OSF with leukoplakia, in our study we did not come across such findings.

The disease covers a wide age range. Various authors believe that young people are usually affected.^[24,26] The patients in our study were in the age group of 18–55 years with the majority of patients (64%) in the age group of 18–38 years, clearly making a case for the younger generation being lured by various harmful addictive habits.

The opinions as regard to sex distribution varied. In our study of 90 cases, there was a male predominance with male to female ratio of 4:1. There is a difference of opinion about predominance in male to female ratio as seen in the studies conducted by Rao,^[21] Pindborg *et al.*,^[24] Gupta *et al.*^[27] whose studies have favored female predominance. Male predominance has been supported by Mukherjee and Biswas,^[19] Wahi *et al.*,^[28] Mani and Singh^[3] and Akbar.^[29]

Histopathology of OSF is characterized by juxta-epithelial hyalinization. The collagen is thick and seen in separate bundles, less number of fibroblasts, fibrosed blood vessels and inflammatory cells are seen. In later stages, the collagen is completely hyalinized and is seen as a smooth sheath, with no separate bundles discernible.^[14,21,22,30-32] Edema is absent, hyalinized areas are devoid of fibroblasts, blood vessels are completely obliterated/narrowed, few inflammatory cells and epithelium shows atrophy.^[7] The histopathological section in our cases did not show much difference from those reported by various authors [Figures 1-4].

NORs are loops of ribosomal DNA found in nucleolus that can transcribe for ribosomal RNA. NORs can be visualized directly by specific methods such as electron microscopy, *in situ*-hybridization, immunolabeling or indirectly by identifying the proteins associated (nucleolar organizer associated proteins [NORAPs]). NORAPs show an affinity to silver stains due to their high electron charge density.^[33] The right choices of fixative, incubation time, reduction of background staining are all intricate steps that have to be synchronized. The interpretation of results depends largely on another factor; counts of silver dots identified as AgNORs.^[12]

In order to analyze the AgNORs both quantitatively and qualitatively, in addition to the total NORs, the count of small and large NORs were also separately recorded. In the present study, the mean of the total count of NORs in normal mucosa was found to be 2.464 ± 0.101 with large being 0.802 ± 0.050 and small being 1.662 ± 0.050 [Figure 5]. The total NORs count is in agreement with investigators such as Rajendran and Nair^[34] and Cabrini *et al.*^[10]

The total count showed a mean of 4.358 ± 0.108 , 3.704 ± 0.106 and 3.279 ± 0.161 in Grades I, II and III of OSF, respectively. It can be observed that there is a slight decrease in the mean of the total count as the grade of OSF increases. However, the statistical analysis of the total count in different grades of OSF showed very high significant values [Figures 6-8].

It was observed that there is a definite increase in the mean total count of AgNORs in different grades of OSF when compared with normal mucosa. This is in accordance with studies conducted by various other investigators. Rajendran and Nair^[34] found the mean AgNOR values to be 5.24 ± 1.23 and 7.26 ± 1.2 in moderately advanced and advanced cases of OSF.



Figure 1: Photomicrograph of normal buccal mucosa (H&E stain, ×100)

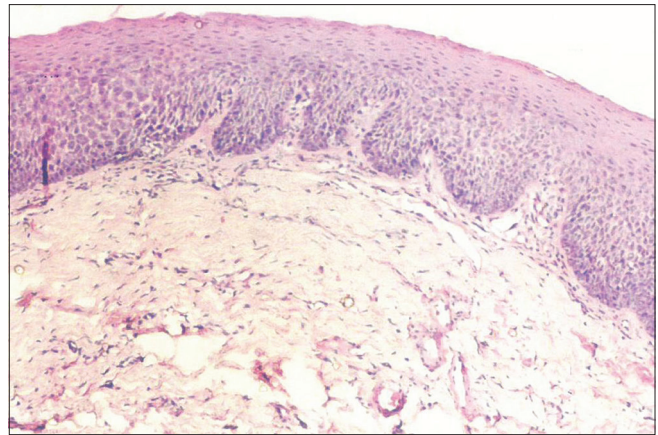


Figure 2: Photomicrograph of grade I oral submucous fibrosis (H&E stain, ×100)

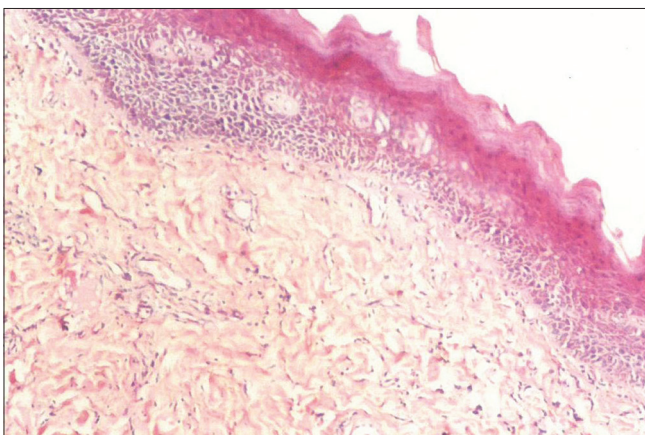


Figure 3: Photomicrograph of grade II oral submucous fibrosis (H&E stain, ×100)

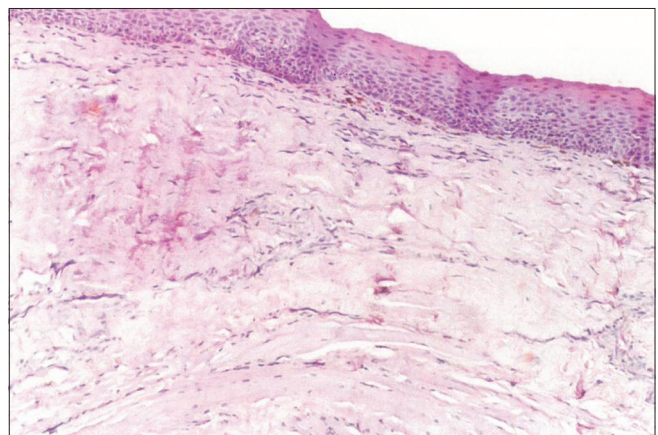


Figure 4: Photomicrograph of grade III oral submucous fibrosis (H&E stain, ×100)

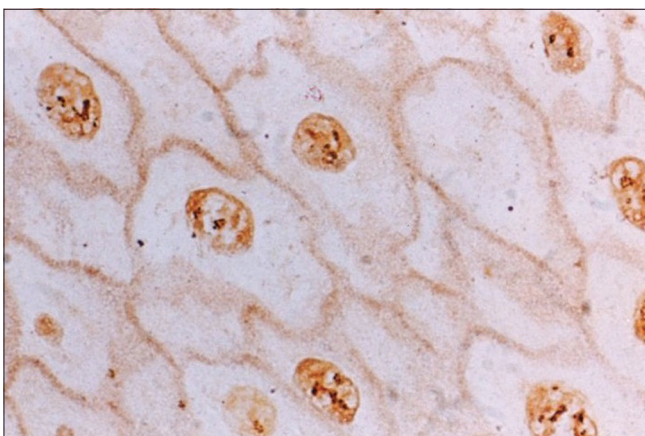


Figure 5: Argyrophilic nucleolar organizer regions in normal buccal mucosa (Silver stain, ×1000)

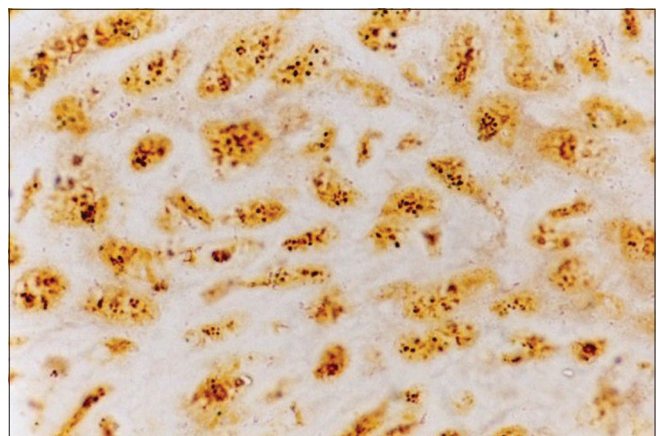


Figure 6: Argyrophilic nucleolar organizer regions in Grade I oral submucous fibrosis (Silver stain, ×1000)

It is said that immediately before and after mitotic division, the NORs disperse and then reaggregate thus leading to an increase in the number of countable AgNORs in the nuclear profiles. The number of countable AgNORs in interphase nuclei is probably related more to their dispersion through the nucleoplasm than to the actual number present.

The AgNOR counts may, therefore, be an index of NORs dispersion rather than the actual number present in the karyotype. Dispersion may in itself reflect the proliferative state of the cells. Before mitosis, the NORs reaggregate and the nucleoli reform. Thus, the AgNOR count rises before and after mitosis.^[35] Thus it is interpreted that the total count

will depend upon the distribution of NORs in the nucleus. As AgNORs represent the cellular proliferative activity, it is expected that there is an increase in the amount of AgNORs in the actively dividing cells.^[10]

In the present study, the qualitative analysis of AgNORs was done taking into consideration the size and presentation of NORs. It is seen that in OSF cases there is a definite difference in the size and presentation of NORs in different grades of OSF. In the qualitative analysis of large and small NORs, the mean of large NORs is found to be 1.174 ± 0.054 , 1.286 ± 0.053 and 1.883 ± 0.119 and mean of small NORs was found to be 3.184 ± 0.054 , 2.418 ± 0.053 and 1.396 ± 0.061 in Grades I, II and III of OSF, respectively [Graph 1].

These results showed a definite change in the pattern of AgNORs as observed in different grades of OSF. It was observed that the size of AgNORs increased as the grade of OSF increased. It is also observed that the NORs tended to cluster as the grades of OSF increased. Due to this counting of NORs posed a problem wherein the intra-nucleolar NORs was so closely present that their count as individual NORs was not possible. These clusters were considered as one large NOR in our study. This could be one of the reasons that there was a decrease in the total count of AgNOR in higher grades of OSF, which is not in accordance with other studies.

Expression of AgNORs depends upon the temperature and the time factor during the staining procedure. In our study, we observed a very high expression of AgNORs, which could be due to the modification done in the staining technique wherein the temperature was maintained at 40°C and staining was carried out for 20 min only.

CONCLUSION

The present study reports an increase in total count of AgNORs in OSF when compared to normal buccal mucosa. A qualitative difference in the form of clustering of NORs also was observed with increasing grades of OSF, resulting in increase in the mean value of large NORs. Therefore, we suggest analysis of AgNORs in OSF may be helpful in identifying cases with higher risk for malignant transformation.

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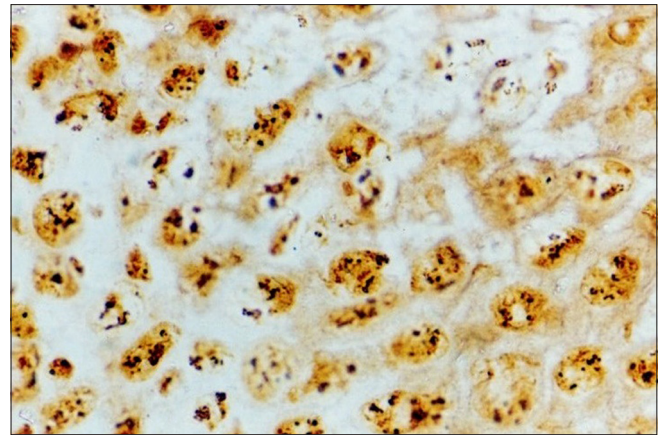


Figure 7: Argyrophilic nucleolar organizer regions in Grade II oral submucous fibrosis (Silver stain, $\times 1000$)

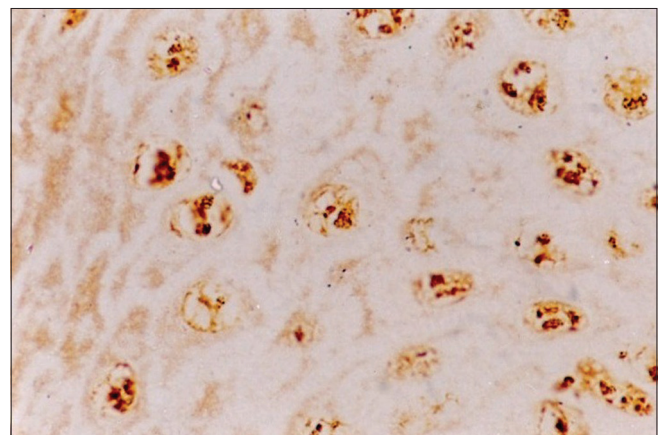
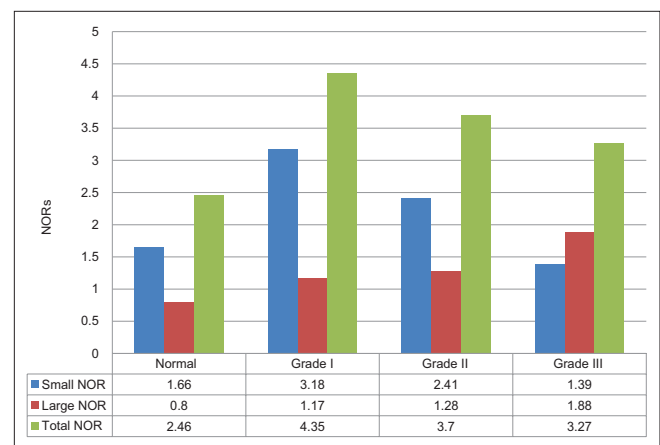


Figure 8: Argyrophilic nucleolar organizer regions in Grade III oral submucous fibrosis (Silver stain, $\times 1000$)



Graph 1: Comparison of mean small nucleolar organizer regions, large nucleolar organizer regions and total nucleolar organizer regions among different groups

Conflicts of interest

There are no conflicts of interest.

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