Original Article

Prognostic potential of AgNORs in oral submucous fibrosis

Sanjay Murgod, Girish Hemadal Channabasaviah, Dyamenahalli Malleshappa Shivamurthy, Lingappa Ashok, Savita Jangal Krishnappa

Department of Oral Pathology, Rajarajeswari Dental College, Bangalore, Karnataka, India

Corresponding author (email: <smurgod@yahoo.com>)
Dr. Sanjay Murgod, Department of Oral Pathology, Rajarajeswari Dental College, No. 14, Ramohally Cross, Kumbalgodu, Mysore Road, Bangalore - 560 074, Karnataka, India.

Abstract

Aim and Objective: The role of prognosis cannot be stressed enough, especially when it comes to potentially malignant lesions. The argyrophilic nucleolar organizer regions (AgNORs), which is simple and cost-effective has been used in diagnostic and prognostic pathologies. This study seeks to identify the nucleolar organizer regions (NORs) in oral submucous fibrosis (OSMF), to correlate the AgNOR count with the histologic grade of OSMF, and to evaluate the prognostic potential of AgNOR. **Materials and Methods:** The sample size consisted of archival paraffin blocks of 35 cases of varying grades of OSMF and 10 cases of squamous cell carcinoma. Normal mucosa samples served as controls for the study. AgNOR staining in accordance with the method of Smith and Crocker was performed and Student's *t*-test was used for statistical analysis. **Results:** The results showed an increase in AgNOR counts with corresponding grades of OSMF, the count being least in normal mucosa and also an increase in AgNOR count with corresponding decrease in differentiation of oral squamous cell carcinoma. **Conclusion:** AgNOR staining is a rapid and inexpensive procedure representing cellular proliferation that can be used to assess the nature of the lesion and therefore, the prognosis.

Key words: *Argyrophilic nucleolar organizer regions, oral submucous fibrosis, potentially malignant disorder, prognosis*

INTRODUCTION

Submucous fibrosis affects any part of the oral cavity, may also involve the pharynx, and is insidious in nature. Paymaster first mentioned the potentially malignant nature of submucous fibrosis and described the occurrence of squamous cell carcinoma in association with submucous fibrosis.[1]

The facts that a high percentage of patients with oral cancer had coexisting submucous fibrosis, that epithelial atypia is present in 13–14% of all cases, and that histologic carcinoma is found in 5–6% of cases without clinical signs of cancer suggest that the disease is a potentially malignant condition.

With regard to the above, varying methods have been employed previously for identifying proliferative cells in tissue sections such as mitotic assessment, DNA fluorocytometry, autoradiographic methods, DNA and RNA applications, *in situ* hybridization, and monoclonal antibodies to identify proliferation-related antigens. The major disadvantages in the above techniques are that they are time-consuming and expensive. Claims that argyrophilic nucleolar organizer regions (AgNORs) are

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significantly increased in malignant cells compared to normal, reactive, and benign neoplastic cells have drawn much attention of late.

Nucleolar organizer regions

Dense nucleolar structure containing ribosomal RNA (rRNA) genes, RNA transcripts, and associated proteins, apparent in thin sections is defined as the chromosomal nucleolar organizer region (NOR).[2] *In situ* hybridization techniques have proved that these sites represent loops of DNA that transcribe to rRNA, later ribosomes, and ultimately proteins.[3] Involvement of NORs in ribosome production and possible qualitative or quantitative modifications in interphase NORs with regard to proliferation or transformation could help in diagnosis or prognosis.[4]

Certain genetic disorders were evaluated by cytogeneticists using NORs for the first time (De La Cruz and Gerald PS, 1981).[5] NORs are seen on the acrocentric chromosomes [13, 14, 15, 21, and 22] and transcription of their genes is thought to play an important role in the production of ribosomes and proteins. A variety of acidic NOR-related proteins can be identified visually due to their argyrophilia. Goodpasture and Bloom developed a two-step method for silver staining NOR-associated proteins (NORAPs) in 1975.[6] Subsequent changes have reduced the technique to a single stage and also more refinements to reduce the problem of nonspecific background staining. The technique has now been transferred to the histopathology laboratory so that it can be used reliably on formalin-fixed, paraffin-embedded tissues. AgNOR analysis does not depend on image analysis and can be easily applied in routine work.[7]

The argyrophilic staining of NORs has practical application in diagnostic pathology for evincing neoplastic potential, prognosis, and aggressiveness of malignant neoplasms.[8] The technique is used to predict the biologic behavior of oral submucous fibrosis (OSMF) in this study. It includes the silver staining of sections from formalin-fixed, paraffin-embedded tissue blocks of OSMF, evaluating silver-binding nucleolar organizer regions (AgNORs) and correlating the data with histologic grading.

The AgNOR method stains NOR-associated proteins: activity condition or indeed malignancy may be shown by the number of nucleolar AgNORs.[9] The advantages of this technique are its simplicity, reliability, and specificity. Numerous studies have shown AgNOR count to be a rapid and easily reproducible method permitting clear distinction between benign and malignant cells.[10]

Methods of visualization

NORs can be visualized directly by such specific methods as electron microscopy, *in situ* hybridization, and immunolabeling or indirectly by identifying the proteins associated [nucleolar organizer associated proteins (NORAPs)]. The argyrophil method is directed against the NORAPs and is the most commonly used. Due to their high-electron charge density, NORAPs, especially nucleoli, show affinity to silver stains. Affinity may also be expressed due to the presence of specific bonds and biochemical configuration of the NORAPs, for example, due to carboxyl and phosphate moieties.

The AgNOR method

The acidic AgNOR proteins were first localized at the electron microscope level by Hernandez-Verdun *et al*. using the usual three-step method of Goodpasture and Bloom (1975). Subsequently, Howell and Black[11] suggested a one-step technique to reduce time. This reaction primarily uses gelatin as a protective colloid to control silver staining and consists of mixing silver nitrate and formic acid in optimal proportions. Various modifications of this technique have been proposed and utilized; preincubation with glycine to reduce incubation time, substitution of gelatin with polyethylene glycol as a protective colloidal developer, primarily used to reduce background staining. Celluloid in film has also been used to reduce nonspecific deposits.[12] Internal controls, period of incubation, control of staining time, and reduction of background deposits are integral to the process.

This study seeks to compare the AgNOR count in histologic grades of OSMF with normal mucosa and also with different grades of oral squamous carcinoma so as to know the possibility of using the AgNOR count in the prognosis of submucous fibrosis. The study differs from the earlier one by Rajendran R (1992) with regard to section thickness and also the comparison is limited to histologic grading only.

Aims and objectives

- To identify the NORs in OSMF
- To correlate the AgNOR count with the histologic grade of OSMF
- To evaluate the potential of AgNOR as a prognostic indicator.

MATERIALS AND METHODS

This study was undertaken by retrieving the archival paraffin blocks of the cases of OSMF over a period of 10 years from the Department of Oral Pathology and Microbiology, Bapuji Dental College and Hospital, Davangere, Karnataka, India. The study included 35 histologically confirmed cases of OSMF and 10 cases of squamous cell carcinoma. Ten samples of normal oral mucosa constituted the controls. The paraffin blocks were sorted out, sections prepared, and stained with hematoxylin and eosin, Van Gieson's stain, and silver colloid stain.

Modified procedure of Smith and Crocker was used for AgNOR staining. 5μ sections from routinely processed paraffin blocks were dewaxed in xylene (3–5 min), and then rehydrated through ethanols to distilled water. Gelatin was dissolved in 1 g/dL aqueous formic acid at a concentration of 2 g/dL to prepare the AgNOR solution, which was mixed [1:2 volumes] with 50-g/dL aqueous silver nitrate solution to obtain the final working solution. The tissue sections were immersed in this solution at room temperature in a dark place for 40 min. Distilled water was used to wash the silver colloid solution, sections were dehydrated through ethanols to xylene, and then mounted in DPX.

Counting procedure

In all specimens, 100 cells were selected randomly and the AgNORs were identified as black dots (100x magnification). The number of individually discernible and separate black dots in each nucleus was noted and the average for each case was computed. In cases where two or more dots were not individually discernible, the score was counted as one.

Histologic grading of oral submucous fibrosis

The OSMF cases were graded according to the grading given by Pindborg and Sirsat.[1]

Statistical analysis

Statistical significance of the values between the different groups was determined by using the Student's *t*-test.

RESULTS

AgNORs were studied in 35 cases of OSMF and 10 cases of squamous cell carcinoma (5 well differentiated and 5 poorly differentiated) [Figures 1-6, and Graph 1]. Ten cases of normal oral mucosa constituted the control group [Figure 7]. The 35 cases of OSMF were further graded histologically [Figures 8-15 and Graph 2] as very early (grade 1), early (grade 2), moderately advanced (grade 3), and advanced (grade 4).

In all specimens, 100 cells were selected randomly and the AgNORs were clearly visible as black dots in the nuclei and the nuclei exhibited a light brown hue. A bar graph showing the mean AgNOR count/nucleus in each category is shown.

In normal mucosa, the mean AgNOR count was 1.57 ± 0.21. The mean AgNOR counts in the moderately advanced and advanced stages of OSMF were higher than those in the very early and early cases. A nonsignificant comparison was noted between early

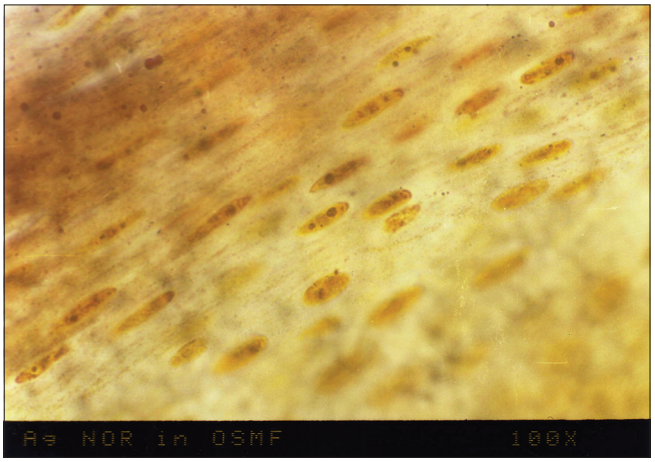
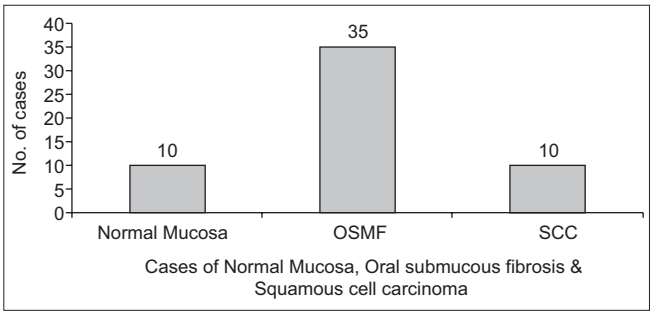
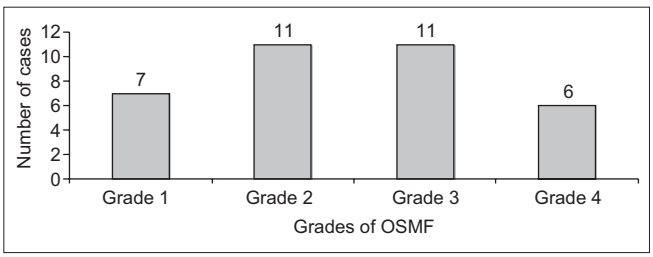


Figure 1: AgNORs in Grade 1 OSMF (100x)



Graph 1: Distribution of cases taken for the study



Graph 2: Gradewise distribution of oral submucous fibrosis cases

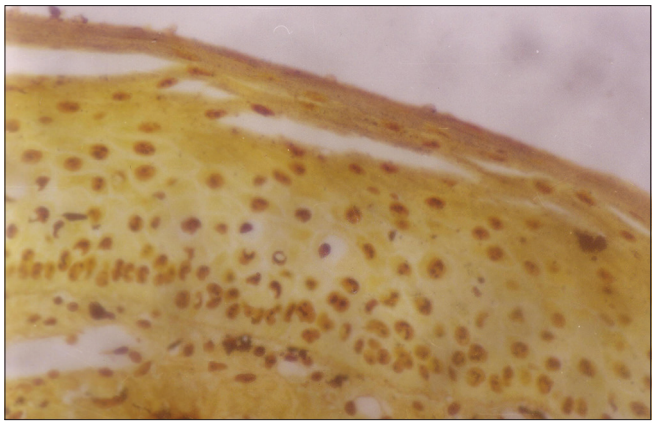


Figure 2: AgNORs in Grade 2 OSMF (40x)

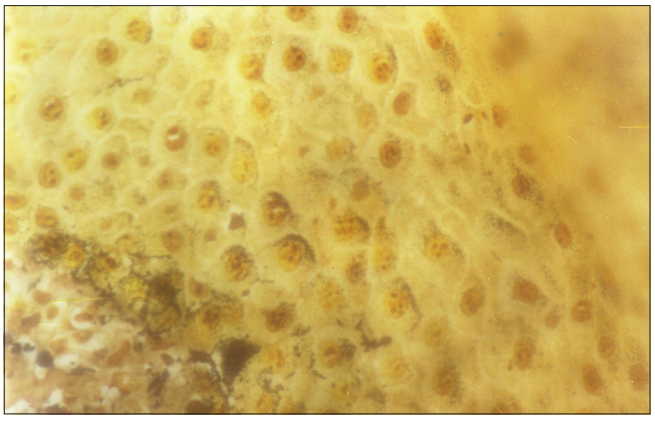


Figure 3: AgNORs in Grade 3 OSMF (40x)

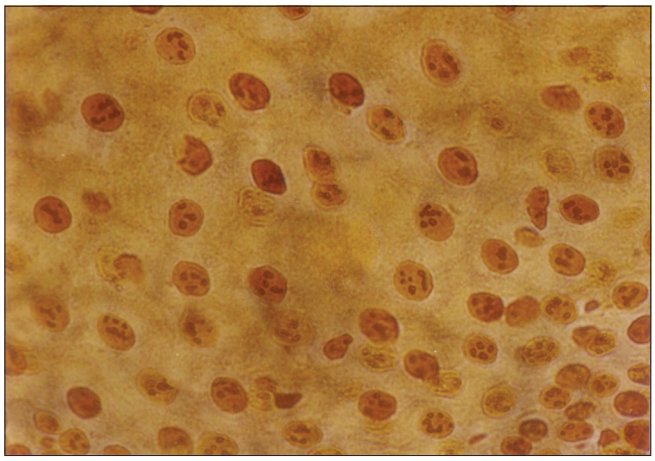


Figure 4: AgNORs in Grade 4 OSMF (40x)

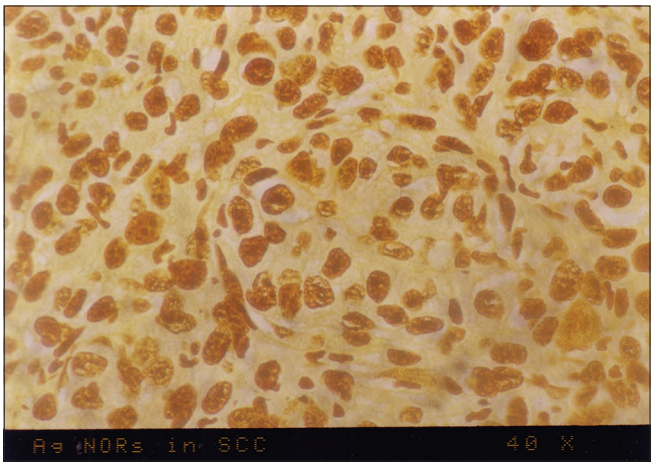


Figure 5: AgNORs in WDSCC (40x)

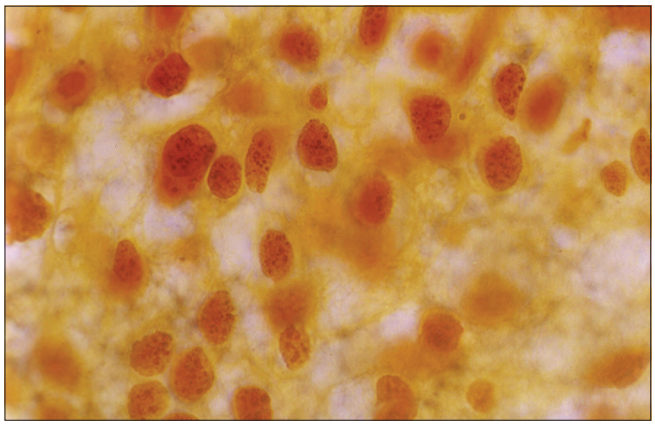


Figure 6: AgNORs in PDSCC (100x)

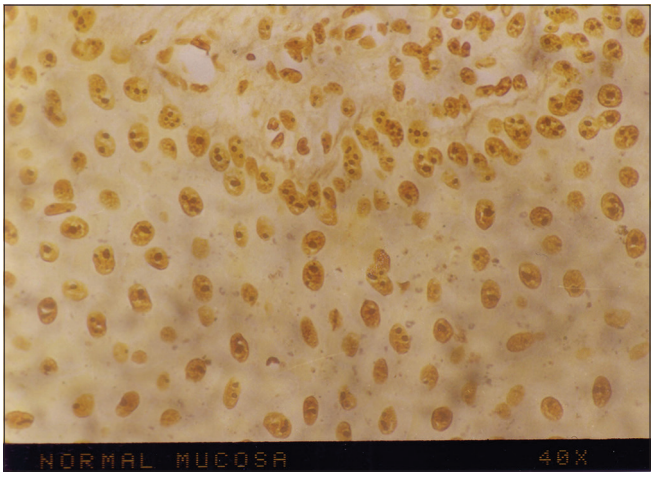


Figure 7: AgNORs in normal mucosa (40x)

OSMF and moderately advanced OSMF and between moderately advanced OSMF and advanced OSMF cases. Comparison of the different groups and the corresponding ranges and mean counts of AgNORs are given in Table 1. The mean AgNOR count was highest in poorly differentiated squamous cell carcinoma and lowest in very early submucous fibrosis ($P < 0.001$, $t = 27.84$). The comparisons of the corresponding t and P values between the rest of the categories were significant [Table 2].

Grading of oral epithelial dysplasia in OSMF and the corresponding AgNOR counts are given in Table 3. Comparison of the scored AgNOR counts and levels

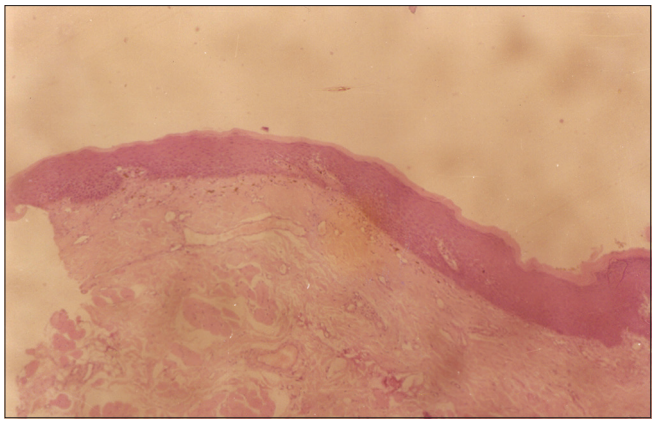


Figure 8: OSMF—H and E—Grade 1 (5X)

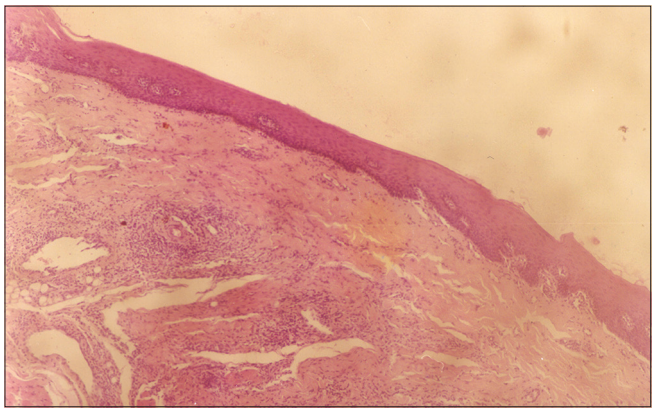


Figure 9: OSMF—H and E—Grade 2 (5X)

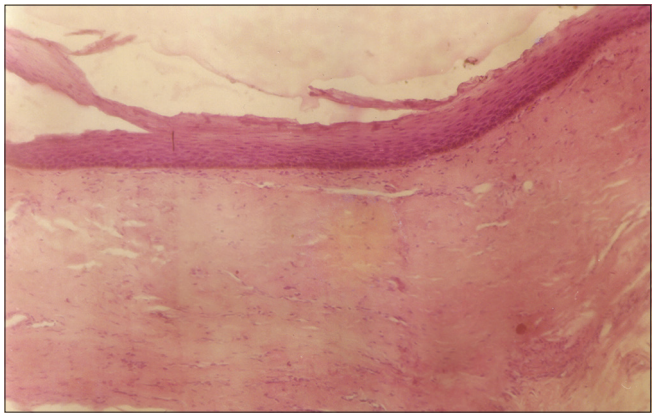


Figure 10: OSMF—H and E—Grade 3 (10X)

of significance between different grades of epithelial dysplasia with normal oral mucosa is given in Table 4.

DISCUSSION

Early detection seems to significantly decrease the morbidity rate in oral cancer. Aberrations in proliferation kinetics of a cell are a crucial factor in the progression of tumors.[13] NORs are useful in the determination of cellular activity and application in neoplastic lesions.[14] Proliferation rates may be assessed by AgNORs on cytologic or histologic preparations.[15] Anticipating survival in human neoplasia is aided by the use of AgNOR number, pattern, and distribution.[16] Differences in the number of visualized AgNORs are based on transcription activity level, chromosome number related to the NORs in karyotype, and phase of cellular cycle as the nucleolus disperses before mitosis and reorganizes later on.[17] Information about the velocity of cell proliferation rate is provided by the number of AgNORs as compared to many proliferation markers that indicate only whether cells are dividing or not.[18] AgNORs have been applied in tumor histopathology

Table 1: Comparison of different groups and corresponding ranges and mean AgNOR counts

Category	Range	Mean±SD
Normal mucosa (*n*=10)	1.36-1.97	1.57±0.21
Grade 1 OSMF (*n*=7)	2.03-3.34	2.32±0.45
Grade 2 OSMF (*n*=11)	2.15-3.89	3±0.6
Grade 3 OSMF (*n*=11)	2.11-6.54	3.59±1.29
Grade 4 OSMF (*n*=6)	3.11-5.98	4.5±0.93
Well-differentiated SCC (*n*=5)	7.12-8.45	7.55±0.55
Poorly differentiated SCC (*n*=5)	8.68-9.62	9.26±0.37

SCC=Squamous cell carcinoma

Table 2: Comparison of AgNOR counts between normal mucosa, grades of OSMF, well-differentiated and poorly differentiated SCC and their corresponding *t* and *P* values

Comparison	*T*	*P*
Normal vs grade 1	4.58	<0.001
Normal vs grade 2	7.02	<0.001
Normal vs grade 3	4.81	<0.001
Normal vs grade 4	9.52	<0.001
Grade 1 vs grade 2	2.57	<0.05
Grade 1 vs grade 3	2.47	<0.001
Grade 1 vs grade 4	5.41	>0.2 NS
Grade 2 vs grade 3	1.37	<0.01
Grade 2 vs grade 4	4.03	>0.2 NS
Grade 3 vs grade 4	1.50	<0.001
Normal vs WDSCC	31.06	<0.001
Normal vs PDSCC	51.78	<0.001
Grade 1 vs WDSCC	18.07	<0.001
Grade 1 vs PDSCC	27.87	<0.001
Grade 2 vs WDSCC	14.4	<0.001
Grade 2 vs PDSCC	21.3	<0.001
Grade 3 vs WDSCC	6.46	<0.001
Grade 3 vs PDSCC	9.01	<0.001
Grade 4 vs WDSCC	6.46	<0.001
Grade 4 vs PDSCC	10.74	<0.001
WDSCC vs PDSCC	5.71	<0.001

WDSCC=Well-differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma

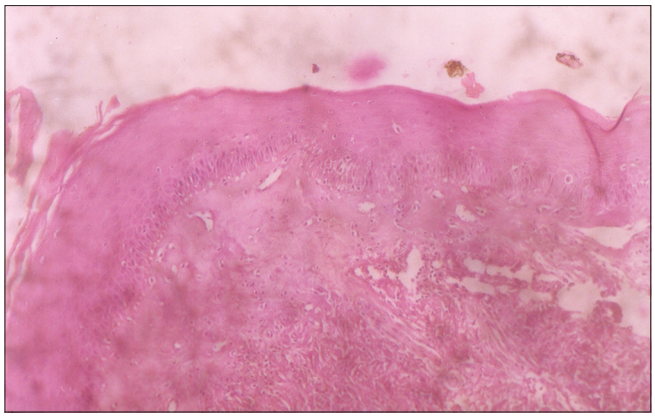


Figure 11: OSMF—H and E—Grade 4 (10X)

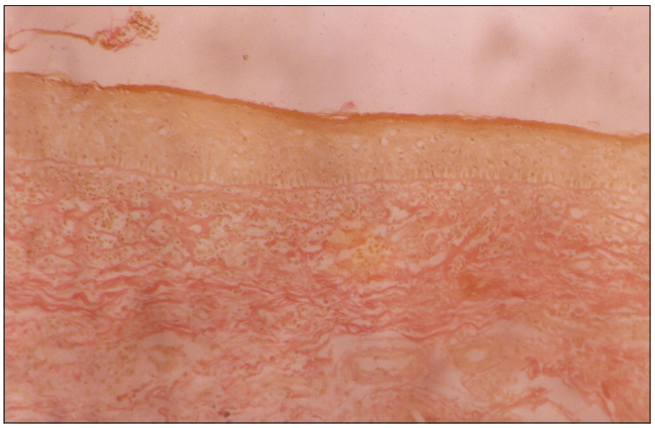


Figure 12: OSMF—Van Gieson's stain—Grade 1 (10X)

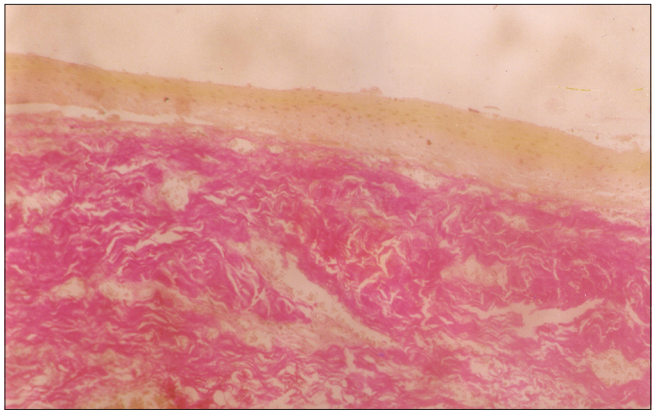


Figure 13: OSMF—Van Gieson's stain—Grade 2 (10X)

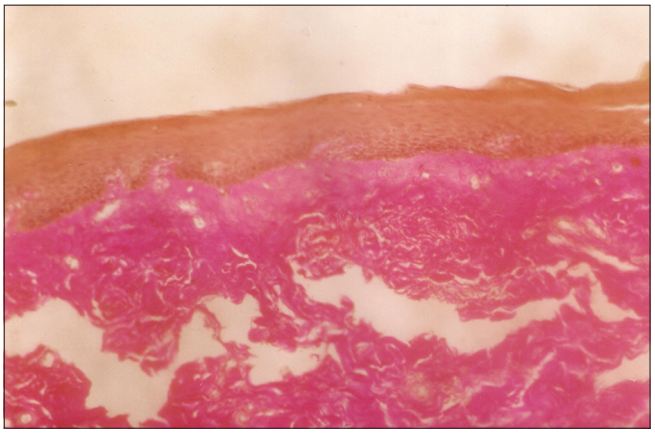


Figure 14: OSMF—Van Gieson's stain—Grade 3 (10X)

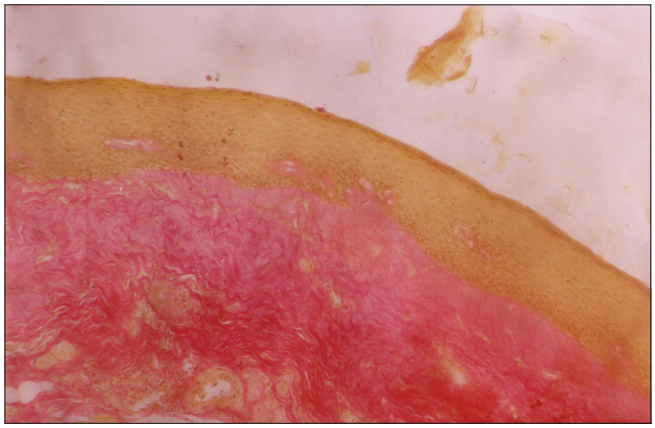


Figure 15: OSMF—Van Gieson's stain—Grade 4 (10X)

for assessing tumor growth and malignant potential, difference between benign and malignant lesions, prognosis, and also recurrence of lesions.[19]

AgNOR numbers are related to cell proliferation and metabolic activity of cells.[20] A higher count of AgNORs may be due to active cell proliferation states, transcriptional activity, and increased cell ploidy.[21] AgNORs are not characteristic of malignancy as such but demonstrate metabolic changes with regard to malignant transformation.[22] The impaired nuclear activity in proliferating cells results in a higher AgNOR count, which relates to the lesion's malignant potential.[23] Quantification of interphase AgNORs is useful in the assessment of cell kinetics.[24] Determination of ploidy, proliferation activity, and metabolic cell activity not associated with proliferation by AgNOR count has been described as a good method.[25]

The mean AgNOR counts in our study were consistent with the findings of Rajendran and Nair (1992)[26] in submucous fibrosis patients in terms of the levels of significance. AgNORs detect cellular alterations before morphologic expression.[27] The number of NORs expressed in a tissue is related to the rate of cellular proliferation, differentiation and its malignant transformation.[28,29]

A higher frequency and scattered dispersion of nucleolar organizer regions NORs are reported in malignancies. The higher counts in tissue section are

Table 3: Comparison between different grades of epithelial dysplasia in OSMF and corresponding AgNOR counts

Grade of epithelial dysplasia	OSMF grade I		OSMF grade II		OSMF grade III		OSMF grade IV		Total cases	Pooled AgNOR count
	No. of cases	AgNOR count (range)	No. of cases	AgNOR count (range)	No. of cases	AgNOR count (range)	No. of cases	AgNOR count (range)		
Absent	1	2.08	1	2.79	1	2.57	-	-	3	2.48±0.36
Low	4	2.03-2.24	6	2.15-2.81	4	2.11-2.83	3	3.11-5.98	17	2.82±1.04
Medium	2	2.23-3.34	4	3.32-3.89	6	3.41-6.54	3	4.13-4.52	15	4.02±0.92

probably due to both increased transcriptional activity and the nucleolus dispersion.[30]

Comparison of AgNOR with degree of epithelial dysplasia

A significant correlation was noted between the control group and the different grades of dysplasia [Table 4].

Only one nonsignificant correlation was noted between the group showing no dysplasia and the one with low dysplasia ($P > 0.4, t = 0.95$).

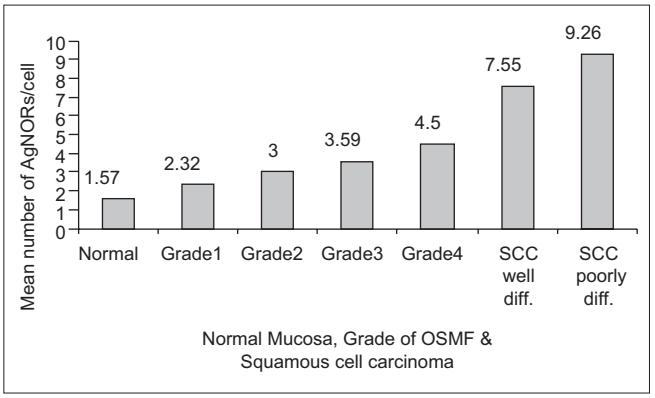
The comparisons of the corresponding t and P values between the rest of the categories were significant (normal vs no dysplasia: $t = 5.7$, $P < 0.001$; normal vs low dysplasia: $t = 3.71$, $P < 0.01$; normal vs medium dysplasia: $t = 8.23$, $P < 0.001$; no dysplasia vs medium dysplasia: $t = 2.82$, $P < 0.02$ and low dysplasia vs medium dysplasia: $t = 3.47$, $P < 0.01$] [Table 4].

Since AgNOR number expressed in a tissue is related to the rate of cellular proliferation, differentiation and malignant change,[28,29] it could be possible that a high AgNOR score would concur with a poor prognosis.

Vuhahula *et al*. (1995)[31] noted increased AgNOR counts with higher histologic grades in their study on the biologic behavior of salivary adenoid cystic carcinoma. They suggested the potential ability of the AgNOR count to portray the biologic behavior of adenoid cystic carcinoma. These findings are consistent with our findings where we noted an increased AgNOR count with higher histologic grades in OSMF [Table 1 and Graph 3]. Variations in AgNOR counts may be attributed to differences in section thicknesses, tissue fixation times, and the judgment of the investigator.[32] In our study, we noticed a progressive increase in the mean AgNOR count in the histologic grades of OSMF cases and with respect to the degree of dysplasia. These findings may

Table 4: Comparison of AgNOR counts between different grades of epithelial dysplasia in OSMF and their corresponding *t* and *P* values

Comparison	*T*	*P*
Normal vs no dysplasia	5.7	<0.001
Normal vs low dysplasia	3.71	<0.01
Normal vs medium dysplasia	8.23	<0.001
No dysplasia vs low dysplasia	0.95	>0.4 NS
No dysplasia vs medium dysplasia	2.82	<0.02
Low dysplasia vs medium dysplasia	3.47	<0.01



Graph 3: Distribution of AgNORs in normal mucosa, oral submucous fibrosis and squamous cell carcinoma

be suggestive of a poor prognosis in concurrence with other studies.[16,33]

However, further studies with comparison of clinical grading and recalls with greater number of patients are required to substantiate these findings and also to obtain a more comprehensive result.

CONCLUSION

AgNOR staining is a rapid, efficient, and inexpensive procedure and provides useful information regarding cellular proliferation. The fact that higher grades of OSMF and poorly differentiated squamous cell carcinoma show higher AgNOR counts may be useful in assessing the aggressive nature of the lesion and hence, the prognosis. Further prospective studies with

more number of cases including patient recall are required to substantiate these findings.

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Conflicts of interest

There are no conflicts of interest.

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