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# Silver binding nucleolar organizing regions in Oral Leukoplakia and Oral Squamous Cell Carcinoma – a clinical histopathological study

**Background:** Nucleolar organizing regions (NORs) are associated with proliferative activity and represents as a diagnostic aid in several neoplastic entities.
**Objective**: The purpose of this study was to determine and compare the AgNOR (Silver binding nucleolar organizing region) count in healthy oral mucosa, oral leukoplakia and oral squamous cell carcinoma (OSCC).
**Methods:** The biopsied tissue specimens were obtained from 15 cases of oral leukoplakia, 15 cases of oral squamous cell carcinoma and 20 cases of normal healthy epithelium followed by staining with silver-nitrate method for the nucleolar organizing regions. Further AgNOR dots were counted by the criteria under 100X oil immersion objective.
**Results:** The mean AgNOR count showed a gradual increase from normal epithelium to leukoplakia to OSCC which was found to be statistically significant.
**Conclusion:** The AgNOR method can be used to provide information on the malignant potential in premalignant lesions and aggressiveness of the malignant lesions.

***Key words*:** normal epithelium; oral leukoplakia; (oral squamous cell carcinoma) OSCC; AgNORs (silver binding nucleolar organizing regions)

## INTRODUCTION

Nucleolar organizing regions (NORs) are loops of DNA, on the short arm of acrocentric chromosome that are associated with ribosomal-RNA activity, protein synthesis and cell proliferation {1}. On routine Haematoxylin and Eosin stained sections, it is easy to identify epithelial dysplasia but its subjective variability and reproducibility are liable. Hence there is a need to have good, objective, quantifiable, economical and consistently reproducible measure for identification of epithelial dysplasia.
It is suggested that quantification of AgNOR is a valuable parameter in tumor pathology {2}. Studies have shown higher number of AgNOR in malignant lesions such as oral squamous cell carcinoma {2}. The purpose of the present study was to count and compare the mean AgNOR count per nucleus and its pattern of distribution in healthy oral mucosa, oral leukoplakia and OSCC.

## MATERIALS AND METHODS

The study was carried out at the department of oral pathology and microbiology, Bapuji Dental College and Hospital, Davangere, Karnataka, India. The biopsied tissue specimens were obtained from 15 cases of oral leukoplakia, 15 cases of oral squamous cell carcinoma and 20 cases of normal healthy epithelium. All specimens were studied for AgNOR characterization. The sections of 5 micrometer thickness were made of the biopsied specimen followed by silver-nitrate staining method as explained by Bancroft and Gamble {3}. Further AgNOR dots were counted by the criteria suggested by Crocker *et al.,* {4} under 100X oil immersion objectives. In all specimens, 100 cells were selected randomly, individually discernible and the separate black dots/blebs were recorded. Average numbers of dots/blebs were calculated in each nucleus. Then the mean AgNOR value for each sample was calculated followed by the mean for each group. Results were expressed as Mean ± S.D and range values. One way ANOVA was used for multiple group comparisons followed by unpaired t-test for group wise comparisons {5}.

## RESULTS

NORs were strictly located within the cell nucleus and these AgNORs were distinctly stained as black-brown dots or blebs within yellow nuclei in a pale, clear background. The present study showed a highly significant increase in OSCC and leukoplakia mean AgNoR count when compared to that in normal epithelium. These findings coincide with the results of Chattopadhyay *et al.,* {6} and Sethi et al., {7}), as shown in Table I and Fig.1
One-way ANOVA was used for multiple group comparisons followed by unpaired 't' test for group wise comparisons. One factor ANOVA between the various groups showed a highly significant difference between the mean AgNOR counts among the three groups ($F = > 8.35$, $p<.001$) as shown in Table II. When one factor ANOVA was applied, it showed a statistically highly significant difference between the different grades of leukoplakia. ($F=65.84$ $p<.001$)(Table III). When the unpaired 't' test was applied to the obtained values, the results were statistically non significant ($t= 1.70$, $p=0.11$) (Table IV).

## DISCUSSION

Deregulated proliferation is considered as a prime characteristic of malignancies. It is thought to reflect the biological aggressiveness of a given tumor, exerting a putative impact on the clinical course of different cancers {8}. Numerous special techniques have previously been studied for their possible application to oral premalignant lesions in order to aid diagnosis and prognosis{9}.Their real value as adjuncts to routine histopathology would be to differentiate those oral lesions that carry a higher risk of subsequent malignancy from those that have a benign outcome{10}.
It has been confirmed that there is an association between cell proliferation and AgNOR count in OSCC. It indicates a useful and inexpensive method for measuring cell proliferation in OSCC that is similar to PCNA or Ki-67 expression {11}.
As regards AgNoR count in OSCC, our data correlated with the studies conducted by Chattopadhyay *et al.,* {6} and Sethi P {7). However, it was in contrast to other studies done by Cabrini et al. {12} and Xie et al., {1}.

Furthermore in cases of leukoplakia, increase in grades showed increase in the mean AgNOR count i.e. dysplastic leukoplakia (moderate leukoplakia 3.93±0.06 and mild leukoplakia 3.51± 0.06) as compared to non-dysplastic leukoplakia (2.39 ±0.42) which was found to be statistically significant (p<.001). These results are in accordance with the study of Chattopadhyay *et al.,* {6}.

However, the mean AgNOR count for dysplastic leukoplakia was 2.65± 0.38 as reported by Cabrini et el., {12} that was in contrast to our study. This could be due to the varying tissue fixation and processing procedure, staining time or method of criteria of counting AgNORs, as all these parameters affect the AgNOR counts {4}.

When cases of OSCC were compared, increase in mean AgNOR count was observed from WDSCC (4.18± 0.54) to MDSCC (4.54 ± 0.31) cases. There was no significant difference between the well-differentiated and moderately differentiated cases of OSCC in our study.

One marked finding in our study was that although increasing group mean AgNOR counts clearly ranked lesions in the order of normal epithelium, leukoplakia and OSCC, there was a slight overlap among individual cases. In leukoplakia, 3 cases have mean AgNOR count values as 1.90, 2.61 and 2.65 and this range falls towards normal epithelium. However, these cases belong to the non-dysplastic category. Similarly, in OSCC, two cases of well-differentiated carcinoma have the mean AgNoR count as 3.39 and 3.65 respectively.

In our study, the AgNOR dots in OSCC and dysplastic leukoplakia were found to be smaller and more widely scattered in the nucleus than in normal epithelium as shown in Fig. (2). The higher AgNOR count, their smaller size, more widely scattered pattern in leukoplakia Fig. (3) and OSCC Fig. (4) appears to be a significant finding. In normal epithelium as seen in Fig (2), the AgNOR dots were found less in number but larger and regular in outline. The same results were obtained by Chattopadhyay *et al.,*{13}.

## CONCLUSION

After all the meticulous deliberations, the following points were concurred:

- The mean AgNOR count was found to be more in OSCC and oral leukoplakia than in normal oral epithelium and showed statistically significant difference.
- The mean AgNOR count showed significant difference in grades of leukoplakia but mean AgNOR count was non-significant in grades of OSCC.
- The difference was also seen in their pattern of distribution; more number of AgNOR dots, small sized, widely scattered over the nucleus were observed in OSCC and leukoplakia cases whereas less number of AgNOR dots, large sized and clustered in the nucleus were present in normal epithelium.

However this suggestion needs to be substantiated by studies with larger sample size and at the same time all the studies with AgNOR should follow a standardized protocol such as fixation, section thickness, counting method, staining reaction time and staining techniques to prevent the differences due to technical sensitiveness.

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Table I - The distribution pattern of AgNOR counts (mean and range) in normal oral epithelium, leukoplakia and OSCC**.**

| Groups | AgNOR counts | | |
|---|---|---|---|
| | Min | Max | Mean± S.D |
| NORMAL | 1.80 | 3.08 | 2.61 ± 0.21 |
| LEUKOPLAKIA | 1.90 | 3.98 | 3.40 ± 0.58 |
| OSCC | 3.39 | 5.14 | 4.40 ± 0.44 |

Table II - Comparison of AgNOR counts in Normal Epithelium, Leukoplakia & OSCC.

| Groups | AgNOR counts | | Difference between groups | | | |
|---|---|---|---|---|---|---|
| | Range | Mean± S.D | Groups compared | Mean diff | t-value | p-value |
| NORMAL | 1.80-3.08 | 2.61±0.21 | I-II | 0.79 | 0.79 | < .001 |
| LEUKOPLAKIA | 1.90-3.98 | 3.40±0.58 | I-III | 1.79 | 16.05 | < .001 |
| OSCC | 3.39-5.14 | 4.40±0.44 | II-III | 1.00 | 5.33 | < .001 |
| ANOVA, F= >8.35 p< .001 HS | | | | | | |

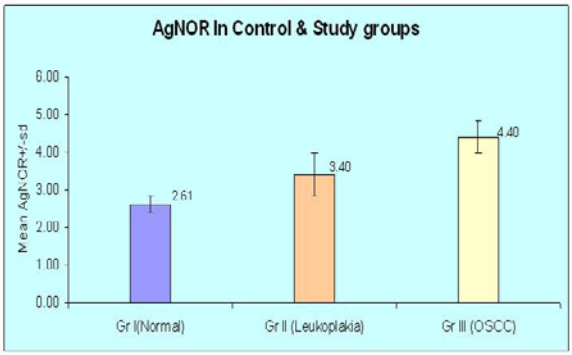


Figure1- Mean AgNOR count in control group, leukoplakia and OSCC.

Table III - Mean AgNOR count of different grades of leukoplakia.

| Group II cases | No. of cases | Mean AgNOR ± S.D |
|---|---|---|
| Non dysplasia | 3 | 2.39±0.42 |
| Mild dysplasia | 8 | 3.51±0.06 |
| Moderate dysplasia | 4 | 3.93±0.06 |
| Total | 15 | 3.40±0.58 |
| ANOVA F=65.84 p< .001 HS | | |

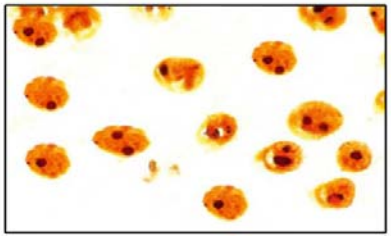

Figure2- AgNORs in normal epithelium exhibiting larger & regular AgNORs dots in outline (100X)

Table IV - Mean AgNOR count among the different grades of OSCC

| OSCC | No. of cases | Mean AgNOR ± S.D |
|---|---|---|
| WDSCC | 6 | 4.18 ±0.54 |
| MSCC | 9 | 4.54± 0.31 |
| Total | 15 | 4.40 ±0.44 |
| Unpaired 't' test t = 1.70 p = 0.11 NS | | |

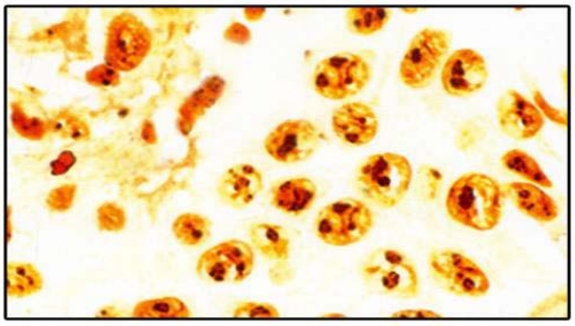

Figure3- AgNORs in dysplastic leukoplakia demonstrating irregular outline and widely scattered pattern (100X)

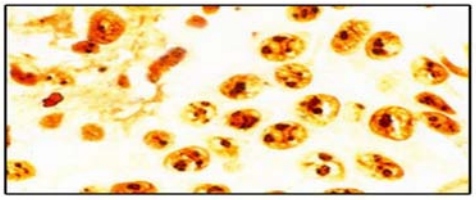

Figure4- AgNORs in OSCC showing more number of dots and widely scattered pattern (100X)