ORIGINAL ARTICLE

# Evaluation of cell proliferation in malignant and potentially malignant oral lesions

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## ABSTRACT

**Aims:** To evaluate the cell proliferation rate by the expression of proliferating cell nuclear antigen (PCNA) and argyrophilic nucleolar organizing region (AgNOR) counts and to assess its usefulness as a marker for malignant potential in oral epithelial lesions. **Materials and Methods:** The study group included 30 cases of leukoplakia, 15 nondysplastic (NDL), 15 dysplastic (DL), 15 cases of oral squamous cell carcinoma (OSCC) and 5 cases of normal oral mucosa. Formalin fixed paraffin embedded tissues were subjected to immunohistochemical staining for PCNA and AgNOR technique. The PCNA labeling index (LI) and the AgNOR dots were evaluated for the entire sample. **Statistical Analysis Used:** ANOVA, Tukey honestly significant difference, Pearson's correlation. **Results:** In this study, the AgNOR count of OSCC was lower than the DL lesions moreover the AgNOR counts were found to be higher in normal mucosa as compared to the DL and the NDL epithelium. The study results also showed that the mean AgNOR count failed to distinguish between DL and NDL lesions. Overall we observed increased PCNA expression from normal epithelium to NDL to DL lesion. **Conclusions:** Based on the findings of the present study on oral epithelial precancerous and cancerous lesions we conclude that mean AgNOR count alone cannot be a valuable parameter to distinguish between the normal, NDL, DL epithelium and OSCC but, on the other hand, we found out that PCNA can be a useful biomarker for delineating normal epithelium from DL epithelium and OSCC.

***Key words:*** Argyrophilic nucleolar organizing region, epithelial dysplasia, oral squamous cell carcinoma, proliferating cell nuclear antigen

## INTRODUCTION

Oral cancer is a major public health issue worldwide; it remains a highly lethal and disfiguring disease. Oral cancer can be defined as a neoplasm involving the oral cavity, which begins at the lip and ends at the anterior pillar of the fauces. The most common intraoral malignancy is squamous cell carcinoma (SCC).[1] Despite the progress in diagnosis and treatment of malignant tumors, the survival index of oral SCC (OSCC) continues to be small.[2] OSCC is often preceded by precursor changes in the oral mucosa. A clinically white lesion that cannot be characterized as any other specific disease entity on the basis of clinical features alone is provisionally designated as "leukoplakia."[3]

Nucleolar organizing region (NOR) are loops of DNA containing ribosomal RNA (rRNA) genes. Argyrophilic NOR (AgNORs) are silver binding NORs. The NOR-associated proteins probably act as regulators of rDNA transcription or

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may have a role in maintaining the extended configuration of rDNA. NOR staining identifies actively transcribing NORs and frequency of NORs per nucleus may reflect cell turnover and may hence, prove a useful replicatory marker.[4]

Malignant tissue is characterized by an uncoordinated proliferating cell nuclear antigen (PCNA) which is an essential component of the DNA replication machinery, required for processive chromosomal DNA synthesis. PCNA is also required for DNA recombination and repair. In addition, PCNA was shown to interact with cellular proteins involved in cell cycle regulation and checkpoint control.[5]

This aim of this study was to assess the proliferative index in potentially malignant and malignant oral lesions using AgNOR count and PCNA expression in order to assess the usefulness of these markers as a predictor of high-risk lesions.

## MATERIALS AND METHODS

The present retrospective study was carried out on a total of 50 biopsy tissues retrieved from the archives of Department of Oral and Maxillofacial Pathology. The study group included 30 cases of leukoplakia (15 nondysplastic [NDL] and 15 dysplastic [DL]), 15 cases of OSCC and 5 cases of normal oral mucosa (NOM), from the retromolar region, were taken as control.

Relevant information (e.g., age, sex, site of the lesion and clinical staging) was obtained from the medical records of the patient. The tissues had been fixed in 10% formalin and processed routinely and embedded in paraffin wax. The diagnosis and grading of dysplasia and carcinoma were reviewed under routine H and E stained sections of 4 μ thickness.

The WHO system (1978) of grading was used to grade cases of epithelial dysplasia. The 30 cases of oral leukoplakia were further divided into two groups as suggested by Warnakulasuriya *et al*.[6]

- NDL group (15 cases) comprised of cases histologically diagnosed as hyperkeratosis, hyperplasia or mild epithelial dysplasia
- DL group (15 cases) comprised of cases histologically diagnosed as moderate epithelial dysplasia. Severe epithelial dysplasia or carcinoma *in situ* cases.

For SCC Anneroth grading system was followed.[7]

### Silver binding argyrophilic nucleolar organizing region staining

The slides were subjected to AgNOR staining according to the method of Ploton *et al*.[8] Briefly the sections were incubated in a mixture of solution A (Silver Nitrate-Qualigens, Deionized water) and solution B (Gelatin Powder, Formic Acid and Deionized water) for 30 min in an incubator. Each time the final working solution was freshly prepared by mixing one volume of solution A and two volume of solution B.

### Proliferating cell nuclear antigen immunohistochemistry

Immunohistochemical detection of PCNA was performed using Biogenex SS polymer HRP-horseraddish peroxidase detection system. For immunohistochemical staining the sections were cut at approximately 3 μm. Sections were floated on to Poly-L-lysine coated slides and incubated for 1 h at 60°C. Later the sections were dewaxed in xylene and rehydrated in descending grades of alcohol.

Positive control consisted of paraffin-embedded sections of tonsils with known antigenic reactivity to PCNA in the lymphoid follicles [Figure 1] and a negative control was performed by omitting the step of primary antibody during the staining procedure which resulted in a lack of staining in all cases.

The slides were kept in a couplin jar filled with TRIS-EDTA buffer (pH 9) and placed in a microwave oven for Heat-Induced Epitope Retrieval. The slides were given two cycles at high (80°C) mode of the microwave for 5 min, one cycle at medium high (60°C) for 5 min and another cycle at low (40°C) mode for 5 min. After every cycle buffer was added to fill the couplin jar.

The sections were then allowed to cool to room temperature and then rinsed with distilled water for 1 min. Endogenous peroxidase activity was blocked by incubating the slides with Peroxide Block (3% hydrogen peroxide in water) for 12–15 min, which was followed by Power Block™ (a highly

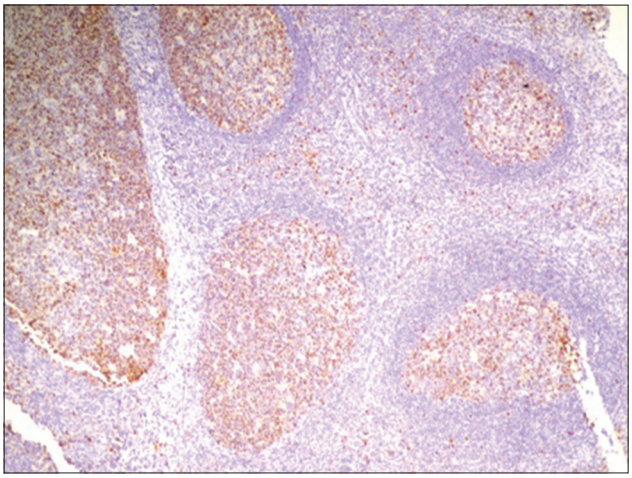


**Figure 1:** Photograph showing proliferating cell nuclear antigen expression in follicular area of Tonsil which was used as positive control (IHC stain, x200)×200 (positive control)

effective universal protein blocking reagent contains casein and proprietary additives in phosphate buffered saline (PBS) with 15 mM sodium azide) for 12–15 min. No washing was done after the incubation in Power Block. Sections were incubated with mouse anti-rat monoclonal PCNA antibody (BioGenex) for 1 1/2 h in a humidifying chamber at 37°C. The sections were then washed thrice with wash buffer for 5 min each and later on incubated with Super Enhancer™ (a reagent that enhances the signal and is used after the primary antibody incubation) for 25 min. After the buffer wash slides were incubated with Super sensitive poly HRP (anti-mouse anti-rabbit IgG labeled with enzyme polymer in PBS with stabilizers, carrier protein and 0.1% Proclin 300) secondary antibody for 25 min in a humidifying chamber. Incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen was done for 1–5 min. Chromogen was freshly prepared by adding 1 ml stable DAB with 1 drop of chromogen and 5 microliters of 30% $H_2O_2$. Slides were rinsed with distilled water and counterstained with Harris hematoxylin for 20 sec.

### Evaluation of argyrophilic nucleolar organizing region staining

All sections were examined under × 400 magnification in oil immersion using Olympus BX51 light microscope and AgNOR dots were counted in 100 randomly selected cells from the basal and parabasal layers. Microscopic fields, representative of the lesion, were identified and photographs of the same were taken using Olympus Live View Digital SLR Camera Olympus E-330. The photographs were analyzed using Image Pro Express 6.0 for Windows (Manufacturer details: Media Cybernetics, Inc.U.S.A). AgNORs from 100 randomly selected nuclei of epithelial cells were assessed at × 400 magnifications for their numbers and the number of AgNOR count was expressed per nucleus [Figures 1-5].

### Evaluation of proliferating cell nuclear antigen staining

The nuclear expression of PCNA was assessed at ×400 magnification and photographs were taken using Olympus E-330 camera. The photographs were then analyzed using Image Pro Express 6.0 for Windows (Media Cybernetics) [Figure 6].

For qualitative assessment the expression of PCNA was observed in basal, parabasal and suprabasal layers in NOM and epithelial dysplasia [Figures 7-9]. In cases of SCC the expression was assessed as peripheral, central or diffuse. [Figures 10 and 11].

The percentages of positively stained nuclei for PCNA were counted in three nonoverlapping ×400 fields.

Total number of positive cells was counted in epithelial dysplasia, NOM and SCC and the labeling index (LI) were calculated.

## RESULTS

### Argyrophilic nucleolar organizing region staining

In all the study groups AgNOR dots were counted in 100 cells. The NORs when stained with silver nitrate appeared as dark brown to black dots or blebs within the yellowish brown nucleus in a yellow background. These were present either as isolated dots, as dots in groups or as a complex, irregular conglomeration of dots within the nuclei. The mean AgNOR count in normal, NDL, DL and OSCC was 2.26, 1.69, 2.10 and 1.76, respectively. No significant intergroup difference was found.

The lowest AgNOR counts were seen in NDL with mean AgNOR count being 1.69 ± 0.27/nuclei while the maximum expression

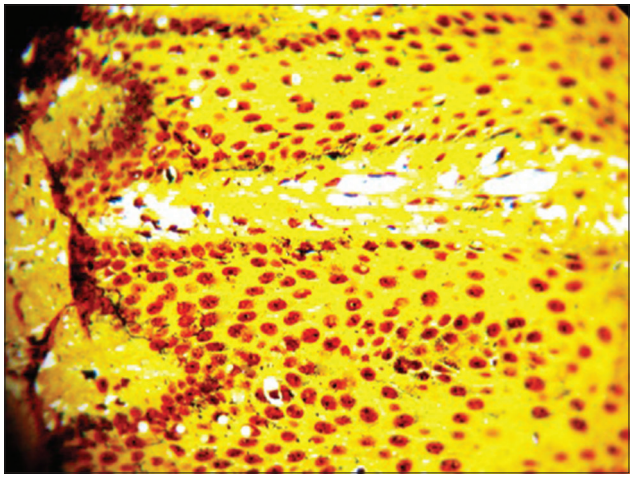


**Figure 2:** Photograph showing argyrophilic nucleolar organizing region staining in normal oral mucosa (AgNOR stain, x400)

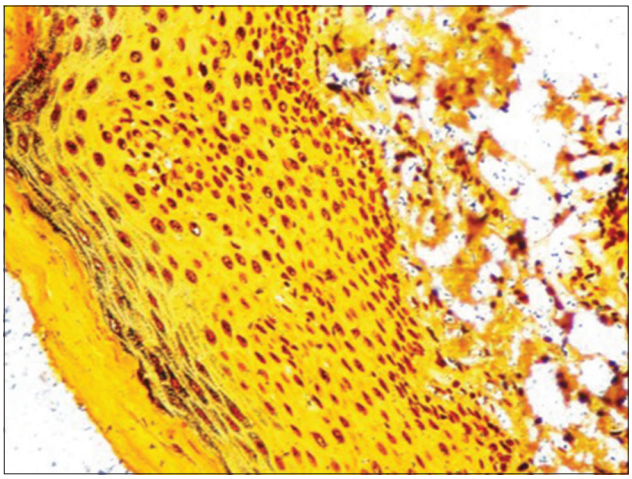


**Figure 3:** Photograph showing argyrophilic nucleolar organizing region staining in nondysplastic epithelium (AgNOR stain, x400)

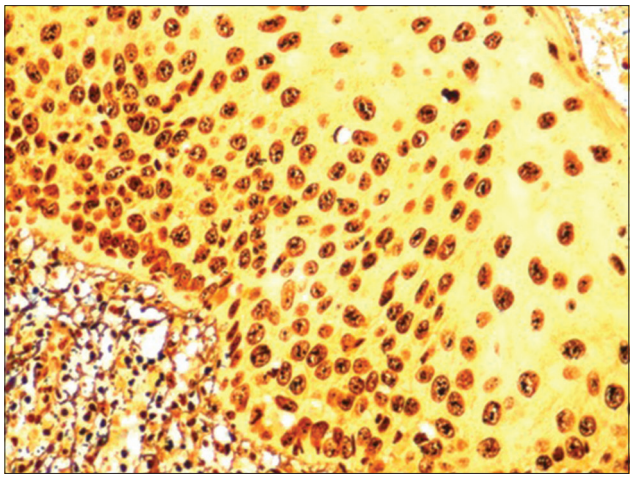


**Figure 4:** Photograph showing argyrophilic nucleolar organizing region staining in dysplastic epithelium (AgNOR stain, x400)

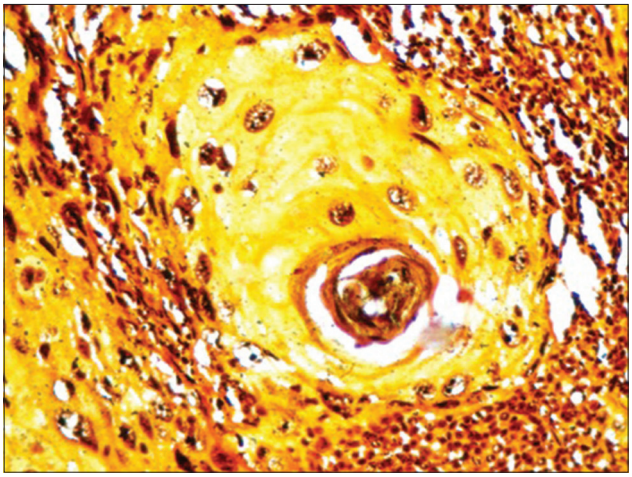


**Figure 5:** Photograph showing argyrophilic nucleolar organizing region staining in oral squamous cell carcinoma (AgNOR stain, x400)

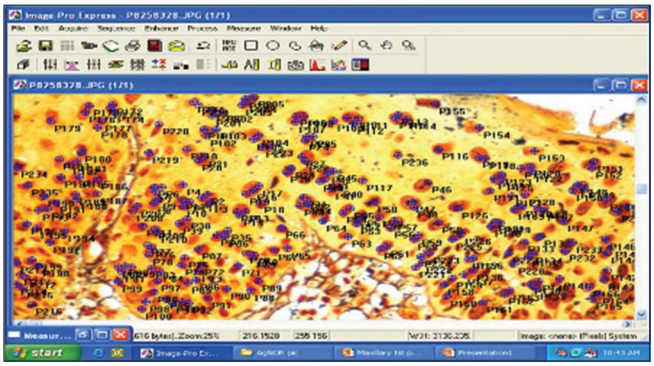


**Figure 6:** Photograph showing argyrophilic nucleolar organizing region counting using Image Pro Express (AgNOR stain, x200)

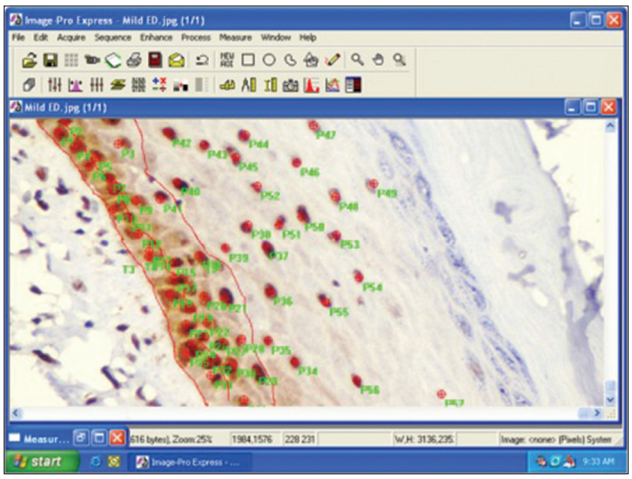


**Figure 7:** Photograph showing counting of proliferating cell nuclear antigen labeling index in basal, parabasal and suprabasal layers of epithelium using Image Pro Express (AgNOR stain, x200

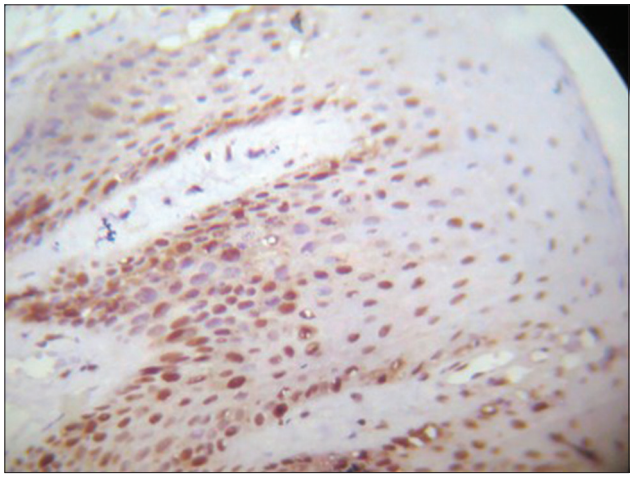


**Figure 8:** Photograph showing proliferating cell nuclear antigen expression in normal oral mucosa (IHC stain, x400)

was seen in NOM (2.26 ± 0.59 AgNOR count/nuclei). The mean AgNOR count for DL and OSCC was in between the two groups with mean AgNOR count of OSCC being lower

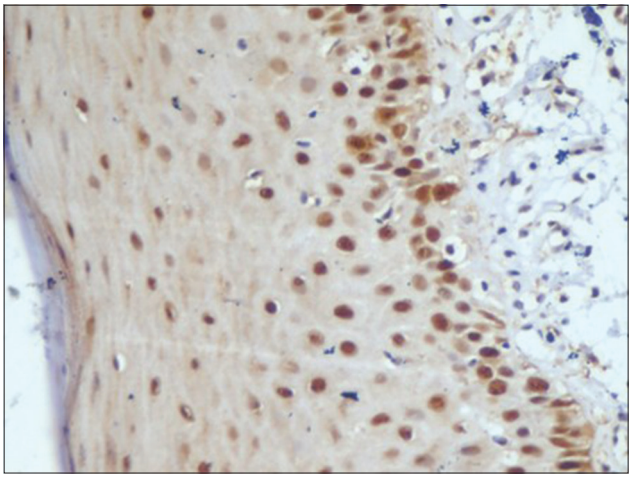


**Figure 9:** Photograph showing proliferating cell nuclear antigen expression in nondysplastic epithelium (IHC stain, x400)

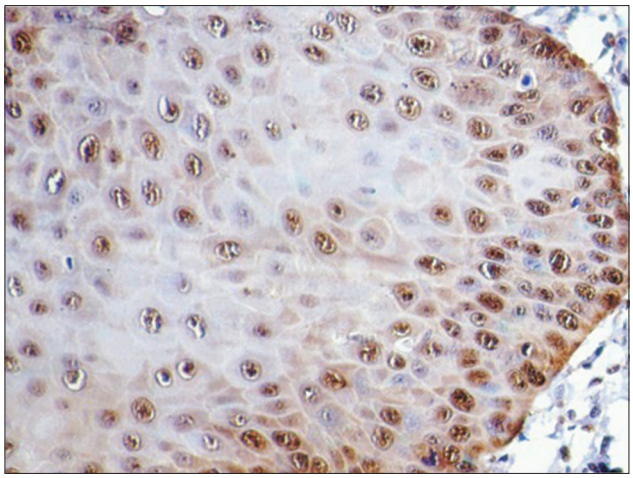


**Figure 10:** Photograph showing proliferating cell nuclear antigen expression in dysplastic epithelium (IHC stain, x400)

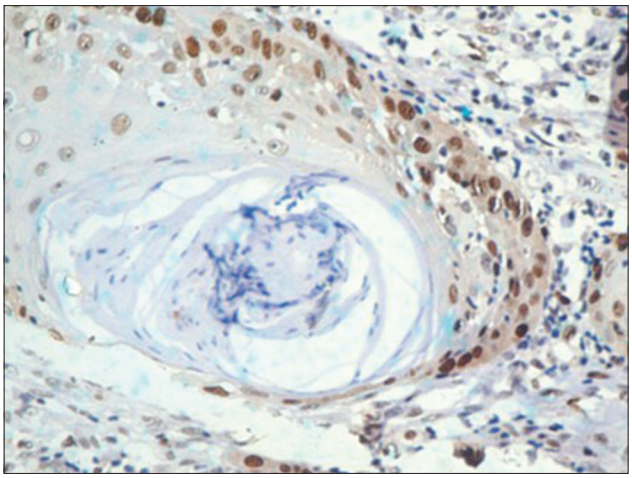


**Figure 11:** Photograph showing proliferating cell nuclear antigen expression in oral squamous cell carcinoma (IHC stain, x400)

(1.76 ± 0.57 mean AgNOR/nuclei) than DL (2.10 ± 0.89 mean AgNOR/nuclei) [Table 1 and Graph 1].

Analysis of variance dose not reveal a statistically significant intergroup difference (F = 1.88; *P* = 0.145). It was observed that NDL and OSCC had the LI values of lower order whereas the same in NOM and DL group were of higher order [Table 2].

Maximum difference was observed between NOM and NDL groups (0.57 ± 0.32) whereas minimum difference was observed between NOM and DL groups (0.15 ± 0.32). The comparisons did not reveal a significant intergroup difference (*P* > 0.05) [Table 3].

On the basis of above observations, the following order of LI was observed:

## Nondysplastic – oral squamous cell carcinoma – dysplastic – NOM

### *Proliferating cell nuclear antigen staining*

The PCNA expression was assessed for NOM, NDL, DL and OSCC. The pattern of staining was strongly positive to granular in some cases. Faint intracytoplasmic staining was also observed in all the cases apart from the strongly positive intranuclear staining.

For all the cases composite PCNA LI was calculated as a percentage of positively stained cells. In general two patterns of PCNA expression were observed in cases of OSCC. Most of the well-differentiated tumors showed PCNA expression mainly restricted to the peripheral layers of tumor islands while the less differentiated lesions showed a more diffuse distribution of PCNA positive cells directly proportional to the increasing degree of anaplasia.

The mean composite PCNA Labeling Index in normal, NDL, DL and OSCC was 25.73%, 40.60%, 44.95% and 80.35%, respectively. On comparing the data statistically, a significant difference was observed between NOM and DL (*P* = 0.020), NOM and OSCC (*P* < 0.001), DL and OSCC (*P* < 0.001)

**Table 1: Mean argyrophilic nucleolar organizing region count in different study groups**

| Group | *n* | Mean±SD |
|---|---|---|
| Normal | 5 | 2.26±0.59 |
| NDL | 15 | 1.69±0.27 |
| DL | 15 | 2.10±0.89 |
| OSCC | 15 | 1.76±0.57 |

SD: Standard deviation, OSCC: Oral squamous cell carcinoma, NDL: Nondysplastic, DL: Dysplastic

**Table 2: Analysis of variance of mean argyrophilic nucleolar organizing region count in various groups**

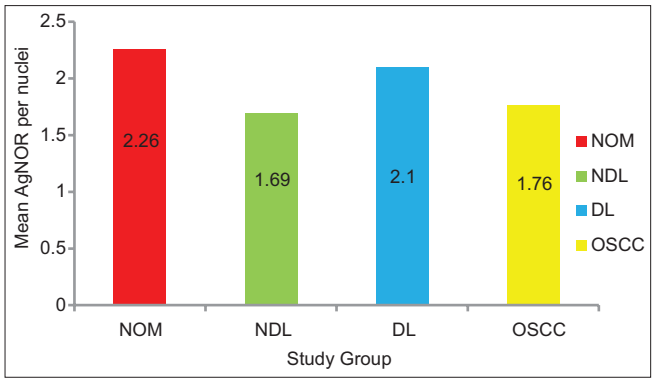
| Mean AgNOR count | *n* | Mean±SD | Mean square | *F* | *P* |
|---|---|---|---|---|---|
| Normal | 5 | 2.26±0.59 | 0.748 | 1.88 | 0.145 |
| NDL | 15 | 1.69±0.27 | | | |
| DL | 15 | 2.10±0.89 | | | |
| OSCC | 15 | 1.76±0.57 | | | |

SD: Standard deviation, OSCC: Oral squamous cell carcinoma, NDL: Nondysplastic, DL: Dysplastic, AgNOR: Argyrophilic nucleolar organizing region

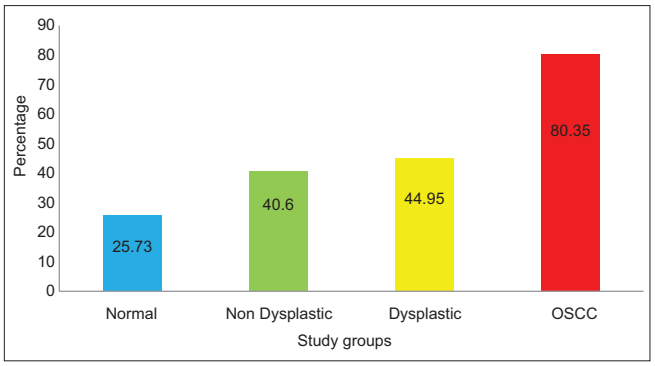
**Table 3: Intergroup comparisons of mean argyrophilic nucleolar organizing region counts between oral squamous cell carcinoma, nondysplastic, dysplastic group and normal healthy controls comparisons**

| Comparison | Mean difference±SE | *P* |
|---|---|---|
| NOM versus NDL | 0.57±0.32 | 0.30 |
| NOM versus DL | 0.15±0.32 | 0.96 |
| NOM versus OSCC | 0.50±0.32 | 0.14 |
| NDL versus DL | −0.41±0.22 | 0.28 |
| NDL versus OSCC | −0.06±0.22 | 0.99 |
| DL versus OSCC | 0.34±0.22 | 0.43 |

SE: Standard error, NOM: Normal oral mucosa, DL: Dysplastic, NDL: Nondysplastic, OSCC: Oral squamous cell carcinoma




**Graph 1:** Mean argyrophilic nucleolar organizing region count in different study groups




**Graph 2:** Mean composite proliferating cell nuclear antigen labeling index in different study groups

and NDL and OSCC ($P < 0.001$) groups. Statistically, the difference between NOM/NDL and NDL/DL was not significant ($P > 0.05$).

The lowest PCNA expression was seen in NOM with mean LI being 25.73 ± 2.80% while the maximum expression was seen in OSCC group (mean LI = 80.35 ± 12.37%). The LI for leukoplakia cases was in between the above two groups with mean PCNA LI of NDL being lower (40.60 ± 8.55%) than DL (44.95 ± 16.31%) [Table 4 and Graph 2].

Analysis of variance thereof reveals a statistically significant intergroup difference ($F = 40.309$; $P \leq 0.001$). It was observed that NOM and NDL had the LI values of lower order whereas the same in DL and SCC group were of higher order [Table 5].

Maximum difference was observed between NOM and OSCC groups (−54.62 ± 6.33) whereas minimum difference was observed between NDL and DL groups (−4.38 ± 4.48). On comparing the data statistically, a significant difference was observed between NOM and DL ($P = 0.020$), NOM and OSCC ($P < 0.001$), DL and OSCC ($P < 0.001$) and NDL and OSCC ($P < 0.001$) groups. Statistically, the difference between NOM and NDL and NDL and DL was not significant ($P > 0.05$) [Table 6].

On the basis of above observations, the following order of LI was observed:

### Normal oral mucosa – nondysplastic – dysplastic-oral squamous cell carcinoma

AgNOR versus PCNA: Pearson's correlation. No significant correlation was found between AgNOR and PCNA [Table 7].

## DISCUSSION

In this study, we used these two proliferative markers, i.e. AgNOR counts and PCNA expression, with the aim to compare the proliferative activity of potentially malignant and malignant oral lesions in order to identify lesions which are at a higher risk for malignant transformation. We also attempted to find out if any correlation exists between AgNOR counts and PCNA expression in these oral lesions.

In our study no significant differences in AgNOR counts were observed with age, gender, tobacco habit and size of the lesion. Moreover, there was no statistically significant difference in

**Table 4: Mean composite proliferating cell nuclear antigen labeling index in different groups**

| Group | *n* | Mean±SD (%) |
|---|---|---|
| Normal | 5 | 25.73±2.80 |
| NDL | 15 | 40.60±8.55 |
| DL | 15 | 44.95±16.31 |
| OSCC | 15 | 80.35±12.37 |

SD: Standard deviation, OSCC: Oral squamous cell carcinoma, NDL: Nondysplastic, DL: Dysplastic

**Table 5: Analysis of variance of composite proliferating cell nuclear antigen labeling index in different groups**

| Composite PCNA LI | *n* | Mean±SD | Mean square | *F* | *P* |
|---|---|---|---|---|---|
| NOM | 5 | 25.73±2.80 | 6070.77 | 40.309 | <0.001 |
| NDL | 15 | 40.56±8.55 | | | |
| DL | 15 | 44.95±16.31 | | | |
| SCC | 15 | 80.35±12.37 | | | |

PCNA: Proliferating cell nuclear antigen, NOM: Normal oral mucosa, NDL: Nondysplastic, DL: Dysplastic, SCC: Squamous cell carcinoma

**Table 6: Between-group comparison of proliferating cell nuclear antigen labeling index (Tukey honestly significant difference)**

| Comparison | Mean difference±SE | *P* |
|---|---|---|
| NOM versus NDL | −14.83±6.33 | 0.104 |
| NOM versus DL | −19.22±6.33 | 0.020 |
| NOM versus OSCC | −54.62±6.33 | <0.001 |
| NDL versus DL | −4.38±4.48 | 0.762 |
| NDL versus OSCC | −39.79±4.48 | <0.001 |
| DL versus OSCC | −35.40±4.48 | <0.001 |

SE: Standard error, NOM: Normal oral mucosa, NDL: Nondysplastic, DL: Dysplastic, OSCC: Oral squamous cell carcinoma

**Table 7: Correlations between mean argyrophilic nucleolar organizing region count and composite proliferating cell nuclear antigen labeling index**

| PCNA composite LI | Mean AgNOR count |
|---|---|
| Pearson correlation | −0.026 |
| Significant (two-tailed) | 0.857 |
| *n* | 50 |

PCNA: Proliferating cell nuclear antigen, LI: Labeling index, AgNOR: Argyrophilic nucleolar organizing region

AgNOR counts of various study groups, i.e. NOM, NDL, DL and OSCC. In fact, the mean AgNOR counts were highest for NOM but since the number of cases of NOM was very small, a higher count in any one case would significantly increase the mean for the group. One of the cases of NOM in our study group exhibited abnormally high AgNOR count as well as a relatively high PCNA LI. Factors like underlying inflammation have been shown to increase AgNOR counts[9] and could be the reason for higher values in NOM. In our study, the normal epithelium showed variable amounts of inflammation because they were obtained from pericoronal tissue, during third molar surgery.

Although many studies have reported the usefulness of AgNOR counts in distinguishing between premalignant and malignant lesions.[10,11] Statistically, no significant difference was seen between leukoplakia and OSCC in our study group.

In this study, the mean AgNOR count of OSCC group was lower than that of DL. We observed that in OSCC the AgNORs were comparatively larger in size and many dots coalesced with each other and hence were counted as single dots according to the counting criteria given by Crocker *et al*.[12] and this could be the reason for lower values in OSCC. On the other hand NORs in leukoplakias, especially DL, were more dispersed and relatively smaller in size and hence the absolute count was higher than OSCC. Recently, emphasis has been given to the morphometric parameters like size, area and contour of AgNORs rather than the absolute count for assessing the proliferative activity of tissues.[11,13,14] Our observations also support this view as measuring area and perimeter of AgNORs would partly negate the error caused by overlapping and coalescing of individual dots.

PCNA expression in our study subjects was found to decrease above the age of 50 years, and this difference was mainly observed in the basal layer. The results suggest that there may be a reduction in PCNA expression with increasing age which may be age-related alteration in a posttranscriptional regulatory function. The decline in the expression of the PCNA gene would possibly contribute to the inability of older cells to initiate replicative DNA synthesis.[15] PCNA expression was also found to be higher among females in this study, but it would be difficult to draw any conclusions from this data because of a very strong male bias in the study sample. No significant difference in PCNA expression was noted between smokers and tobacco chewers. The values were slightly higher in the smokeless category, which could be attributed to the direct contact of the carcinogens with the oral mucosa in the case of chewers.[16] Statistically, no significant difference in PCNA expression was seen between lesions of different sizes either for leukoplakia or SCC.

The results of this study showed that PCNA expression was seen in all cases of NOM, NDL, DL and OSCC. The overall LI increased gradually from NOM, through leukoplakia to OSCC with significantly higher PCNA expression in cases of carcinoma. These results are in concordance with previous studies which have shown an increased PCNA expression in malignancy, both of oral cavity as well as other regions.[17-20] These results confirm that an increased cell proliferation is a characteristic feature of malignancy. Higher the cell proliferation rate, higher is the risk of cells suffering mutations and effect of carcinogens during mitosis which could result in a malignant phenotype.[19]

In NOM, PCNA positive cells were observed in basal, parabasal, as well as suprabasal compartments with LI gradually decreasing from basal to suprabasal layers, although there was no significant difference in the LI of any of the layers. In general, a slightly increased PCNA expression was seen in the NOM as compared to some of the previous studies.[17,18] One of the reasons for this finding could be because of the effect of inflammation as discussed earlier. An increased PCNA expression secondary to inflammation has been reported previously and hence may have led to such a finding in our study.[21,22] Moreover, PCNA is characterized by a longer half-life (around 20 h) and results in staining of cells which have recently left the cell cycle.[23] Deregulated expression of PCNA has also been reported in normal tissues and specimens adjacent to tumors. Other factors, such as epitopic differences, effect of fixation protocols and the involvement of PCNA in other cellular processes such as DNA repair, may have contributed to the generally higher values of PCNA positivity noted in this and other studies.[20]

Cases of leukoplakia showed increased PCNA LI as compared to normal mucosa which is similar to some of the previous studies.[18,23] Although increased expression was evident in all layers of epithelium, the difference was significant, especially for the basal compartment. Studies have shown that percentage of PCNA immunoreactive cells, as well as DNA content increased progressively in the basal layers with an increasing grade of dysplasia and it has been suggested that the malignancy-related cellular alterations seen in the DL lesions of oral cavity generally occur in the basal cell layers.[24]

In this study, no significant differences were observed between NDL or DL leukoplakic lesions though mean PCNA LI was slightly higher in the DL group. This suggests that although PCNA expression may be higher in leukoplakia when compared to NOM, it may not be able to differentiate

between DL and NDL epithelium. Similar observations were made by Soares *et al*. who found that the quantitative and tissue distribution of PCNA immunostaining are unable to distinguish progressive proliferative status among oral lesions independent of histological grade. They suggested that PCNA has a long half-life resulting in staining of even those cells which have recently left the cell cycle.[25] Factors such as involvement of PCNA in other cellular processes such as DNA repair may contribute in reducing the specificity of this protein as a proliferative marker and it has been shown that Ki-67 derived growth fraction allows for better determination of the proliferative activity than does PCNA.[20] On the contrary, the lack of significant differences in PCNA expression between NDL and DL may also indicate the inadequacy of routine histomorphological features in identifying tissue changes at the molecular level. While morphological criteria seems to be the most reliable marker for the diagnosis of malignancy it appears that a certain period is required before the degree of morphological atypia is manifested histopathologically to allow the diagnosis of premalignancy even after the formation of cancer clones.[18]

Our results show that though PCNA may have some usefulness in the diagnosis of OSCC and differentiation of DL from NDL, its absolute reliability is questionable. PCNA was previously considered to be a marker for cell cycling, but it is now known that nonproliferating cells also express PCNA and Ki-67 is now considered to be a more specific marker for cell proliferation. Therefore, PCNA expression may not be directly related to cell proliferation a view that is further strengthened by the observation that cells which are unable to regenerate may show PCNA positivity but are Ki-67-negative.[26]

In this study, no significant correlation was found between AgNOR and PCNA. Similar findings were reported by Kobayashi *et al*.[27] and it is suggested that the inadequacy of statistical correlation between PCNA index and NORs counting has been attributed to the fact that these methods quantify different aspects of the proliferative activity or that PCNA and NORs are not synchronized in the cell cycle.[28] In addition, the number of interphase AgNORs is thought to be related to rRNA transcriptional activity and to cell doubling time and consequently to the rapidity of cell proliferation, a relation was not found with PCNA.[29]

## CONCLUSIONS

Based on the findings of our study, it can be concluded that mean AgNOR count is of limited value if it is not supplemented with the morphmetric analysis. On the other hand, PCNA can differentiate between NOM and OSCC but it is not a good marker in delineating NDL from DL. Moreover, we did not find any correlation between the AgNOR count and PCNA labeling index which suggest that they are not well synchronized in the cell cycle and they mark cells at different stages of proliferation.

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## Conflicts of interest

There are no conflicts of interest.

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