ISSN (print) : 2589-9120
ISSN (online) : 2589-9139

International Journal of Medical Science and Applied Research (IJMSAR)

Available Online at: https://www.ijmsar.com

Volume – 2, Issue – 5, September – October - 2019, Page No. : 08 - 13

Efficiency of Agnor as a Proliferative Marker

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Type of Publication: Original Research Article

Conflicts of Interest: Nil

Abstract

This study was undertaken to assess the aggressiveness of leukoplakia and squamous cell carcinoma of the oral cavity by determining the mean number of Argyrophilic nucleolar organizing region-AgNORs and comparing them with those of normal mucosa. The study sample consisted of total 50 subjects. Biopsy specimens of 15 oral squamous cell carcinomas-OSCC 30 leukoplakia (15 Dysplastic-DL and 15 Non dysplastic-NDL) and 5 healthy oral mucosa were taken. AgNORs were assessed quantitatively. The data were analyzed using ANOVA and Tukey honesty significant difference. The study results depict that the AgNOR counts fail to differentiate between DL and NDL. Based on the results we conclude that AgNOR counts are not a very useful marker in differentiating between premalignant and malignant lesions.

Keywords

Argyrophilic nucleolar organizing region, leukoplakia, epithelial dysplasia.

Introduction

Nucleoli are the prominent contrasted structures of the cell nucleus. In the nucleolus, ribosomal RNAs (rRNAs) are synthesized, processed and assembled with ribosomal proteins. The size and organization of the nucleoli are directly related to ribosome production. The organization of the nucleolus reveals the functional compartmentation of the nucleolar machineries that depends on nucleolar activity. In addition, nucleoli are dynamic structures in which nucleolar proteins rapidly associate and dissociate with nucleolar components in continuous exchanges with the nucleoplasm.[1]

Nucleolar organizing region (NORs) are loops of DNA containing ribosomal RNA (r-RNA) genes. In humans, they are located on the short arm of the five acrocentric chromosomes which are chromosomes number 13, 14, 15, 21 & 22. Argyrophilic Nucleolar organizing region (AgNOR) are silver binding NORs. The NOR associated proteins probably act as regulators of r-DNA transcription or may have a role in maintaining the extended configuration of r-DNA. NOR staining identifies actively transcribing NORs & frequency of NORs per nucleus may reflect cell turnover & may hence, prove a useful replicatory marker. [2]

Giri et al (1985) had illustrated that in an inactive cell the AgNOR’s are seen in close approximation and form a smoothly outlined nucleolus. On the contrary, in an actively proliferating cell the AgNOR distribution is generally disorganized. This results in the formation of a dispersed and multiple nucleoli. Based on these findings attempts are made to distinguish between the premalignant and malignant lesions. [3]

This study was done to assess the aggressiveness of Dysplastic, Non Dysplastic Leukoplakia and Oral Squamous cell carcinoma (OSCC) by analyzing the mean number of AgNORs and comparing them with the normal mucosa. This study also tries to assess the usefulness of AgNORs in differentiating between the premalignant and malignant 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, Saraswati Dental College & Hospital, Lucknow. The study group included 30 cases of leukoplakia (15 non dysplastic-NDL and 15 dysplastic-DL), 15 cases of oral squamous cell carcinoma(OSCC) and 5 cases of normal oral mucosa(NOM), from the retro molar region, were taken as control.

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

Grading of Dysplasia

W.H.O. 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.[4]

1. **Non dysplastic group** (15 cases) comprised of cases histologically diagnosed as hyperkeratosis, hyperplasia or mild epithelial dysplasia.
2. **Dysplastic group** (15 cases) comprised of cases histologically diagnosed as moderate epithelial dysplasia, severe epithelial dysplasia or carcinoma in situ cases.

For squamous cell carcinoma Anneroth grading system was followed.[5]

Agnor Staining

The slides were subjected to AgNOR staining according to the method of Peloton et al. The working solution was freshly prepared every time before staining. [Figure 1]

Figure 1: Photograph showing reagents used in AgNOR staining.

Counting Procedure

All sections were examined under x400 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. AgNORs were seen as separate dark brown to black dots or "blebs" of varying size, observed in a light brown stained nucleus within a pale yellow cytoplasm. Minor artifactual background staining was also seen in some cases observed as extraneous silver deposits in the cytoplasm. Care was taken to distinguish between AgNORs dots and artifacts. Microscopic fields, representative of the lesion, were identified & 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, (Media Cybernetics). [Figure 2-5] AgNORs from 100 randomly selected nuclei of epithelial cells were assessed at x400 magnification for their numbers and the number of AgNOR

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count was expressed per nucleus. Statistical analysis of the data obtained was performed using ANOVA and Tukey+ honesty significant difference.

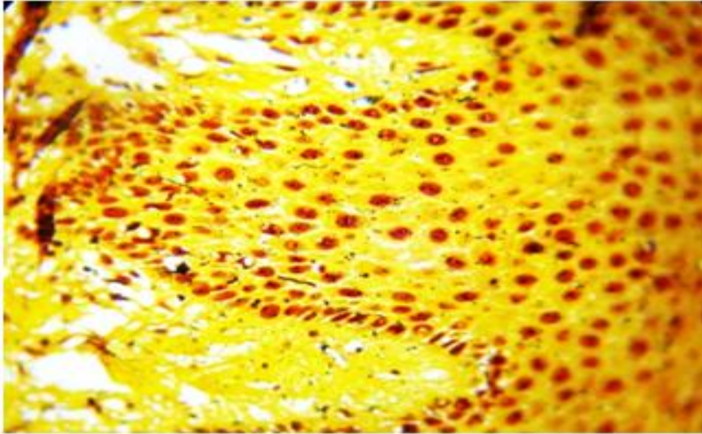

Figure 2: Photograph showing AgNOR staining in NOM (AgNOR stain x400)

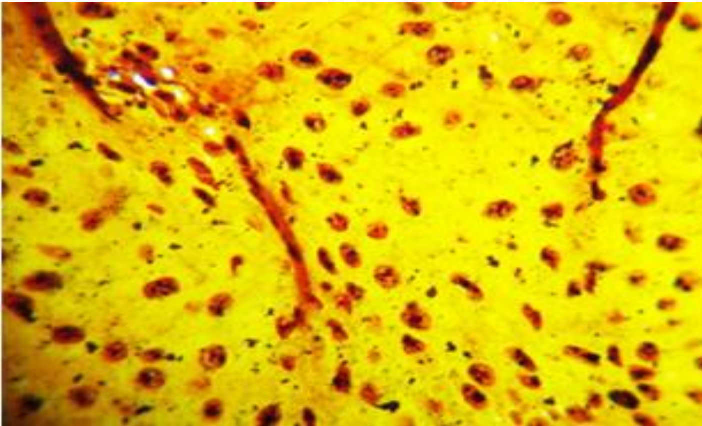

Figure 3: Photograph showing AgNOR staining in NDL (AgNOR stain x400)

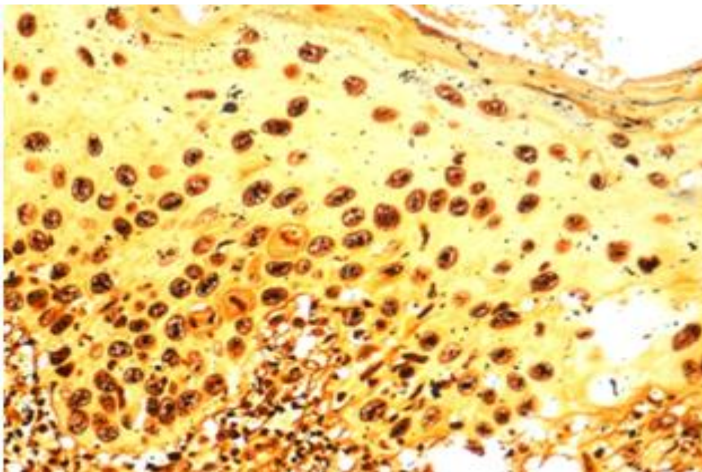

Figure 4: Photograph showing AgNOR staining in DL (AgNOR stain x400)

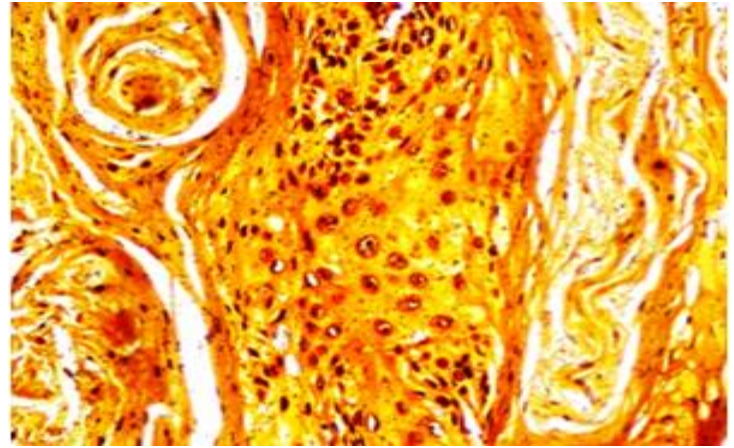

Figure 5: Photograph showing AgNOR staining in OSCC (AgNOR stain x400)

Results

AgNOR counts in NOM, NDL, DL and OSCC

The NORs when stained with silver nitrate appeared as dark brown to black dots or blebs within 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.

Mean Agnor Count Per Nucleus In Various Groups [Table 1]

Mean AgNOR count		N	Mean	SD	Mean Square	F	P value
	NORMAL	5	2.36	0.69	0.758	1.98	0.155
	NON DYSPLASTIC	15	1.79	0.37			
	DYSPLASTIC	15	2.20	0.99			
	OSCC	15	1.86	0.67			

The lowest AgNOR counts was seen in NDL with mean AgNOR count being 1.79±0.37/nuclei while the maximum expression was seen in NOM(2.36±0.69 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 (1.86±0.67 mean AgNOR/nuclei) than DL (2.20±0.99 mean AgNOR/nuclei).

[Table 1_]

Between oral squamous cell carcinoma, non-dysplastic, dysplastic group and normal healthy controls comparisons [Table 2]

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S.No.	Comparison	Mean Difference	SE	"p"
1.	NOM vs NDL	0.67	0.42	0.40
2.	NOM vs DL	0.25	0.42	0.96
3.	NOM vs OSCC	0.60	0.42	0.24
4.	NDL vs DL	-0.51	0.32	0.38
5.	NDL vs OSCC	-0.07	0.23	0.99
6.	DL vs OSCC	0.44	0.32	0.53

Maximum difference was observed between NOM and NDL groups (0.67±042) whereas minimum difference was observed between NOM and DL groups (0.25±0.42). The comparisons did not reveal a significant intergroup difference (p>0.05).[Table 2]

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

NDL ~ OSCC ~ DL ~NOM

Discussion

Proliferation is considered to be a fundamental biological process because of the role it plays in the growth and maintenance of tissue homeostasis. It is well understood that transition of the normal oral epithelium to dysplasia to malignancy is featured by increased cell proliferation. Discovery of various proliferation markers has enabled the detection of the hyperactive state of the epithelium and has been suggested to be of prognostic significance.

Epithelial dysplasia is characterized by a number of cell and tissue alterations, visible with a light microscope, that reveal an alteration of the cellular maturation in the epithelium and an increase of the proliferative suprabasal activity (for example, presence of mitosis in the upper half of the epithelium). The factor that has the most influence on the potential for malignant transformation of a premalignant lesion is the presence and severity of the epithelial dysplasia. Cellular kinetics has been extensively studied in oral epithelium using both monoclonal and polyclonal antibodies among which reported work on various proliferative marker deserve attention. [2]

The proliferative markers used in our study are AgNOR, with the aim to compare proliferative activity of potentially malignant and malignant oral lesions in order to identify lesions which are at a higher risk for malignant transformation.

In our study AgNOR counts did not show any significant differences amongst age, gender, tobacco habit, and size of the lesion. Similarly, no statistically significant difference was observed in counts of various study groups i.e. NOM, NDL, DL, and OSCC. [Table no:2] It was observed that the mean AgNOR count was highest in the NOM, the reason could be a small sample size, higher count in any one case would significantly increase the mean for the group. The NOM tissue was obtained from the pericoronal area at the time of third molar extraction. In our sample, the pericoronal tissue showed higher degree of inflammation in one particular case. It has been reported earlier that underlying inflammation can show increased AgNOR counts. [6] We were unable to arrange the normal mucosal tissue due to the ethical issues but healthy or uninflammed mucosa would have been idea for the control group. Although many studies have reported usefulness of AgNOR counts in distinguishing between premalignant and malignant lesions but [7],[8] statistically no significant difference was seen between leukoplakia and OSCC in our study group. Sousa et al has also reported similar findings, their study had shown no significant statistical difference in AgNOR counts between epithelial dysplasia and oral squamous cell carcinoma. [9]

Warnakulasuriya had reported that though AgNOR counts were significantly different between carcinoma and epithelial dysplasia, counts in each diagnostic group

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overlapped so much that they were of no practical value in distinguishing between individual lesions. [10]

In the present study we observed that the mean AgNOR count of OSCC group was lower than that of DL. AgNORs in OSCC were comparatively larger in size and many dots coalesced with each other hence we had to count them as single dots according to the counting criteria given by Crocker et al. [11] This could be the reason for lower values in OSCC. On the contrary, NORs were more dispersed and smaller in size in DL hence the absolute count was higher than OSCC. 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. [8],[12],[13]

Our study also supports this view as measuring area and perimeter of AgNORs would partly negate the error caused by overlapping and coalescing of individual dots. Several technical difficulties were encountered during this study and it was felt that the staining method must be meticulously established as regards the duration of staining, temperature, purity of water, reagents, etc. (all these parameters had a great influence on the final result). Authors have also reported that increased number of AgNOR dots were noticed when the sections were stained in the dark as compared to the number of dots which were seen when the staining was done in the day light.[14]

Other problems which can be faced during the AgNOR staining are: limited reliability and reproducibility; general background staining of tissue making weakly stained NORs difficult to identify and making NORs harder to resolve; staining of granules of cytoplasm that can be confused with NORs; black precipitates scattered over the slide that can be confused with NORs; fading of the sections often within days; poor staining with some tissue fixatives; and variation in staining intensity and configuration among NORs. Several authors have proposed variations in the original procedure to reduce these problems, including: distaining of slides before silver staining, pre or post incubation of sections with alcoholic acid, other acids, or glycine to reduce background staining, variations in solution concentration; increase in pH; variation in temperature; staining slide while inverted or coating the sections with collodion, to reduce precipitates; conversion of the silver precipitate to a dye complex; combination with other staining procedures; replacement of gelatin component with other colloids and use of sodium thiosulfate, and gold toning to improve permanence. [15]

These technical hindrances limit the usefulness of AgNOR technique in being employed as an objective marker for differentiating high risk oral lesions with those having lower risk of malignant transformation and our results support this view.

Conclusion

Based on the findings it can be concluded that AgNOR counts are of limited value if not supplemented with the Morphometric analysis.

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