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Research Article

The Significance of Quantification of AgNORs in Different Oral Mucosal Lesions

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Abstract

Aim: To evaluate the role of AgNOR stain (both mAgNOR and pAgNOR) in differentiating different oral mucosal lesions.

Methods: Total 100 oral biopsy samples were taken from January 2014 to December 2015 for this study. All the samples were processed for HandE and AgNOR stains. Then they were categorized into benign, premalignant and malignant groups based on histopathology report. Among the malignant cases, most common were oral squamous cell carcinoma (OSCC) and they were graded as I, II and III. After analyzing AgNOR dots, association of AgNOR dots with different oral lesions were calculated. The data was analyzed by using nonparametric method (Kruskal Wallis, ANOVA) for the intergroup comparisons.

Results: Out of 100 cases 28 cases were benign (Group-I), 20 cases were premalignant (Group-II) and 52 cases were malignant (Group-III) oral lesions. The AgNOR (mAgNOR and pAgNOR) status (count and size) were seen to be gradually changing from benign to premalignant and from premalignant to malignant lesions and showed significant association ($p < .001$) when different groups were compared. Similar findings of mAgNOR and pAgNOR were also found among different grades of OSCC.

Conclusion: This study concludes that AgNOR counts have significant association among benign, premalignant and malignant lesions of the oral cavity and also between different grades of OSCC. As AgNOR count is simple and cost-effective, it can be used routinely in histopathology laboratory for diagnosis of different borderline oral mucosal lesions.

Keywords: *AgNORs; Oral Squamous Cell Carcinoma (OSCC); Grading of SCC; Squamous Cell Carcinoma; Oral Cancer*

Introduction

Carcinoma of the oral cavity is considered as a major health problem in many parts of the world [1]. Worldwide, oral cancer is the sixth commonest causes of cancer-associated deaths, even though most of the people are not aware of its prevalence [1]. The incidence of oral cancer is relatively low in most western countries but the incidence is quite alarming in few developing countries in Southeast Asia like India, Pakistan, and Bangladesh. In those regions, oral squamous cell carcinoma (OSCC) is one of the most common forms of oral cancer [2]. Oral squamous cell carcinoma (OSCC) is responsible for over 95% of oral carcinoma cases and a percentage of these cases are

believed to develop from precancerous lesions like- leukoplakia, erythroplakia, and oral submucousal fibrosis etc [3,4]. Leukoplakia is the commonest form of the malignant oral lesions and may also potentially undergo malignant transformation ranging from 3 - 33% over 10 years [4]. As OSCC is preceded by premalignant lesion with dysplastic epithelium, evaluation of the degree of dysplasia is important for predicting the malignant transformation of these lesions [4]. Several studies have suggested that quantification of argyrophilic nucleolar organizer regions (AgNORs) can be an important parameter in tumor pathology [5-9]. AgNORs have been drawing much attention because of claims that their frequency within the nucleus has been significantly higher in malignant cells when compared with normal reactive and benign neoplastic cells [10]. In general, highly malignant tumor presents with an increased number of smaller sized AgNOR dots and benign or low-grade malignant tumors presents with few numbers of AgNOR dots of larger sizes [11]. AgNOR dots can also determine the histological grade of malignant tumors in many other organs including lung, skin, thyroid, cervix, breast etc [12-14]. AgNOR technique marks the proteins associated with the nucleolar organizer regions (NORs). NORs are actually the loops of DNA that transcribe for ribosomal RNA. The higher the number of NORs, the lower is the duration of the cell cycle and the higher the velocity of cell proliferation. Therefore the quantification of NORs is a good indicator of the proliferative activity of the cells that can predict the prognosis of tumors [15].

Purpose of the Study

The purpose of the present study was to find out the count of AgNORs dots (both mAgNOR and pAgNOR) in the benign, premalignant and malignant lesions of the oral cavity and also to find out the role of this AgNORs dot count in differentiating different grades of the malignancy.

Materials and Methods

This study was carried out in the department of pathology, SSMC and Mitford Hospital during the period of January 2014 to December 2015. Ethical clearance was obtained from the ethical committee of Sir Salimullah Medical College. Informed written consent was taken from all the patients after explaining the study procedure. An incisional or excisional biopsy was performed at the dental unit of Sir Salimullah Medical College and Hospital for collecting the samples from 100 patients having oral lesion indicated for biopsy. Then the specimen was preserved in 10% neutral buffered formalin. Gross examination of the specimen was done and biopsied tissue was processed according to routine histology slide preparation procedure. From each paraffin block, two unstained slides were prepared. One of the slides was stained with haematoxylin and eosin (H and E) stain and the other remaining slide was stained with AgNOR stain. The H and E stained slides were then examined under light microscope (Leica DM750M-German) and samples were categorized into three groups according to Gulia., *et al.* (Table 1) [16].

Group	According to Histopathological Report
I. Benign	Histopathologically normal oral mucosa
	Inflammatory lesions without dysplasia
II. Pre-malignant	Leukoplakia, Erythroplakia, Lichen planus
	Mild, moderate and severe dysplasia
III. Malignant	Squamous cell carcinoma (well differentiated)
	Squamous cell carcinoma (Moderately differentiated)
	Squamous cell carcinoma (poorly differentiated)
	Others

Table 1: *Categorization of the oral mucosal lesions according to benign, premalignant and malignant.*

The AgNOR stained slides were also examined under light microscope. From each slide, AgNOR dots were counted from hundred randomly selected cells. Individually detectable and separate black dots were counted and recorded. The average numbers of dots were calculated in each nucleus. Where two or more dots were closely related and were not clearly separated, they were counted as a single AgNOR dot. These criteria's for counting AgNOR dots were suggested by Crocker., *et al* [17].

Two types of counts were performed on AgNOR stained slides. One is average of the count of AgNOR dots per cell or per nucleus of the cell (mAgNOR) and the other one is a percentage of cells showing positive number of AgNOR dots in that particular slide. AgNOR represents the DNA content of the cell. As DNA content of the cell increases during the S phase of cell division, mAgNOR and as well as the pAgNOR both reflects the proliferative activity of the cells. The mAgNOR is the mean of AgNOR dots in 100 nuclei and it is expressed as arithmetic mean SD. The pAgNOR is the percentage of nuclei exhibiting five or more AgNOR dots in one nucleus and it is expressed in % SD. The pAgNOR dot count represents the proliferative activity of the tumor cells. Tumors having a pAgNOR count of 8% or above is considered to have high proliferative activity [18].

AgNOR variables (both mAgNOR and pAgNOR) determined on oral lesions were studied statistically. Associations among the mean values of benign, premalignant and malignant groups were tested by the nonparametric method. Kruskal Wallis, ANOVA is used for the intergroup comparisons. P value < 0.05 was considered as statistically significant. For statistical analysis, SPSS (version 20) were used.

Results

In the present study, biopsy samples from 100 patients were studied. After histopathological analysis by hematoxylin and eosin stain, 28 cases were categorized under benign (Group-I), 20 cases were categorized under premalignant (Group-II) and 52 cases were categorized under malignant oral lesions (Group-III). Both mAgNOR and pAgNOR count showed significant association among the three groups (Table 2 and Table 3).

Group	Median	Minimum	Maximum	QD	P
Benign	2	1	6	0.8	< 0.001
Premalignant	6	4	8	1.0	
Malignant	14	9	23	2.38	

Table 2: *The median, minimum and maximum count (pAgNor) vs. types of oral mucosal lesions.*

Group	Median	Minimum	Maximum	QD	P
Benign	1.41	1.05	2.54	0.36	< 0.001
Premalignant	2.34	1.98	2.78	0.23	
Malignant	3.58	2.33	4.76	0.51	

Table 3: *The median, minimum and maximum mAgNor vs. types of oral lesions.*

As the data is not following the normal distribution hence we use nonparametric method (Kruskal Wallis, ANOVA) to find whether there is any difference in the distribution of benign, premalignant and malignant conditions. Kruskal Wallis test shows there is statistically significant (p < .001) difference in the distribution of various types of oral lesions.

Pairwise comparison using Kruskal Wallis, ANOVA

Group	Medians	P
Benign Vs. Premalignant	2 Vs. 6	< 0.05
Premalignant Vs. Malignant	6 Vs. 14	< 0.001
Benign Vs. Malignant	2 Vs. 14	< 0.001

A pairwise comparison shows there is a statistically significant difference in the distribution of benign vs premalignant ($p < .05$), premalignant vs malignant ($p < .001$) and benign vs malignant ($p < .001$).

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Pairwise comparison using Kruskal Wallis, ANOVA

Group	Medians	P
Benign Vs. Premalignant	1.41 Vs. 2.34	< 0.05
Premalignant Vs. Malignant	2.34 Vs. 3.58	< 0.001
Benign Vs. Malignant	1.41 Vs. 3.58	< 0.001

A pairwise comparison shows there is a statistically significant difference in the distribution of benign vs. premalignant ($p < .05$), premalignant vs. malignant ($p < .001$) and benign vs. malignant ($p < .001$).

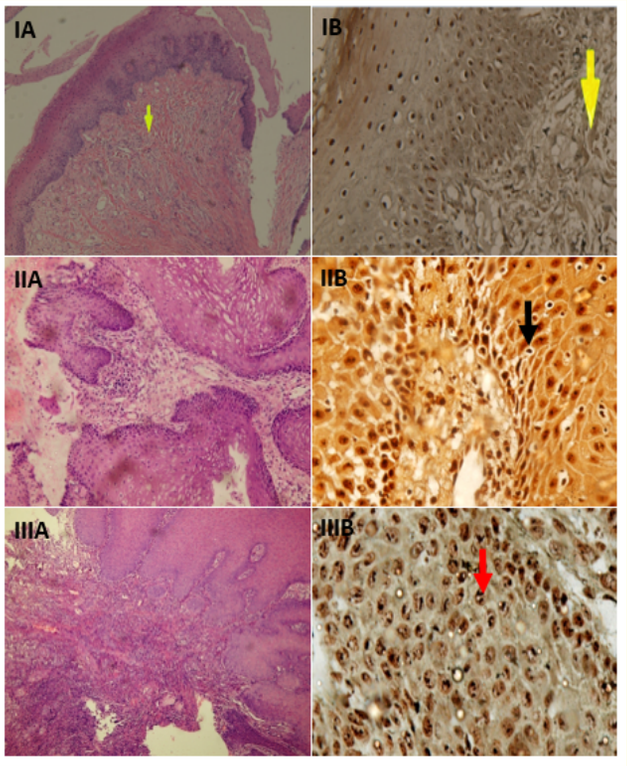

Figure 1: *IA: A Photomicrograph showing H & E stained Section of fibro epithelial polyp (H&E, x100). IB: Photomicrograph showing AgNORs in fibro epithelial polyp (yellow arrow) (AgNOR, x400).*

II A: A Photomicrograph showing H & E stained sections of moderate dysplasia (H&E, x100). IIB: Photomicrograph showing AgNORs in moderate dysplasia (black arrow) (AgNOR, x400).

IIIA: A Photomicrograph showing H & E stained sections of severe dysplasia (H&E, x100). IIIB: A Photomicrograph showing AgNORs in severe dysplasia (red arrow) (AgNOR, x400).

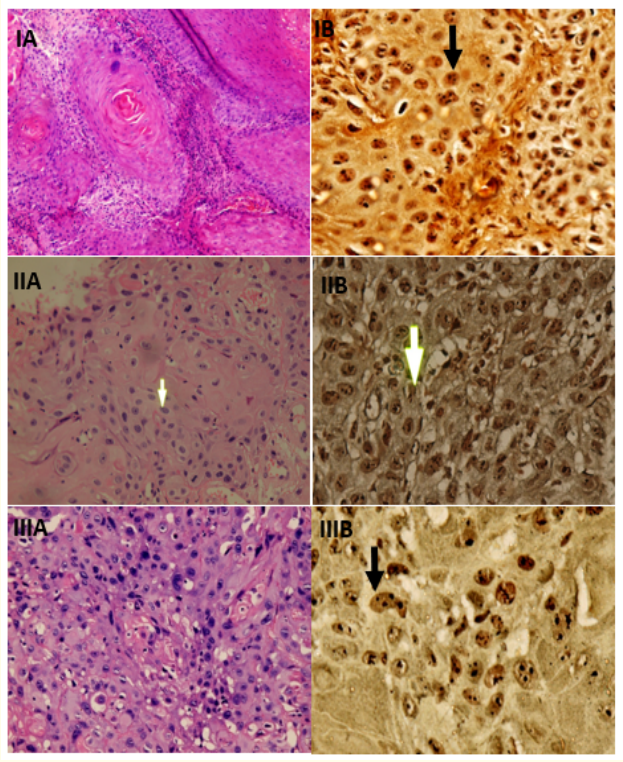

Figure 2: *IA: A Photomicrograph showing H & E stained sections of well-differentiated squamous cell carcinoma (H&E, x100). IIB: A Photomicrograph showing AgNORs in well-differentiated squamous cell carcinoma. (AgNOR, x400).*

IIA: A Photomicrograph showing H & E stained sections of moderately differentiated squamous cell carcinoma. (H&E, x100). IIB: A Photomicrograph showing AgNORs in moderately differentiated squamous cell carcinoma. (AgNOR, x400).

IIA: A Photomicrograph showing H & E stained sections of poorly differentiated squamous cell carcinoma. (H&E, x100). IIIB: A Photomicrograph showing AgNORs in poorly differentiated squamous cell carcinoma. (AgNOR, x400).

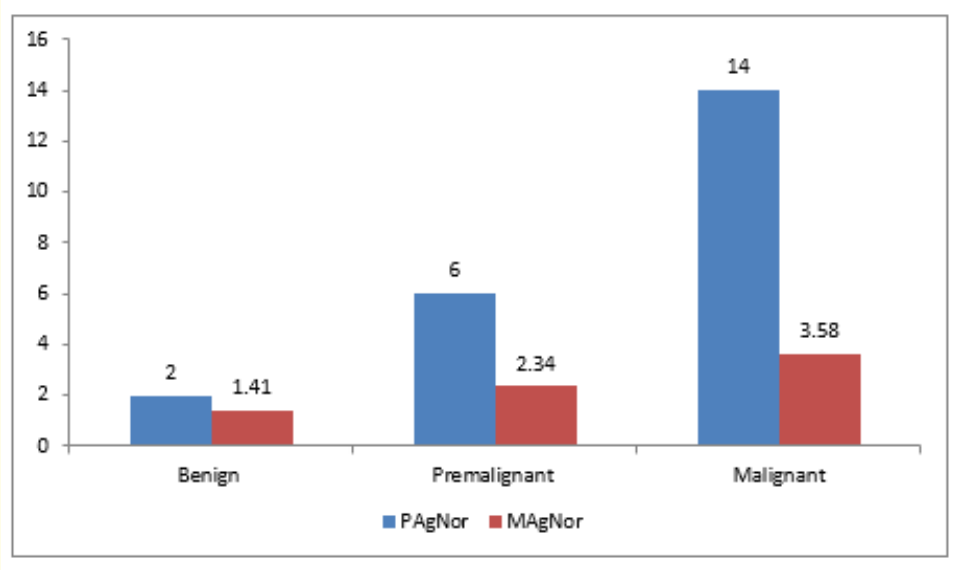

Figure 2: *Bar diagram shows significant association of pAgNor and mAgNor between benign, premalignant and malignant oral lesions.*

Pairwise comparison using Kruskal Wallis, ANOVA

Group	Medians	P
Grade 1 Vs.2	13 Vs. 15	< 0.01
Grade 1 Vs.3	13 Vs. 20	< 0.001
Grade 2 Vs.3	15 Vs. 20	NS

All 52 malignant cases were diagnosed as oral squamous cell carcinoma (OSCC) and among them, 23 cases were categorized under grade I, 19 cases were categorized under grade II and 7 cases were categorized under grade III. Both mAgNOR and pAgNOR count showed significant association among these three grades of oral squamous cell carcinoma (Table 4 and Table 5).

Group	Median	Minimum	Maximum	QD	P
Grade 1	13	9	15	1.5	< 0.001
Grade 2	15	11	23	2.0	
Grade 3	20	16	23	2.5	

Table 4: *Association between pAgNOR dots count and different grades of OSCC.*

Group	Median	Minimum	Maximum	QD	P
Grade 1	3.43	2.33	3.89	0.1	< 0.001
Grade 2	3.76	3.32	4.24	0.22	
Grade 3	4.14	3.89	4.76	0.11	

Table 5: *Association between mAgNOR dots count and different grades of OSCC.*

Pairwise comparison using Kruskal Wallis, ANOVA

Group	Medians	P
Grade 1 Vs.2	3.43 Vs. 3.76	< 0.001
Grade 1 Vs.3	3.43 Vs. 4.14	< 0.001
Grade 2 Vs.3	3.76 Vs. 4.14	NS*

**NS: Not significant*

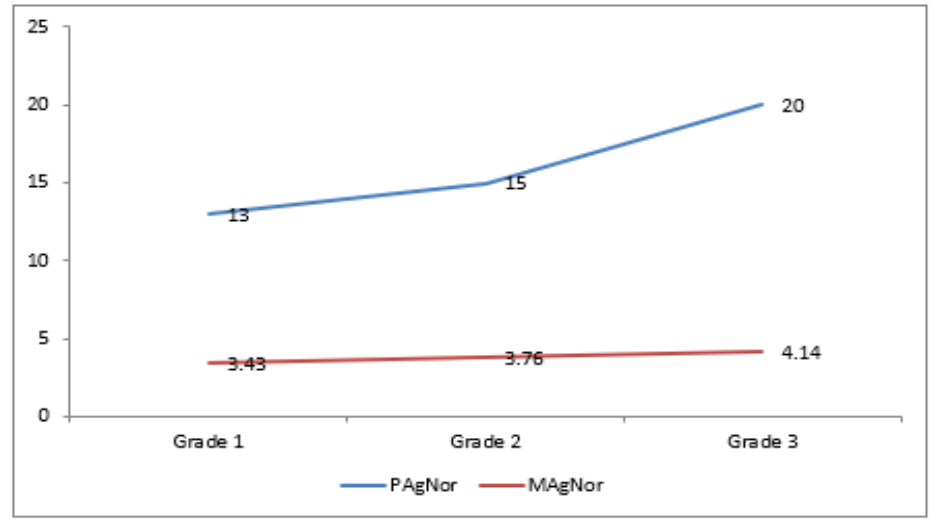

Figure 4: *Line diagram shows the trend of median values for grade 1, 2 and 3 oral mucosal lesions.*

Discussion

Dysregulated proliferation is regarded as a main characteristic feature of malignant neoplasms. It is predicted that the biological aggressiveness of a specific tumor is responsible for clinical course and prognosis of different cancers [19]. About 50% of oral squamous cell carcinomas (OSCC) are suspected to be associated with or preceded by leukoplakia of oral mucosa. Early detection and subsequent proper management of patients with oral leukoplakia may prevent the development of OSCC [20]. It was discovered that the count of AgNOR is high in malignant neoplasms compared to their benign counterparts and also to that of the normal tissue of origin [21]. This has been confirmed that there is an association between cell proliferation and AgNOR counting in OSCC. It indicates a useful and inexpensive method for detecting and measuring cell proliferation in OSCC that contributes similarly to proliferating cell nuclear antigen (PCNA) or Ki-67 expression [21-24]. But PCNA or Ki-67 is costly and not widely available.

The present study revealed highly significant mAgNOR count in OSCC (malignant) and leukoplakia (premalignant) compared to benign oral tumors. This finding was in concordance with the several previous studies as compared in table 6 [11,22,25,26].

Authors	mAgNOR count			'P' value
	Normal epithelium (Benign)	Leukoplakia (Premalignant)	OSCC (Malignant)	
Kobayashi., *et al*.	1.83 ± 0.66	2.52 ± 0.47	4.41 ± 0.82	< .05
Yue., *et al*.	1.67 ± 0.19	2.39 ± 0.59	3.58 ± 1.15	< .05
Manu., *et al*.	1.56 ± 0.42	2.61 ± 0.48	4.26 ± 0.96	< .05
Chattopadhyay., *et al*.	2.26 ± .32	2.69 ± .49	3.48 ± .42	< .05
Present study	1.52 ± 0.43	2.38 ± 0.26	3.86 ± 0.37	< .05

Whereas some investigators have reported increased mAgNOR count in cases of OSCC in their studies 6.2 ± 1.5 (Xie., *et al.* 1997), 8.04 ± 2.44 (Cabrini., *et al.* 1992) that contrasted to the present study [27,28]. This difference can be due to the automatic counting of dots. In the present study, mAgNOR was calculated manually.

Regarding pAgNOR count in the present study, the statistically significant difference was noted among benign, premalignant and malignant lesions ($p < .001$). Similar findings have been observed by Manu., *et al.* and Ashraf., *et al.* 2010 [24,26]. Comparison of the pAgNOR values between well differentiated, moderately differentiated and poorly differentiated carcinoma was also found significant in their studies ($p < .001$). Similarly, the pAgNOR count showed a significant association between different grades of OSCC in this present study which is concordance with Hildebrand., *et al* [29].

Conclusion

Based on the findings of the present study, it appeared that the AgNORs staining for the NOR associated proteins, can act as a marker for cell proliferation and thereby can detect the pre-malignant condition of the oral cavity. It is found to be a useful and inexpensive technique for measuring cell proliferation and progression in different grades of OSCC. Hence, the AgNOR count can be a used as a prognostic tool for various types of oral malignancies. It is also helpful in detecting an early malignant lesion guiding the treatment protocol. It has been highlighted that patients with the higher AgNOR count in their tumors should get special attention from the physician and should be subjected to careful follow-up and timely intervention.

We believe that more studies should be done in the future to investigate the role of AgNORs in diagnosis of different oral mucosal lesions with increase the sample size to confirm the results in our study.

Conflict of Interest

There is no any financial interest or any conflict of interest exists.

Bibliography

1. Ferlay J., *et al*. "GLOBOCAN 2002. Cancer incidence, mortality and prevalence worldwide". IARC Cancer Base (2002 estimates). Lyon: IARC Press (2004).

2. Moore SR., *et al*. "The epidemiology of mouth cancer: a review of global incidence". *Oral Diseases* 6.2 (2000): 65-74.

3. Freitas MD., *et al*. "Clinicopathologic aspects of oral leukoplakia in smokers and non-smokers". *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 102.2 (2006): 199-203.

4. Gupta PC., *et al*. "Epidemiology of cancer by tobacco products and the significance of TSNA". *Critical Reviews in Toxicology* 26.2 (1996): 183-198.

5. Kademani D. "Oral Cancer". *Mayo Clinic Proceedings* 82.7 (2007): 878-887.

6. Al-Rawi NH and Talabani NG. "Squamous cell carcinoma of the oral cavity: a case series analysis of clinical presentation and histological grading of 1,425 cases from Iraq". *Clinical Oral Investigations* 12.1 (2008): 15-18.

7. Tuccari G., *et al*. "Standardized use of the AgNOR method". *Journal of Oral Pathology and Medicine* 29.10 (2000): 526-527.

8. Lindner LE. "Improvements in the silver-staining technique for nucleolar organizer regions (AgNOR)". *Journal of Histochemistry and Cytochemistry* 41.3 (1993): 439-445.

9. Crocker J and Nar P. "Nucleolar organizer regions in lymphomas". *Journal of Pathology* 151.2 (1987): 111-118.

10. Newbold KM., *et al*. "Nucleolar organiser regions and proliferative index in glandular and squamous carcinomas of the cervix". *Journal of Clinical Pathology* 42.4 (1989): 441-442.

11. Chattopadhyay A and Ray JG. "AgNOR cut-point to distinguish mild and moderate epithelial dysplasia". *Journal of Oral Pathology and Medicine* 37.2 (2008): 78-82.

12. Adeyemi BF., *et al*. "A study of the utility of silver nucleolar organizer regions in categorization and prognosis of salivary gland tumors". *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 102.4 (2006): 513-520.

13. Alaeddini M., *et al*. "Argyrophilic proteins of nucleolar organizer regions (AgNORs) in salivary gland mucoepidermoid carcinoma and its relation to histological grade". *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 105.6 (2008): 758-762.

14. Hanemann JAC., *et al*. "Histologic grading and nucleolar organizer regions in oral squamous cell carcinomas". *Journal of Applied Oral Science* 19.3 (2011): 280-285.

15. Bukhari MH., *et al*. "Modified method of AgNOR staining for tissue and interpretation in histopathology". *International Journal of Experimental Pathology* 88.1 (2007): 47-53.

16. Gulia SP., *et al*. "The Role of Silver Staining Nucleolar Organiser Regions (AgNORs) in Lesions of the Oral Cavity". Journal of Clinical and Diagnostic Research 5.5 (2011): 1011-1015.

17. Crocker J., *et al*. "How should we count AgNORS? Proposals for a standardized approach". *Journal of Pathology* 158.3 (1989): 185-188.

18. Ploton D., *et al*. "Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level". *Histochemical Journal* 18.1 (1986): 5-14.

19. Piffkò J., *et al*. "Standardized AgNOR analysis of the invasive tumour front in oral squamous cell carcinomas". *Journal of Pathology* 182.4 (1997): 450-456.

20. Schepman K., *et al*. "Concomitant leukoplakia in patients with oral squamous cell carcinoma". *Oral Diseases* 5.3 (1999): 206-209.

21. Kanitakis J., *et al*. "Keratinocyte proliferation in epidermal keratinocyte disorders evaluated through PCNA/cyclin immunolabelling and AgNOR counting". *Acta Dermato-Venereologica* 73.5 (1993): 370-375.

22. Kobayashi I., *et al*. "The proliferative activity in oral epithelial dysplasia analyzed by proliferating cell nuclear antigen immunostaining and argyrophilic nucleolar organizer region staining". *Human Pathology* 26.8 (1995): 907-913.

23. Costa A de L., *et al*. "PCNA/AgNOR and Ki-67/AgNOR double staining in oral squamous cell carcinoma". *Journal of Oral Pathology and Medicine* 28.10 (1999): 438-441.

24. Ashraf MJ., *et al*. "Comparison of Ki67 and modified AgNOR in dysplastic epithelial lesion, primary squamous cell carcinoma and metastatic squamous cell carcinoma of head and neck: Comparison of Ki67 and modified AgNOR". *Basic and Applied Pathology* 3.4 (2010): 121-125.

25. Yue L., *et al*. "Evaluation of argyrophilic nucleolar organizer regions in tongue squamous cell carcinomas". *Oral Oncology* 35.1 (1999): 70-76.

26. Manu V., *et al*. "Value of Silver Binding Nucleolar Organiser Regions (AgNOR) in Squamous Cell Carcinomas of Upper Aero-digestive Tract". *Medical Journal Armed Forces India* 62.2 (2006): 123-128.

27. Xie X., *et al*. "Diagnostic and prognostic value of nucleolar organizer regions in normal epithelium, dysplasia, and squamous cell carcinoma of the oral cavity". *Cancer* 79.11 (1997): 2200-2208.

28. Cabrini RL., *et al*. "Morphometric study of nucleolar organizer regions in human oral normal mucosa, papilloma and squamous cell carcinoma". *Journal of Oral Pathology and Medicine* 21.6 (1992): 275-279.

29. Hildebrand LC., *et al*. "Evaluation of cell proliferation rate in non-dysplastic leukoplakias". *Medicina Oral Patologia Oral y Cirugia Bucal* 15.2 (2010): e328-334.

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