Research Article

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Analysis of Cell Proliferation Rate in Oral Leukoplakia and Oral Squamous Cell Carcinoma

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ABSTRACT

Background: This study was conducted to analyse the distribution of the silver stained Nucleolar Organiser regions (AgNOR) in leukoplakia(epithelial dysplasia) and oral squamous cell carcinoma (OSCC), and in their various histological grades, and to assess the role of AgNOR distribution, that if it could give information about the malignant potentiality in premalignant lesions and aggressiveness of the malignant lesions.

Material and method: The study specimens comprised of 70 archival cases, of which 30 cases were of leukoplakia and 40 cases of OSCC. The samples were stained by H&E and modified silver staining method of Ploton et al. for the Nucleolar Organiser Regions. The specimens were evaluated by the two different observers.

Results: The mean AgNOR count in leukoplakia was 2.80 ±0.50 and in cases of OSCC was 5.71± 1.08. The mean AgNOR count in leukoplakia cases of mild dysplasia was 2.59 ±0.66, in moderate dysplasia was 2.92± 0.43 and in severe dysplasia was 2.79. The mean AgNOR count in cases of well differentiated OSCC was 5.73± 1.62 and in cases of moderately differentiated OSCC was 5.67±1.19.

Conclusion: The mean AgNOR count was higher in cases of oral squamous cell carcinoma as compared to cases of leukoplakia(epithelial dysplasia), and the AgNOR counts increased with the increase in the grades of dysplasia indicating a higher proliferative rate with increase in dysplasia.

Keywords: Leukoplakia, AgNORs, squamous cell carcinoma, cell proliferation.

INTRODUCTION

Squamous cell carcinoma accounts for over 90% of oral cancer cases and a small percentage of these cases are thought to develop from precancerous lesions and conditions like leukoplakia, lichen planus, oral submucous fibrosis etc. Strikingly there is a difference in the incidence and the mortality rates across the world, and the highest rates were generally registered in developing countries like India, Pakistan and Bangladesh, where this is the most common form of cancer. In some parts of the world, including the Indian subcontinent, oral cancer is a major health problem accounting for about 10% of the estimated new cancers that occur in all parts of the body every year[1]. Therefore, a prompt and early diagnosis along with management of precancerous lesions and conditions is important. various methods have been used to identify the proliferation cells in tissue sections with the aim to

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use them as a marker for approaching malignancy, one among them is Silver binding Nucleolar Organiser region (AgNOR) technique. This is a simple one step staining technique which overcomes the disadvantages of other techniques such as requirement of sophisticated equipment, technical expertise, cost and time consumption[2,3]. Nucleolar Organiser Regions (NOR) are loops of DNA that encode ribosomal RNA and are considered important in the synthesis of protein[4]. They are located on the short arms of acrocentric chromosomes - 13, 14, 15, 21 and 22[5]. It has been suggested that the number of AgNORs in a nucleus may reflect the state of activation and the proliferation activity of the cells[6] and / or degree of malignant transformation of certain tissues[7]. The amount of AgNOR is related to the cell cycle, increasingly progressive from Go to S-phase and is proportional to the proliferative activity of neoplastic cells[8]. A rapidly dividing tumor population is more likely to have a greater proportion of cells in the early stages of G1 before individual NORs have associated and are therefore more likely to be observed in greater numbers. Conversely tumors with low rate of cell proliferation are more likely to display a single NOR (8). The silver staining technique neither identifies rRNA nor rDNA but the acidic proteins associated with these sites of rRNA transcription, these proteins are designated as B23, C23, 'AgNOR' proteins and RNA polymerase I . NORs have recently attracted much attention because of the claims that their frequency within the nuclei is significantly higher in malignant cells than in normal, reactive or benign neoplastic cells[9]. The aim of this study was to assess the cell proliferation rate with the use of the AgNOR technique in OL and OSCC. The objectives of the study were to analyze NOR related parameter in OL and its various histological grading and in OSCC and its various histological grading; and to assess the correlation in the difference between nucleolar organizer related parameters in OL and OSCC.

MATERIALS AND METHODS

The archival study specimens were taken from the outpatient subjects of the college Hospital, and comprised of 70 paraffin embedded tissue specimens, of which were 30 specimens of leukoplakia and 40 specimens of OSCC. The approval of the ethical committee of the college was obtained prior to the study. The paraffin embedded tissue specimens were sectioned using a Semi-automatic microtome and were subsequently stained with hematoxylin and eosin, and silver stain and mounted on glass slide using DPX as mounting media. The counting of stained cells was done under binocular microscope. The paraffin embedded archival tissue specimens of 30 specimens of leukoplakia were grouped histologically as no dysplasia, mild dysplasia (Fig. 1)moderate dysplasia (Fig. 2), severe dysplasia (Fig. 3), and that of 40 specimens of OSCC as grade I (Well differentiated) (Fig. 4), grade II (Moderately differentiated) , grade III (Poorly differentiated). Preparation of the AgNOR solution was done according to the method proposed by Ploton et al[11]. by mixing 1 *part of 'Solution A' with 2 parts of 'Solution B' (Solution A*-2g of Gelatin in 100ml of 1% Formic acid; Solution B-50% solution of Silver nitrate in distilled water). The solution was poured immediately after mixing on the slides and placed in a dark chamber for 40 minutes. The silver nitrate solution deteriorates on standing, so the solution had to be freshly prepared each time before use. AgNOR counting was done as per the method proposed by Giri et al[5], where in counting of all the specimens was done independently by two observers.200 cells were counted using a X100 objective, under oil immersion. The number of individually discernable and separable black dots in each nucleus was recorded and the average for each case computed; where two or more dots were so closely aggregated within a nucleolus that the precise number within the aggregate could not be counted, the aggregate was recorded as one. Inter-observer differences were recorded and cases with a difference in excess of one was reassessed. The average of that obtained by the two observers represented the final mean AgNOR count for a given case. This data was further statistically analysed. In cases of leukoplakia NOR's presented as small round dots which were aggregated within the nucleolus as compared with cases of OSCC where the NOR's presented with larger contours and were dispersed within the nucleolus which could be easily counted.

Statistical Analysis

The data collected was analysed independently by two observers and the average of both the values obtained by two observers was obtained for each case, and this was tabulated as the average AgNOR

count. The mean AgNOR count and the standard deviation (SD) were calculated in cases of leukoplakia and OSCC.

Table 1: Interobserver variation.

Groups	Total AgNOR count observer 1	Total AgNOR count observer 2	Statistical analysis
Leukoplakia	42.69	40.25	p<0.0045
Oral squamous cell carcinoma	114.51	109.75	p<0.0001

Table 2: Histological grades of oral leukoplakia and oral squamous cell carcinoma.

	Histological grading	No. of cases	AgNOR range	Mean AgNOR and standard deviation in different grades
Leukoplakia	mild dysplasia	10	2.14 – 3.66	2.59 ±0.66
Leukoplakia	Moderate dysplasia	18	2.45 – 3.63	2.92± 0.43
Leukoplakia	Severe dysplasia	02	2.79	2.79
Oral squamous cell carcinoma	Well differentiated	24	3.48-8.55	5.73 ±1.62
Oral squamous cell carcinoma	moderately differentiated	16	4.33-8.27	5.67 ±1.19

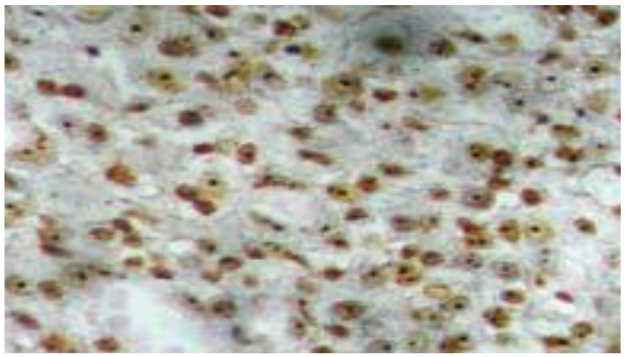

Fig 1: Photomicrographs of silver stained sections of oral leukoplakia (100x) - mild dysplasia.

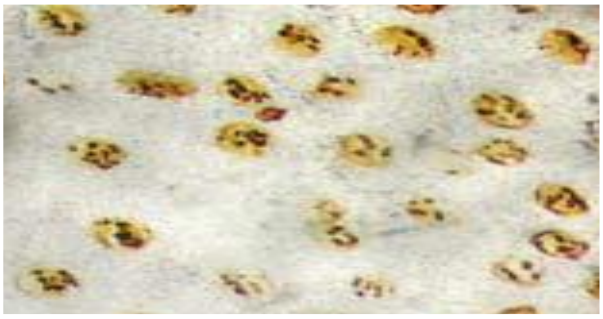

Fig 2: Photomicrographs of silver stained sections of oral leukoplakia (100x) - moderate dysplasia (1b).

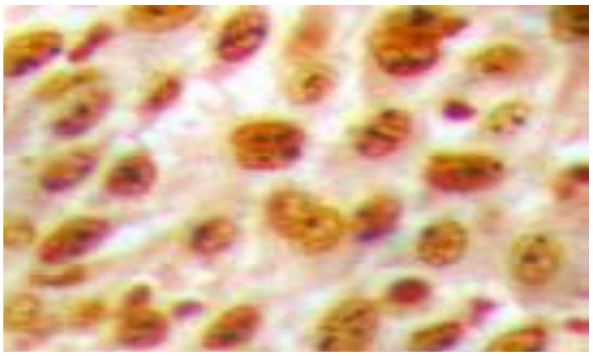

Fig 3: Photomicrographs of silver stained sections of oral leukoplakia (100x) - severe dysplasia

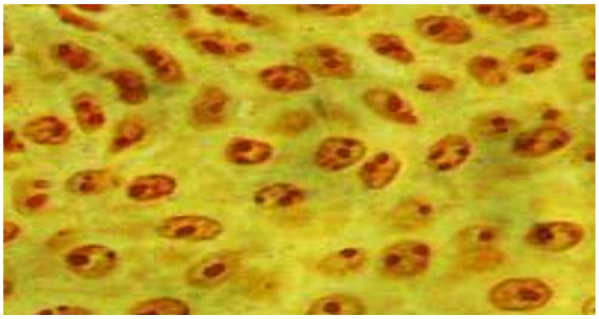

Fig 4: Photomicrographs of silver stained sections of oral squamous cell carcinoma (100x) - well differentiated

RESULTS

The inter observer variation between the two observers was calculated for both leukoplakia and OSCC after calculating the total AgNOR count in each group for each observer using the Pearsons correlation coefficient and was found to be highly statistically significant (p<0.0001) (Table 1). Correlation of the mean AgNOR counts in 30 Archival specimens of leukoplakia was 2.80±0.50 (20 males and 10 females; age range of 40 to 65years) and in 40 archival specimens of OSCC was 5.71±1.08 (10 males and 30 females; age range of

45 to 69years). Comparison of AgNOR count in leukoplakia and OSCC was done using the "t test" and was found to be highly statistically significant ($p<0.0001$). Comparison of AgNOR count in different grades of leukoplakia and in OSCC: The three groups in 30 specimens of leukoplakia was compared using the "ANOVA" test and showed statistically no significant results ($p> 0.51$). The two grades in 40 specimens of OSCC were compared using the "t" test and showed statistically no significant results ($p> 0.97$) (Table 2). Diagnostic validity of AgNOR count in predicting cancerous lesion in our study was done by establishing a cut-off value, where in a cut-off value of greater than 3.5 AgNOR count can be considered to differentiate between leukoplakia and OSCC.

DISCUSSION

The present study comparatively assessed the difference in the cell proliferation rates in the various histological grades of leukoplakia and OSCC by the AgNOR technique. The mean AgNOR count was higher in the cases of OSCC as can compared to cases of leukoplakia, with a statistically significant difference ($p<0.0001$) indicating a high proliferative activity in former cases. The mean AgNOR count increased as the grade of dysplasia increased in cases of OL, where as in the cases of OSCC there was no statically significant difference between well and moderately differentiated OSCC. The AgNOR count has been thought to be a proliferation marker; therefore one would find a difference in normal mucosa, leukoplakia and OSCC as reported in literature[12-16]. The inter observer variation between the two observers in our study was highly statistically significant ($p<0.0001$) indicating that the AgNOR counting method employed in our study is reliable. Diagnostic validity of AgNOR count in predicting cancerous lesion was estimated by determining the cut-off value of AgNOR between precancer and cancer. In our study a cut-off value of 3.5 has been suggested, an AgNOR value greater than 3.5 is suggestive of OSCC. In one study the cut-off value of 4.8 was proposed to differentiate between benign and malignant lesions in brush biopsy cases of suspicious lesions of oral cavity[17]. In another study a cut-off point of 2.37 was proposed to differentiate between nondysplastic and dysplastic OL[16]. Another study proposed that a mean AgNOR count >2.8 concurred with poor prognosis in OSCC and that T3 and T4 tumors >2.8 mean AgNOR counts were aggressive and may exhibit resistance to current treatment protocols[12]. The higher AgNOR counts found in many cancers may be due to dispersion of AgNORs in the nucleoplasm, so the AgNOR type may be a helpful in making such a distinction, whether this indicates increased risk of malignant transformation awaits longitudinal study[11]. The AgNOR dots in benign lesions shows a large, central, regularly contoured nucleolus containing single or multiple AgNOR dots, where as the malignant neoplasms had multiple small nucleoli with prominent AgNORs distributed throughout the nucleus[5] as seen in our study. As with most histochemical markers of malignant transformation, AgNOR would evidence the metabolic alterations associated with malignant transformation rather than characterize malignancy. AgNORs detect cellular alterations before morphologic expression. In one study the degree of keratinisation and nucleolar activity was assessed in smokers by obtaining cytological smears from the lateral aspect of the tongue using a wooden spatula and endo brush and also evaluated the depth of the cytological smears obtained by the two methods on the lateral aspect of the tongue. The samples were stained with papanicolaou and the silver stain. Smokers with clinically normal mucosa showed a greater percentage of keratinized cells and greater nucleolar activity, and the cytological smears obtained from the endo brush revealed deeper cells, suggesting that cigarette smoking influences the cellular activity of the mucosa of the lateral tongue[18]. In another study the percentage of the number AgNORs present in the nucleus was analyzed, and was proposed that the pAgNOR >1 or>2 reflects the size of the growth fraction as well as its proliferative activity. This is consistent with the observation that high proliferative activity coincides with poor prognosis. They proposed that the pAgNOR>1 were strong indicators with regard to disease recurrence and short survival in T1 and T2 OSCC[14]. In our study two cases of well differentiated (8.55, 8.55) and one case of moderately differentiated (8.27) OSCC showed high Mean AgNOR counts, which were well beyond the Mean AgNOR count of 5.73± 1.62 for well differentiated and 5.67±1.19 for moderately differentiated OSCC. The hematoxylin and eosin sections showed high mitotic figures even though they were well and moderately differentiated OSCC. Sano et al[19]. proposed that the 5-year survival rate

of cases with high AgNOR counts (≥6.5) was significantly lower than that of counts (<6.5). Therefore, they proposed that AgNOR counts might be a useful marker in assessing the prognosis of the malignant lesion. Based on this proposition the AgNOR counts in our study showing very high values may be marked as having poor prognosis. Therefore, the AgNOR method can be used to provide information on the malignant potentiality in premalignant lesions and aggressiveness of the malignant lesions. To draw substantial conclusions as to whether AgNOR counts indicate increased risk of malignant transformation awaits longitudinal study with larger samples.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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