Quantitative Assessment of Argyrophilic Nucleolar Organizer Regions in Nonsmokers, Smokers and Oral Submucous Fibrosis: A Pilot Study

GV Sowmya, BN Padmavathi, Mohitpal Singh, Prashant Nahar

ABSTRACT

Background: Nucleolar organizer regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods. After silver-staining, the NORs can be easily identified as black dots exclusively localized throughout the nucleolar area and are called AgNORs. AgNORs have been used to assess biologic aggressiveness of lesions, such as smokers, submucous fibrosis and squamous cell carcinoma of oral cavity.

Materials and methods: The study consisted of 42 individuals, of which 15 healthy controls who neither had any habit nor mucosal lesions, 15 were smokers with leukoedema and 12 were smokers with oral submucous fibrosis. The cytosmears obtained were subjected to the silver-staining method for AgNOR proteins. The difference in AgNOR counts were calculated among and between groups using the one-way ANOVA with post hoc and Scheffe's test.

Results: The mean AgNOR count ranged from 3.11 in normal, 5.13 in leukoedema to 6.73 in oral submucous fibrosis. When comparison of mean AgNOR counts was prepared between the normal and study groups, it was also found to be statistically significant (p < 0.05).

Conclusion: The mean AgNOR counts was higher in smokers with oral submucous fibrosis and leukoedema when compared to normal individuals without habits. This significant increase in AgNOR counts in smoker’s mucosa indicates changes that could be assessed at cellular level before any structural changes in the mucosa could take place.

Keywords: Argyrophilic nucleolar organizer region associated proteins, Cytosmears, Oral submucous fibrosis.


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Conflict of interest: None

INTRODUCTION

Oral cancer is the most common cancer and accounts for almost 40% of all cancers in the Indian subcontinent.1-3 During the long incubation period between the initiation of carcinogenic changes due to tobacco habits and the development of oral cancer, well-defined oral precancerous lesions occur. The progression of epithelial carcinoma has been broadly classified into normal, hyperplastic (nondysplastic), dysplastic and carcinoma. During this progression, the cells exhibit a variety of biochemical and genetic alterations and recent studies have concentrated on the evaluation of such alterations as diagnostic and prognostic markers of cancer.4-6

The most crucial factor in the cells during tumor progression is aberrations in their proliferation kinetics. Several different methods have been proposed to determine the proliferative rate in tumors. Silver staining of argyrophilic nucleolar organizer regions (AgNORs) is considered to be the best and most cost-effective marker to assess the proliferative behavior of a lesion.4,7,8

This study was conducted to assess the biologic behavior or aggressiveness of smoking tobacco habits on smoker’s mucosa with leukoedema and oral submucous fibrosis; by determining the mean number of AgNORs and comparing them with those of normal mucosa without any habits. This would help to determine the biologic aggressiveness of cells before and after presentation of oral mucosal lesions.

MATERIALS AND METHODS

Total number of 42 individuals were included from the Outpatient Department of Oral Medicine and Radiology and they were divided into three groups as follows: Group 1: The control group consisted of 15 individuals with normal mucosa without habits. Group 2: Consisted of 15 smokers with leukoedema. Group 3: Consisted of 12 smokers with oral submucous fibrosis.

Individuals with systemic and other oral mucosal diseases were excluded from the study. Each subject signed an informed consent form.

After clinical examination, they were asked to rinse their mouth with normal saline and the area to be sampled was wiped with sterile gauze to remove excessive saliva and surface debris. The smears were obtained with a cytobrush scraped firmly across the mucosa. The brush was rotated along the clean glass slide to transfer the sample. It was then fixed with 95% ethanol for 2 hours. Fixed smears were later subjected to the silver-staining method for AgNOR proteins as described by Ploton’s method9 (Table 1).

The AgNOR counting was established in 100 cells for each cytologic smear as proposed by Crocker et al.10 The cells were examined at 1000× magnification under oil immersion. AgNORs were strictly located within the nucleus and distinctly stained in black, visible as dots that were either
round or oval. Where two or more dots were closely aggregated within a nucleus, the aggregate was counted as single. Rest of the nucleus stained pale.

RESULTS

Number of cells studied was approximately 1,500 in group 1 (n = 15), 1,500 in group 2 (n = 15) and 1,200 in group 3 (n = 12) (Table 2). Mean and standard deviation of AgNORs in group I was 3.61 ± 0.27, in group II, i.e. smoker with leukoedema was 5.13 ± 0.73 and in group III, i.e. OSMF was 6.25 ± 0.25. The difference in AgNOR counts were assessed using the one-way ANOVA with post hoc and Scheffe’s test. A significant difference in AgNOR counts among study groups (p < 0.000) was also noted (Table 3).

When comparison of mean AgNOR counts was done between the three study groups, it was found to be statistically significant (p < 0.000) (Table 4).

DISCUSSION

Alcohol consumption and tobacco smoking are the most important risk factors in oral cancer. The mutagenic capacity of tobacco has been extensively studied. The particular action of these carcinogens that exert their effect from the surface of the mucosa induces the phenomenon known as ‘field cancerization’. The concept was introduced by Slaughter et al to explain the increased risk of malignant transformation in large areas of the epithelial lining of the upper aerodigestive tract. This hypothesis was based on the high incidence of second primary tumors or multifocal cancer and was proved by the demonstration of molecular changes in clinically healthy mucosa of smoking patients.

Furthermore, the sequential or simultaneous development of oral premalignant and malignant lesions in a single patient evidences progressive genotypic and phenotypic alterations associated to field cancerization. The search for markers of field cancerization before the appearance of premalignant morphological alterations is of biological interest and clinically relevant in terms of early diagnosis and prevention of oral cancer.

Several methods have been used in the past for identification of the proliferating cells in the tissue sections, such as the assessment of mitosis, use of DNA flow cytometry, autoradiographic methods, applications of DNA and RNA, and use of monoclonal antibodies to detect proliferation-related antigen, Ki 67, proliferating cell nucleolar antigen (PCNA) and silver-staining nucleolar organizer regions (AgNORs) staining of which AgNORs have attracted much attention.

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Steps</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Preparation of specimen</td>
<td>Cytological smears; cells are smeared, air dried. Sections are post-fixed in 95% ethanol and hydrated through graded alcohols to ultrapure water.</td>
</tr>
<tr>
<td>2.</td>
<td>Preparation of the staining solution</td>
<td>Two solutions are needed the first one (solution A) is a 2% gelatin solution dissolved in ultrapure water, to which formic acid is then added to make a final 1% solution; the second one (solution B) is a 50% silver nitrate solution in ultrapure water. The staining solution is obtained instantly by rapidly mixing one part of solution A with two parts of solution B into a glass cylinder. The staining solution (&lt;0.3 ml) is immediately poured on each slide. Staining takes 14 to 20 minutes at room temperature, depending on the desired intensity of the reaction. Although protection from light is not necessary, it is better to avoid direct sunlight during silver-staining. After staining, the solution is poured off and the slides are washed in several baths of ultrapure water, placed for 10 minutes in a 5% thiosulfate solution, washed again in several baths of ultrapure water, dehydrated and mounted.</td>
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<tr>
<td>3.</td>
<td>NOR silver-staining</td>
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<table>
<thead>
<tr>
<th>Groups</th>
<th>Approximate number of cells studied</th>
<th>Total number of AgNORs</th>
<th>Mean AgNOR</th>
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<tbody>
<tr>
<td>Group 1</td>
<td>1,500</td>
<td>5,428</td>
<td>3.16</td>
</tr>
<tr>
<td>Group 2</td>
<td>1,500</td>
<td>7,699</td>
<td>5.13</td>
</tr>
<tr>
<td>Group 3</td>
<td>1,200</td>
<td>7,505</td>
<td>6.25</td>
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<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Mean AgNOR</th>
<th>F-value</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Group 1</td>
<td>15</td>
<td>3.11-4.04</td>
<td>3.61 ± 0.27</td>
<td>99.658</td>
</tr>
<tr>
<td>Group 2</td>
<td>15</td>
<td>3.81-5.99</td>
<td>5.13 ± 0.73</td>
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<tr>
<td>Group 3</td>
<td>12</td>
<td>5.85-6.73</td>
<td>6.25 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>3.11-6.73</td>
<td>4.91 ± 1.17</td>
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</tbody>
</table>
Thus, when comparisons among different groups were made, it was found to be statistically significant indicating AgNOR count in control group to be significantly less, indicating increased proliferative activity in study group. When comparison between groups was done, it revealed significantly more AgNOR counts in groups 2 and 3 than in control group. AgNORs were also significantly higher in group 3 than in group 2. Significant increase in AgNOR counts in group 2 indicates changes that could be assessed at cellular level before any clinically obvious premalignant lesions appear indicating changes toward increased mitotic activity and a progression step toward hyperplasia, dysplasia and carcinoma.

**CONCLUSION**

On the basis of AgNOR count, it appears that NOR associated proteins act as markers of cell proliferation in normal appearing smoker’s mucosa and in premalignancies,

<table>
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<th>Groups</th>
<th>Mean</th>
<th>SD</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Group 1 vs group 2</td>
<td>1.5140</td>
<td>0.17814</td>
<td>0.000</td>
</tr>
<tr>
<td>Group 1 vs group 3</td>
<td>2.6355</td>
<td>0.18895</td>
<td>Significant</td>
</tr>
<tr>
<td>Group 2 vs group 3</td>
<td>1.1215</td>
<td>0.18895</td>
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Nucleolar organizer regions (NORs) are ribosomal DNA loops and associated proteins that run on the dense fibrillary components of cell nucleolus during interphase and are responsible for ribosomal RNA copy. NORs associated nonhistone proteins can bind silver ions and are selectively visualized by silver methods in routinely processed cytological samples. At light microscopy, the AgNOR proteins appear as well-defined black dots, which in interphase cells are exclusively located within nucleoli. These NOR associated proteins are RNA polymerase I (RPI), C23 protein (nucleolin), topoisomerase I, B23 protein (numatrin) and fibrillarin. C23 protein is the main silver binding NOR-associated protein, important in the handling of new RNA transcripts and may be involved in the kinase sequence of the cell cycle. Its phosphorylation endows it with strong argyrophil properties and it is a well-defined AgNOR protein. Topoisomerase I acts to diminish stress in the rDNA helix as it is ‘read’ by RNA polymerase I and in the past been regarded as a marker of cell proliferation status. B23 protein is one of the proteins first recognized as being associated with NORs and increases in mitogenic responses. Fibrillarin has been shown to be associated with U3 rRNA and may thus play a role in the cleavage.

It has been suggested that the number of AgNORs in nuclei might reflect their state of activation and ultimately degree of malignancy of lesion. In a resting or relatively inactive cell, the acrocentric chromosome bearing AgNORs orientate in close apposition to each other to form a central smoothly outlined nucleolus. On the other hand, in a proliferating cell, the chromosomal and the AgNOR distribution remains disorganized. This results in the formation of dispersed and multiple nucleoli. On this basis attempts have been made to distinguish benign from malignant lesions and differentiate between various degrees of malignancy. Interest in AgNOR proteins increased greatly around the end of 1980s following the observation that malignant cells frequently exhibit a greater AgNOR protein amount as compared with the corresponding benign or normal cells.

In the present study, the approximate number of cells studied in group 1 was 1,500 with a total number of 5,428 AgNORs and a mean value of 3.16 (Fig. 1), group 2 included 1,500 cells under study with a total number of 7,699 AgNORs and a mean value of 5.13 and group 3 included 1,200 cells under study with a total number of 7,505 AgNORs and a mean value of 6.25 (Figs 2 and 3).

**Table 4:** Comparison of the mean AgNOR counts and standard deviations and test of significance between the control and different groups

**Fig. 1:** The photomicrograph in high power (100x) view showing silver-staining method for AgNOR proteins visible as dark brown dots within the exfoliated cells from the healthy controls who neither had any habit nor mucosal lesions

**Fig. 2:** The photomicrograph in high power (100x) view showing silver-staining method for AgNOR proteins visible as dark brown dots within the exfoliated cells from the patients who were smokers having leukoedema
such as oral submucous fibrosis. Thus, AgNORs have a major role in screening and prevention of premalignancies and malignancies. Many studies have suggested the use of exfoliative cytology in an oral cancer prevention program for early diagnosis. Our study underlines the importance of AgNOR counts depicting changes at cellular level demonstrating aggressiveness in the mucosa before any demonstrable premalignant change using exfoliative cytology.

REFERENCES


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