Full Length Research Paper

Silver binding nucleolar organiser regions in oral submucous fibrosis, lichen planus, leukoplakia and squamous cell carcinoma

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Accepted, 6 November, 2009

This study was undertaken to assess the biologic aggressiveness of sub mucous fibrosis, lichen planus, leukoplakia and squamous cell carcinoma of the oral cavity by determining the mean number of AgNORs and comparing them with those of normal mucosa. The study sample consisted of total 93 subjects. Biopsy specimens of 26 squamous cell carcinomas, 22 sub mucous fibrosis, 15 lichen planus, 15 leukoplakia and 15 healthy oral mucosa were taken. AgNORs were assessed both quantitatively and qualitatively. The data were analyzed using Student's independent t-test. Quantitatively significant difference existed in the number of AgNORs between the normal mucosa, premalignant lesions and carcinomas. AgNOR quantity is strictly proportional to the proliferative activity of the cell and does not necessarily indicate malignancy. It is the qualitative characteristics of AgNOR that help to differentiate premalignant and malignant lesions.

Key words: AgNORS, nucleolar organizer regions, oral cancer, potentially malignant disorders.

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of ribosomal DNA (rDNA) that occur in the nucleoli. The NOR DNA possesses ribosomal DNA genes which are transcribed by RNA polymerase I and ultimately direct ribosome formation and protein synthesis. As rRNA molecules are the main sites of protein synthesis, the number of NORs in each cell nucleus reflects the cellular activity.

NORs can be readily identified with the help of a silver staining technique by which they are visualized in the nuclei of cells as brown or black dots by virtue of argyrophilia of NOR associated proteins which are believed to include B23, C23 and RNA polymerase I. The silver binding by NORs has been attributable to the above mentioned acidic, non-histone proteins which are selectively stained. The amount of silver deposited within the nucleolus in this cyto-chemical reaction is a reflection of the transcriptional activity of the ribosomal genes. It has been suggested that the number of argyrophilic nucleolar organizer regions (AgNORs) in nuclei might reflect their state of activation and ultimately degree of malignancy of lesion. Giri et al. (1985) pointed out that 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

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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.

Elangovan et al. (2008) conducted a study and found quantitatively significant difference in the number of AgNORs between the normal mucosa, inflammatory lesions and carcinomas, but the premalignant lesions failed to differ significantly from the normal mucosa.

This study was conducted to assess the biologic aggressiveness of sub mucous fibrosis, lichen planus, leukoplakia and squamous cell carcinoma of the oral cavity by determining the mean number of AgNORs and comparing them with those of normal mucosa and also to evaluate the significance of number and dispersal pattern of AgNORs in determining the malignant potential of potentially premalignant disorders.

**MATERIALS AND METHODS**

For the present study, 93 subjects were selected randomly reporting to the department of Oral Medicine and Radiology, Government Dental College, Nagpur, Maharashtra, India and divided into 3 groups:

**Group 1:** The control group consisting of 15 individuals with healthy oral mucosa.

**Group 2:** Consisted of individuals with premalignant lesion or condition, which has further divided in to 3 sub groups:

**Group A:** Consisting 22 cases clinically and histopathologically confirmed oral sub mucous fibrosis.

**Group B:** Consisting 15 cases of clinically and histopathologically confirmed diagnosis of lichen planus.

**Group C:** Consisting 15 cases of clinically and histopathologically confirmed diagnosis of leukoplakia.

**Group 3:** Comprised of 26 cases of clinically and histopathologically confirmed diagnosis of leukoplakia.

Each subject signed the detailed consent form. After clinical examination, subjects were referred for routine hematological investigations. Punch biopsies from the lesion were taken and fixed in 10% formalin, they were processed and embedded in paraffin. Histopathological diagnosis was done and 5 micrometer thick unstained sections of the same were stained with silver nitrate solution. Counter staining was carried out with Mayer’s haematoxylin solution.

**AgNOR counting**

50 nuclei were randomly examined with a 100 × oil immersion objective, by the method proposed by Giri et al. (1985). Number of individually discernible and separate black dots in each nucleus was recorded and the average for each case computed. Where two or more dots were so closely aggregated with in a nucleus that the precise number within the aggregate could not be counted, the aggregate was counted as one as shown in Figure 1. AgNORs were visible as black dots whereas nucleoli stained light brown.

Three types of AgNOR patterns were identified, as defined by Warnakulasuriya et al. (1993), in histological sections. Type 1: Single or few large dots with in the nucleolus. Type 2: Discrete small dots with in the nucleolus. Type 3: Numerous small dots dispersed through out the nucleoplasm. Mean AgNOR values were calculated for each case and group means were derived. Statistical analysis of the data obtained was performed using Student’s independent t-test.
Table 1. Showing mean AgNOR counts with standard deviation of control group, Oral Sub Mucous fibrosis, Lichen planus, Leukoplakia and Squamous cell carcinoma groups.

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Control group</th>
<th>Oral sub mucous fibrosis</th>
<th>Lichen planus</th>
<th>Leukoplakia</th>
<th>Malignant group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers</td>
<td>15</td>
<td>22</td>
<td>15</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>Mean</td>
<td>1.64</td>
<td>2.55</td>
<td>2.54</td>
<td>2.9</td>
<td>3.78</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.10</td>
<td>0.47</td>
<td>0.60</td>
<td>0.58</td>
<td>0.78</td>
</tr>
</tbody>
</table>

**RESULTS AND OBSERVATION**

**Quantitative**

The mean AgNOR count of the control group, oral submucous fibrosis, oral lichen planus, oral leukoplakia and squamous cell carcinoma are shown in Table 1. The pooled mean AgNOR count (±S.D) of the control group was 1.64 (±0.10) and that of the premalignant group was 2.64 (±0.54). The control group thus, had lower AgNOR counts than that of the premalignant group.

When student t test was applied to compare the differences between the pooled mean AgNOR count of the control group with that of the premalignant group, it was found to be significant (t = -4.35, df = 65, p < 0.01). Comparison of the control group with that of the malignant group was also found to be significant (t = -6.9, df = 39, p < 0.01). Comparison of the premalignant group with that of the malignant group was also found to be significant (t = -7.6, df = 76, p < 0.01).

Amongst the premalignant group, leukoplakia showed to have the highest pooled mean AgNOR count (2.9 ± 0.58) followed by oral sub mucous fibrosis (2.55 ± 0.47) and lichen planus (2.54 ± 0.60). In 46.66% cases of leukoplakia, 26.66% of lichen planus and 9.09% of oral sub mucous fibrosis cases, the number of AgNORs was found to be above 3 per nucleus.

**Qualitative**

The morphological assessment of AgNORs based on their size, shape and the pattern of distribution revealed certain differences and characteristics among the various study groups. In case of control (Group I), the individual AgNOR dots were medium sized, uniformly round or oval (Figure 1) and belonged to the Type I pattern of distribution. In case of submucous fibrosis (Group A), lichen planus (Group B) and leukoplakia (Group C), the AgNORs were not so uniformly round or oval (Figures 2, 3 and 4, respectively). Some of them were slightly larger in size, irregular in shape and exhibited mixed Type I and II patterns of distribution. This variation in size and shape appeared to be due to overlapping/clumping of the otherwise discrete and smaller AgNOR dots seen in Type II. In case of SCC, the AgNORs were distributed throughout the nucleus and predominantly belonged to the Type III pattern of distribution (Figure 5).

**DISCUSSION**

The first observation of the nucleolus is attributed to...
Figure 4. High power micrograph (1200 ×) showing nucleoli in Oral Lichen Planus, bearing 3 - 7 AgNORs.

Figure 5. High power micrograph (1200 ×) showing nucleoli in Oral Leukoplakia, bearing 4 - 7 AgNORs.

Fontana in 1781 and silver staining was first described by Good Pasture and Bloom in 1975. Hubbel et al. (1977) carried out a study to identify NORs in normal and neoplastic cells by the silver staining method.

Nucleolar organizer regions are focal aggregates of intranuclear non-histone proteins that are associated with potential sites of ribosomal DNA transcription. These proteins are easily localized by virtue of their argyrophilia, hence the acronym AgNOR (argyrophilic nucleolar organizing region). The mean number of AgNORs per nucleus accurately correlates with mitotic rate in tumor cell lines. Most of the studies on AgNOR have been done focusing on its application in neoplastic and related conditions only. A thorough search of the dental literature does not show the application of this technique for studying more than one commonly occurring potentially malignant disorders and its comparison with the normal. With this in mind, this study aimed to assess AgNORs in normal tissue and in the commonly occurring potentially malignant disorders and malignant oral lesions. Special attention was given to assess AgNORs both quantitatively and qualitatively.

Elangovan et al. (2008) was unable to found significant difference in the number of AgNORs between the normal mucosa and the premalignant lesions but in contrast our study found significant difference in the number of AgNORs.

It was observed that the pooled mean AgNOR counts gradually increases from the control towards the malignant group. This clearly indicates that the AgNORs increase in number with increased mitotic activity. Though they are not of much use in distinguishing between different lesions, they certainly are of great value in determining the prognosis of an individual case. The leukoplakia had highest pooled mean AgNOR counts as well as highest percentage of AgNORs above 3 per nucleus. It has been recognized as the most frequent pre-cancerous lesion of the mouth. The lesions of leukoplakia in this study had comparable and even higher AgNOR counts than some of the cases of malignant group. It indicates that certain leukoplakic lesions have mitotic activity comparable or even greater than that of squamous cell carcinoma. Hence whenever a leukoplakic lesion is encountered, before initiating the treatment, its biological aggressiveness should be evaluated by the AgNOR technique.

Though the pooled mean AgNOR counts of oral sub mucous fibrosis (2.55 per nucleus) was higher than those of lichen planus (2.54 per nucleus), the percentage of AgNORs above 3 per nucleus was much higher in lichen planus (26.66%) as compared to oral sub mucous fibrosis (9.09%). This study does indicate that lichen planus is more liable to malignant transformation than oral sub mucous fibrosis.

Qualitative assessment of AgNORs based on their size, shape and pattern of distribution showed significant difference among the different study groups and these findings seem to give more information about the malignant status of the lesions. The appearance of the individual AgNOR dots in Groups 1 were regular and appeared uniformly round or oval. On comparing the normal mucosa with the premalignant group, there were noticeable differences in the appearance of the individual dots. AgNOR dots in the premalignant group comprised of mixed Type I and II patterns of distribution and were not uniformly round or oval as in normal mucosa. They were of slightly different shapes and sizes. In the malignant lesions, AgNORs were fragmented and smaller in size and their abnormal aggregation had resulted in
significant AgNOR pleomorphism, with atypical appearances like the signet ring or spidery web appearance. In addition, squamous cell carcinomas exhibited a predominantly Type III pattern, with AgNORs distributed throughout the nucleus.

As our study has shown that there is no much difference in AgNOR count in all potentially malignant disorders but it is the size, shape and pattern of distribution which showed significant difference among the different study groups so from the present findings we conclude that AgNOR counts alone can not distinguish between control group, potentially malignant disorders and malignant lesions, rather it is the qualitative characteristics that are more important in assessing cellular changes occurring in premalignant and malignant lesions and in distinguishing them from the normal.

REFERENCES


