

Nucleolar organizer regions in a chronic stress and oral cancer model

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Abstract. This study aimed to examine the role of chronic restraint stress (RS) on oral squamous cell carcinomas induced by 4-nitroquinoline-1-oxide (4-NQO) in CF-1 mouse tongues, measured by the expression of argyrophilic staining of nucleolar organizer regions (AgNOR). Thirty one samples of lingual epithelial tissue of CF-1 mice with a diagnosis of oral squamous cell carcinoma (OSSC) were assigned to two experimental groups: the RS/4-NQO group, where animals received RS and induction of oral chemical carcinogenesis (n=17); and the 4-NQO group, where animals received induction of chemical carcinogenesis without restraint stress (n=14). The mean number and distribution pattern of AgNOR were recorded. The mean AgNOR number per cell was found to be slightly higher in the 4-NQO group. AgNOR in the RS/4-NQO group revealed a higher tendency to be arranged in a clumped distribution compared to the 4-NQO group. No statistically significant difference was found between the groups. In conclusion, the induction of chronic restraint stress in CF-1 mice does not increase the number or affect the distribution pattern of AgNORs in OSSC induced by 4-NQO.

Introduction

A number of studies have demonstrated a positive association between stress and cancer (1,2). However, using conventional microscopy analysis, we recently showed that this correlation does not appear to apply to chronic stress and oral squamous cell carcinoma (OSCC) (3).

The initial changes that occur at the cell nucleus are not detected by the conventional H&E staining technique (4). Changes in the cell nucleus can be detected early through analysis of the nucleolar organizer regions (NORs) by silver staining, used to evaluate proliferative and malignant activities. In this manner, benign tumors are distinguished from malignant ones, and tumor grade and prognosis are determined (5-8). NORs are loops of DNA occurring within nucleoli that encode for ribosomal RNA. NORs are closely associated with non-histone proteins, known as argyrophilic staining of nucleolar organizer regions (AgNOR), which may be visualized by a histochemical technique relying on their argyrophilic properties.

The AgNOR number is directly proportional to the speed of the cell cycle. For this reason, cell proliferation has a prognostic value, since high proliferative activity is associated with poor prognosis (9). Various AgNOR parameters have been established to objectively evaluate the variations that occur between one tissue condition and another. These include number and distribution pattern (the most commonly used parameters), as well as mean nucleus area, size, shape and AgNOR location, which have proven to be significant factors in previous studies on oral mucosa lesions (10).

The number of AgNORs per nucleus has been widely used and described in the literature (6); this parameter varies in the nucleoli according to the transcriptional activity of ribosomal RNA (11). Various studies have linked higher levels in the number of AgNORs with the degree of malignancy of the lesions (4,8,12,13) and with poor prognosis (13).

In terms of their distribution pattern, AgNORs have been suggested to form groups or clusters of malignant lesions when compared with benign tumors (8,13,14). Two distribution parameters have been established: clustered and dispersed. When AgNORs have points that are in contact, they are considered to be clustered, whereas those that have no points of contact are classified as dispersed (8).

The aim of this study was to analyze the number and distribution pattern of NORs using the AgNOR staining technique on samples collected in a previous study by Rivera *et al* (3) that combined a model of chronic stress and chemical carcinogenesis in CF-1 mouse tongues.

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Table I. AgNOR analysis in OSCC tongue lesions induced by 4-NQO.

Treatment	Samples	AgNORs in OSCC		
		AgNOR number by cell (mean \pm SD)	AgNOR distribution pattern	
			Clustered	Dispersed
RS/4-NQO	14	2.41 \pm 0.45	9	5
4-NQO	17	2.44 \pm 0.56	7	10

RS, restraint stress; 4-NQO, 4-nitroquinoline 1-oxide; OSCC, oral squamous cell carcinoma; AgNOR, argyrophilic staining of nucleolar organizer regions.

Materials and methods

A retrospective study design was used to conduct this investigation. The independent variable was stress exposure; the dependent variables were the number and distribution of NORs.

Samples. This study involved 31 cases from a previous investigation (3), which examined the role of chronic restraint stress on the incidence and severity of lingual SCC induced by 4-nitroquinoline-1-oxide (4-NQO) in CF-1 mice (Fig. 1). The study was approved by the Bioethics Committee of the University of Talca.

The samples belonged to two experimental groups. The RS/4-NQO group (n=17) received two treatments: restraint stress and induction of chemical carcinogenesis. The 4-NQO group (n=14) received induction of chemical carcinogenesis without restraint stress. In all cases, we evaluated NORs in tumor cells (AgNOR count and distribution) using a semi-quantitative method.

AgNOR staining. The AgNOR staining technique was applied to all paraffin-embedded specimens, with some modifications to the method reported by Ploton *et al* (15). The working solution consisted of two parts 50% silver nitrate solution and one part mixture of 2% gelatin and 1% formic acid, prepared immediately prior to the staining procedure. Sections were hydrated in decreasing concentrations of alcohol, rinsed in distilled water and subsequently incubated in freshly prepared working solution for 30 min at room temperature in the dark. Sections were then washed in distilled water for 1 min, dehydrated in increasing concentrations of alcohol, cleaned and mounted in Disterine Plastiser Xylene (DPX) medium. NORs were counted in tumor cells, appearing as brownish black intranuclear dots on a pale yellow background.

Number and distribution of AgNORs. In the areas of squamous cell carcinomas, the plates were photographed at a magnification of x100, using a Micrometrics® camera (Micrometrics® 122CU 1.3-megapixel 1/2") attached to an optical microscope. Individual microphotographs were morphometrically evaluated using a semi-automatic electronic image analyzer (Microscope Software AxioVision LE), for which measurements were performed in 2 fields of 100 cells each to record the mean number of AgNORs per cell and the distribution pattern

of AgNORs. Fig. 2 shows how the counting was performed manually using the software.

Analyses were performed by an oral pathologist (B.V.) from the University of Talca. The observer was unaware of the group to which the study samples belonged (single-blinded). We determined the number and distribution pattern of AgNORs per cell, according to the categories used in a previous study (8): clustered, AgNORs in contact; and dispersed, no AgNORs in contact.

Statistical analysis. Qualitative data were analyzed using the Chi-square statistical test with Pearson's correlation. Quantitative data were studied using the Mann-Whitney U test. $P \leq 0.05$ was considered to be statistically significant for all tests.

Results

Severity of invasive carcinoma according to the analysis of AgNORs. Restraint stress did not increase the severity according to the number of AgNORs per cell of 4-NQO-induced OSCC tongue lesions in CF-1 mice. Table I shows that the two groups of mice demonstrated similar results for the number of AgNORs per cell. The RS/4-NQO group had a mean of 2.44 AgNORs per cell, compared to 2.41 for the 4-NQO group. The Mann-Whitney U test was used to evaluate the difference between the groups. No statistically significant differences were found between the groups ($p=0.97$).

Restraint stress did not increase the severity according to the distribution pattern of AgNORs of 4-NQO-induced OSCC tongue lesions in CF-1 mice. Fig. 3 shows the distribution pattern prevailing in each group. The RS/4-NQO group revealed 9 (64.3%) AgNOR cases with a clustered distribution pattern and 5 (35.7%) dispersed ones. The 4-NQO group had 7 (41.2%) clustered and 10 (58.8%) dispersed cases (Table I). The Chi-square test with Pearson's correlation revealed that the difference between the two groups was not statistically significant ($p=0.2$).

Discussion

The histochemical technique with silver staining has been used in a number of investigations since the early 1980s (16) for the purpose of visualizing NORs found in acrocentric chro-



Figure 1. (A) Mouse tongue lesions induced by 4-NQO (4-nitroquinoline-1-oxide). (B) Oral squamous cell carcinoma induced by 4-NQO.

mosomes. Since its beginnings, this technique has undergone various changes under the enhanced expression of AgNORs, in an attempt to eliminate or reduce those artifacts that may interfere with viewing them.

The application of morphological methods to study cell proliferation is affected by various factors that can escape any observer (13). During the observation of samples in this study, background staining was detected that hindered to a certain extent the process of analysis and identification of AgNORs, which is consistent with observations described by Lindner *et al* (17).

It has been reported that the number of AgNORs per cell increased, and this increase is associated with significant changes in the tissue (6).

In a similar study conducted by Venegas *et al* (18), the mean number of AgNORs per cell in the tongue epithelium of healthy CF-1 mice was found to be 2.1. Our values were slightly higher, with a mean of 2.44 for mice in the 4-NQO group and 2.41 in the RS/4-NQO group.

Notably, although a higher number of AgNORs per cell was expected in plaques of mice with cancer and stress, the results demonstrated otherwise. This finding may be correlated with the distribution adopted by the AgNORs, as there was a greater tendency for the AgNORs to cluster and overlap,

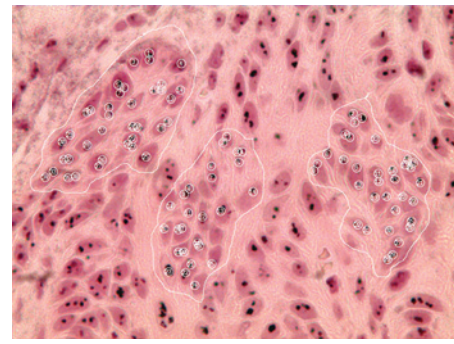


Figure 2. Example of manual counting of AgNORs (argyrophilic staining of nucleolar organizer regions) using the software.

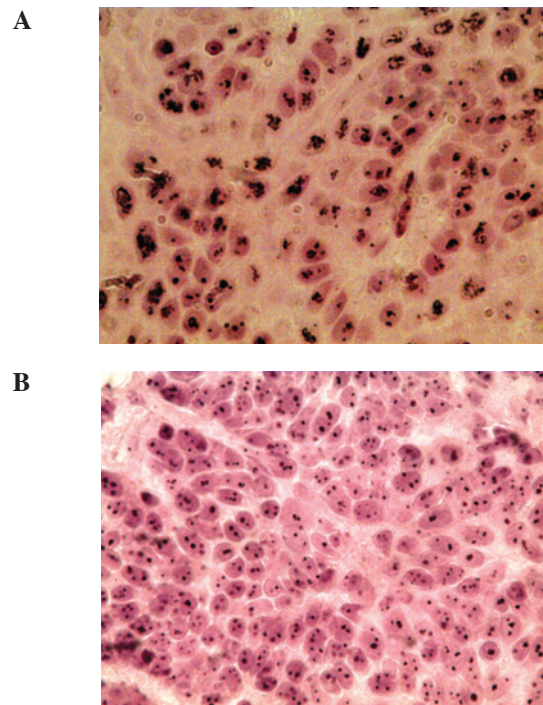


Figure 3. AgNOR distribution pattern. (A) RS/4-NQO group, predominantly clustered pattern. (B) 4-NQO group, mostly dispersed pattern. No significant difference was observed between mice in the RS/4-NQO and 4-NQO groups. RS, restraint stress; 4-NQO, 4-nitroquinoline 1-oxide; AgNOR, argyrophilic staining of nucleolar organizer regions.

and after counting, one unit of a group of AgNORs could not be individually identified.

The second parameter used in this study was the pattern of AgNOR distribution, for which a classification was selected due to its simplicity and easy application (8). According to this analysis, the clustered distribution corresponded to 64.3% in the RS/4-NQO group. In mice in the 4-NQO group there was a predilection for the dispersed distribution pattern, observed in 58.8% of all specimens in this group. However, in spite of this finding, and although there was a slightly higher trend towards a grouped distribution in the cancer group receiving stress treatment, no statistically significant difference was noted when comparing the distribution patterns between the two groups of mice.

Although the AgNOR technique is described by certain authors as being simple, cost-effective and easy to perform (12,13,19), it should also be taken into account that this technique is sensitive to various factors, including the thickness of histological sections, the reaction temperature of the solutions, the concentration of silver nitrate, incubation times and even the use of glass that is not perfectly clean (9). Such factors may hinder the identification of the AgNORs. Furthermore, the interpretations of these data are less reliable if not supported by a computational method, which is a limitation of the research protocol.

The findings, in our view, indicate that the AgNOR procedure is not a technique sufficiently sensitive to measure the biological functions, at least related to the methods of our research.

The methodology used in this study demonstrates that chronic restraint stress has no involvement in the severity of lingual carcinomas of CF-1 mice, according to the parameters analyzed, consistent with our previous report (3). However, of note is that more sensitive markers were analyzed by immunohistochemistry and molecular biology methods.

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