Original Study

Significance of AgNOR Evaluation in Oral Carcinoma — A Morphometric Study

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Abstract

Nucleolar organizing regions (AgNORs) are loops of DNA which are transcribed to ribosomal RNA which contribute to regulation of protein synthesis and the number of NORs in each cell nucleus reflects cellular kinetics including the proliferative activities. The estimation of NORs helps in prognostication and clinical management of cancer. In order to have high precision, reproducibility and good inter –observer correlation a morphometric study of oral cancers were studied and statically analyzed.

50 cases including normal (n=10), dysplastic (n=10) and carcinoma (n=30) of various histological grade and stage were studied. Relevant clinical data were considered. Various morphometric parameters including the(1)nuclear area, (2)nuclear perimeter, (3)nuclear diameter, (4)cytoplasmic area, (5) form factor and (6) nuclear-cytoplasmic (N:C) ratio of 50 normal and 50 abnormal cells were assessed in each case along with their (7)mean AgNOR count. Increased values were noted in all morphometric parameters in lesions ranging from the normal, dysplastic to malignant cases. Carcinomas had a significant higher values than the benign lesions ($p<0.005$). Among carcinomas the values increased with grade and stage of the disease ($p<0.05$). Morphometric analysis conclusively proves the role of AgNORs as a useful prognostic parameter and a marker for tumor progression.

Keywords

AgNORs, Oral carcinomas

Introduction

The biological behaviour of a neoplasm is determined mainly by the proliferative activity of its cellular population. Mitotic counts, AgNOR counts, flow cytometric analysis of S-phase fraction, determination of incorporated radiolabel led thymidine and immunohistochemical methods-PCNA, and Ki 67 estimation represents current methods to assess proliferative activity. While immunohistochemistry and flow cytometry are fairly accurate, their expensive methodology and stringent standardization requirements make them unaffordable for regular laboratory use. On the other hand the mitotic count is highly subjective with poor reproducibility, making morphometric estimation of AgNORs a more reliable method of assessing proliferative activity.

The present study is aimed at (1) detecting the efficiency of AgNORs in distinguishing between normal, dysplastic & malignant lesions, (2) to correlate the histopathological grade of oral squmaous cell carcinomas with AgNOR counts (3) to detect the correlation,if any, between AgNOR counts and tumor stage at diagnosis

Material & Methods

50 specimens received by the department of Pathology at R.L. Jalappa Hospital, Tamaka, and Kolar were taking

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up for the study. Relevant clinical data for the cases were collected from the records. For each case, a minimum of two sections were made from the blocks. One section was stained with Hematoxylin and Eosin(H&E) to give a morphological diagnosis and to identify the grade of the tumor.The other section was stained for AgNOR. .Based on the grade 30 cases were divided into three groups namely well differentiated, moderately differentiated &poorly differentiated.Suitable specimens of squamous epithelium,cervical tissue and skin were studied to standardize the staining technique.Similarly 50 controls of normal squamous epithelium were also evaluated for AgNOR values.

Method of AgNOR Staining

Formalin fixed 5 mm paraffin sections were stained with a.solution made up of 50% silver nitrate(freshly prepared) and 2% gelatin solution (stored at room temperature). Working solution was freshly prepared using 2 parts of silver nitrate and one part of gelatin solution. To carry out AgnoR staining the cut sections were deparaffinized & rehydrated using distilled deionized water.They were then placed in a plastic jar (slide container) filled with the staining solution and incubated at 37 degree celcius in dark for 45 minutes. Slides were washed thoroughly with distilled deionized water & dehydrated. After drying, the slides were cleared in xylene and mounted in DPX.

Method of Morphometry (Image analysis)

The basic principle of morphometry or image analysis to transform microscopic images into numbers, which can be analyzed in computers. Image analysis was done on the AgNOR stained sections by counting at least 50 cells per slide in all cases of normal (category-1), dysplastic (category-2), well differentiated (category-3), moderately differentiated (category-4) & poorly differentiated (category-5) of squamous cell carcinomas. The use of lymphocyte AgNOR area as an internal control for the standardization of AgNOR evaluation in malignant tissues was used because lymphocyte AgNOR area is almost constant in tumors independent of age and sex.

The image analysis system used for the present study consisted of the following components-(a) Olympus Bx 50 research microscope(Olympus Corporation, Japan), (b)Xilix micro imager (Xilix Technologies Corporation, Canada), (c) Optima's image analysis software (Optima's 5.3 version, December 1995, Optima's Corporation,USA), (d) F-64 oculus image grabber card, (e)Pentium IBM personal computer(Celsbris XL, Digital Corporation,USA).

The following morphometric parameters were considered

1. Cytoplasmic area. 2. Nuclear area. 3. Perimeter of the nucleus. 4. Nuclear cytoplasm (N: C) ratio. 5. Form factor.

The slides were scanned at the microscope using an x 100 oil immersion lens. The selected image was captured into digital memory and on the monitor of the image analyzer. The silver-stained NORs appeared as dark structures uniformly distributed throughout the nucleolus & easily distinguishable from the lighter background. Looking at the monitor, the operator interactively defined the grey threshold which permitted the selective identification of the dark spots corresponding to interphase AgNORs. The measurements were automatically performed by the computer after defining which cells to evaluate. By means of this morphometric technique 50 cells from each of the ten normal cases were measured. In the dysplastic and malignant samples 50 cancer cells were evaluated with lymphocytes as internal control.

Tumor staging was performed according to American Joint Committee Criteria (AJCC), with 7 stage I, 13 stage II, 6 stage III & 4 stage IV tumors.

Results

Table 1 shows distribution of squmaous cell carcinoma of the oral cavity according to the site and grade. Ten cases each belonging to category 3, category 4 & category 5

Table 1
Distribution of squmaous cell carcinoma of the oral cavity according to the sites and grades

Sl No.	Site	Well different-tiated	Moderately differen-tiated	Poorly diffren-tiated
1	Mandible	4	6	7
2	Tongue	4	3	1
3	Lip	2	1	2

Table 2
Results of image analysis

Sl No.	Criteria	Normal(I)	Dysplasia(II)	Well diff(III)	Moderately diff(IV)	Poorly diff(V)	p values Ratio I & II Ratio I&III Ratio I&IV Ratio I&V
1	Cytoplasmic area	15.246+2.5905	16.866+3.1583	44.091+12.815	48.071+14.816	50.246+15.816	< 0.05
2	Nuclear area	6.957+1.267	9.0759+1.412	23.898+5.1146	29.947+6.1178	32.848+7.1476	< 0.05
3	Perimeter	11.467+1.667	13.721+2.08604	28.680+2.7244	31.747+2.8471	34.864+2.9641	< 0.05
4	F.F.	0.350+0.0131	0.0596+0.0175	0.0613+0.0007	0.0813+0.0007	0.0979+0.0008	< 0.05
5	N.C. Ratio	0.5222 +0.8869	0.5246+0.078	0.5534+0.1140	0.6120+0.1207	0.7648+0.1207	< 0.05

were considered. **Table 2** shows the results of various morphometric parameters in all categories of patients along with their statistical significance. All the morphometric parameters were statistically significant. **Table 3** shows the mean AgNOR counts for normal epithelium and those for stages I-IV tumors are shown as mean values and

Table 3
Mean Nucleolar Organizer Regions Versus Tumor Stage

Sl No.	Stage	No. of cases	Mean	95%confidence interval for mean
1	Stage I	7	9.47	7.48- 11.41
2	Stage II	13	13.07	11.06- 14.55
3	Stage III	6	14.07	12.21- 14.67
4	Stage IV	4	16.07	14.10-16.07
5	Control	-	3.88	3.40 - 4.41

95% confidence intervals for those mean values. Normal epithelium differed significantly from tumors at all stages. The mean AgNOR count for all stages of squamous cell carcinomas was 12.47 per nucleus, compared with a mean AgNOR count of 2.66 in controls. Thus a significant difference exists between the AgNOR count of normal squamous epithelium and that of squamous cell carcinomas. No normal specimen had a mean AgNOR count higher than the lowest tumor stage. Low stage tumors (stage I & II) differed significantly from high stage tumors (stage IV) in mean AgNOR count, while mid stage tumors (stage III) showed mild overlap with both lower stage (stage II) & high stage (stage IV) tumors.

Discussion

Nucleolar organizer regions are collections of nucleolar proteins associated with ribosomal genes that can be visualized in histologic sections using a silver colloid stain, thus the term silver staining nucleolar organizer region, (AgNOR). The nucleolar organizer regions (NORs) are chromosomal segments which contain ribosomal genes[1]. During interphase, the NORs are located in the fibrillar centres & surrounding dense fibrillar components of nucleoli (interphase NORs).

Interphase NORs can be selectively visualized in paraffin embedded sections by the silver staining method according to Ploton *et al*[2].

The distribution of interphase AgNORs is considered to represent a new, additional diagnostic tool in tumor pathology because malignant cells can be distinguished from corresponding normal/ dysplastic on the basis of a higher quantity of interphase AgNORs[3].

In view of the simplicity & the rapidity of the silver staining technique for AgNOR proteins, the parameter related to interphase AgNORs distribution should be introduced into routine histopathology for diagnostic & prognostic purposes. However, data on the distribution of interphase AgNORs in the same type of cancer were not comparable due to lack of standardization of the technique for both the visualization and quantification of interphase AgNORs[4]. Some of the important drawbacks regarding AgNORs were "NORs were difficult to identify, time consuming and do not have a consistently proven correlation with other measures of proliferative activity or prognosis"[5]. Lack of consistency of data were due to multifactorial reasons

ncluding the fixative used for tissue processing and the ime of staining reaction[6].

The correlation between certain chromosomal regions and nucleoli was first recognized by Clintok in 1934 who called these regions nucleolus organizing regions "(NOR)".[1] Ruschoff *et al* (1990) have made the use of automatic image analysis for the evaluation of AgNORs. this provided good reproducibility as determined by the standard cumulative means and interobserver agreement. The area of silver stained proteins associated with inter phase AgNORs was compared with labeling data obtained by bromodeoxyuridine (BrDU) in corporation and Ki-67 immunostaining AgNOR protein area measurement is therefore proposed as a simple inexpensive and reliable method of evaluating the prol ative activity in routinely processed tumor samples. Hence a morphometric study was performed on various parameters to obtain a scientific, consistent & reproducible data on oral cancer[7].

Statistical analysis were performed on various parameters of different histological grades and showed significiant p values (**Table 2**). Statistical analysis of the cases were done which showed significiant differences between the control benign squamous epithelium and tumors at all stages. Significiant differences were found between stage I & stage III & IV and between stages II & IV (**Table 3**).

In this series of 50 cases we examined AgNOR number per cell versus clinical stage and various morphometric parameters of different histological grades(**Fig. 1,2,3**). The

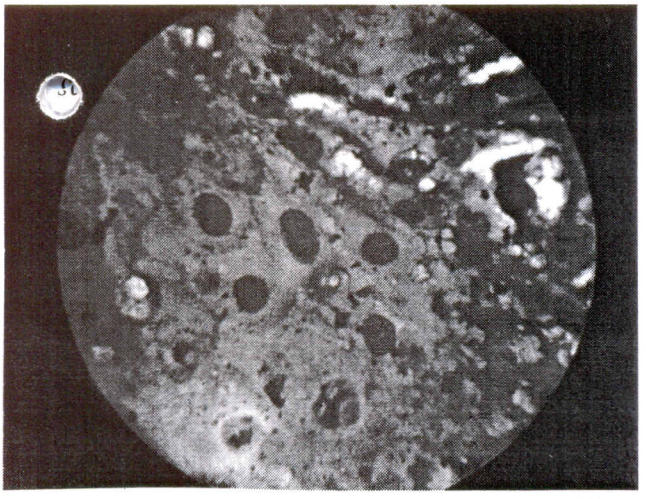

Fig. 1
Well Differentiated Squamous Cell Carcinoma
(AgNOR stain x 1000)(oil)

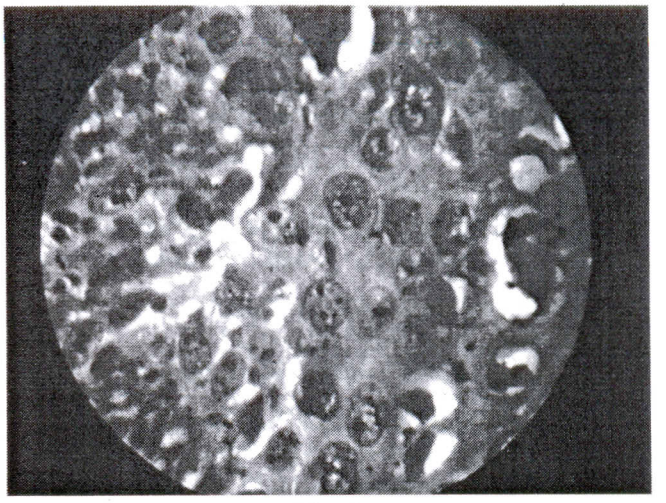

Fig. 2
Moderately Differentiated Squamous Cell Carcinoma(AgNOR stain x 1000)(oil)

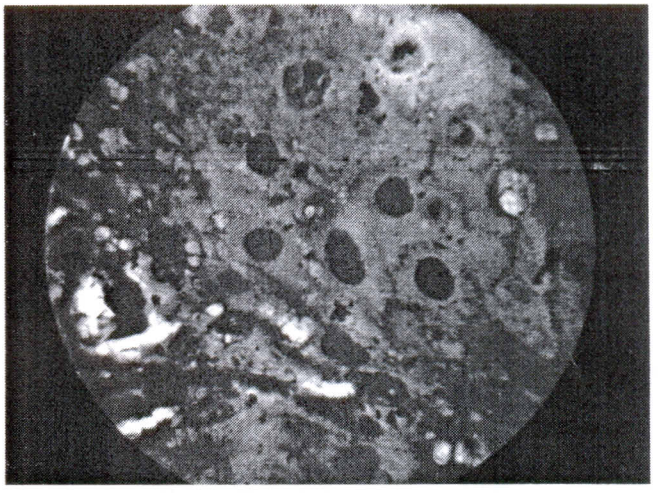

Fig. 3
Poorly Differentiated Squamous Cell Carcinoma
(AgNOR stain x 1000)(oil)

mean number of AgNORs per nucleus was significantly less for benign lesions as compared to low stage tumors and there was no overlap in the range of values for benign versus malignant epithelium. This suggests that AgNOR count is of great value for differentiating benign from malignant squamous epithelial lesions of oral cavity. A significiant correlation was noted between the mean AgNOR number per cell and tumor stage.

This suggests that AgNOR values is related to the

biological aggressiveness of malignant squamous lesions.[8] This also suggests that AgNORs has a prognostic significance for tumors of equal stages, since, conceivably, AgNOR number could change as tumor progress[9]. The fact that AgNOR can be readily determined on routine paraffin sections adds to its potential usefulness.This aspect has been highlighted in our study[10].

The potential use of AgNOR stain at the time of initial diagnosis needs to be evaluated. This may allow tumour specific prognostic classification according to the potential for local recurrences or metastatic disease[11]. Alternatively, AgNOR number may allow more precise tumor staging if it can be shown that it is a marker for tumor progression[12]. Increasing tumor stage specificity in such a manner would allow more precise therapeutic planning[13] and would add to the treatment profile of the carcinomas.

Conclusion

Two methods are usually employed for the quantitative evaluation of the interphase AgNORs counting the number of the AgNORs per cell directly at the microscope and measurement of the area occupied by the AgNORs in each nucleus by means of a computer assisted image analysis system.The possibility of standardizing the AgNOR indicies with the use of image analysis along with the possibility of having consistent and reproducible results is of great importance particularly in view of the current application of cell kinetics evaluation in tumor pathology.The AgNOR parameter has, in fact, been shown to be as as reliable as other well established parameters of cell kinetics such as the bromodeoyuridine labeling index and Ki-67 immunostating. On the other hand, the AgNOR parameter has the advantage over these other methods that it can be determined on routinely processed paraffin – embedded samples.

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