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ORIGINAL REPORT

Tongue Squamous Cell Carcinoma: Relationship Between Argyrophilic Nucleolar Organizer Regions (AgNORS) and Histopathologic Grading

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ABSTRACT

Current available data from the literature have suggested correlation among histopathological differentiation (tumor grade), treatment, and prognosis in oral malignant tumors. Cell proliferation is an important factor in the prognosis of malignant neoplasia. Argyrophilic nucleolar organizer regions (AgNORs) quantification is strongly associated to cell proliferation. OBJECTIVE: The aim of this study is to verify the relationship between total AgNORs mean value (mAgNOR) and / or the percentage of cells with 1, 2, 3, 4 or more AgNORs (pAgNOR) with histopathologic grading of tongue squamous cell carcinoma according to Wahi. MATERIAL AND METHODS: Eighteen cases of tongue squamous cell carcinoma were selected, histopathological evaluation grading was performed and AgNORs were counted on 100 epithelial tumoral nuclei. RESULTS: AgNOR mean value / nucleus has ranged according to the histopathologic grading; the mean value has increased as the histopathologic grading increased. A negative correlation among pAgNOR series (1,2,3,4 or more) was obtained between grade I and grade III lesions, whereas grade II lesions have shown intermediate values of correlation. CONCLUSION: A direct relationship between AgNORs / nucleus mean, percentage of AgNORs / nucleus and malignancy grading was observed.

Key words: Tongue. Mouth mucosa. Carcinoma, Squamous Cell. Proliferating Cell Nuclear Antigen. Cell proliferation.

INTRODUCTION

In 1941, Broders[1] related the extent of malignancy in neoplasms and emphasized the correlation between tumor histopathological differentiation, their treatment and prognosis. Wahi,[2] in a World Health Organization (WHO) report, has commented that oral primary carcinomas prognosis should be established always taking into consideration the histological grading, tumor site and their clinical features. The histological malignancy grading system developed by that author is subdivided into three grades: grade I, II and III and this classification is mainly based on tumoral proliferation and differentiation.[2]

Altered proliferative activity is one of the major characteristics of cancer and it is strongly correlated with the prognosis of a lesion.[3-5] Consequently, proliferative rates are useful indicators for prognosis.[5]

Silver staining of active nucleolar organizer regions (NORs), which are known as AgNORs, has been applied to a variety of lesions in order to provide more information on their nucleolar activity.[6]

This silver staining technique is directly related to the level of r-DNA (ribossomal DNA) transcription,[6-8] and increased NORs counts will be expected in cells with active proliferation.[5,7,9-12]

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Therefore the AgNORs study provides understanding of the tumoral behavior since their frequency, size and cell arrangement have been reported as a discriminatory factor between certain benign and malignant lesions, reflecting the cell proliferative activity, cell cycle phase, cell ploidy and DNA content.[6,13,14-18]

A correlation between increased AgNORs, decreased differentiation, and increased malignancy is known. It has been observed in non-Hodgkin lymphomas, melanocytic skin lesions, breast tumors, cutaneous tumors, maxillary bone neoplasia, nasopharyngeal carcinoma and salivary glands tumors.[7,8,14,19-22]

Pillai et al.[23] have analyzed the AgNORs counting as a biological marker of tumoral progression on oral lesions and have concluded that AgNORs counting is greater in malignant lesions, mainly in those less differentiated.

Yue et al.[24] using AgNOR and PCNA (Proliferation Cell Nuclear Antigen) on oral squamous cell carcinoma have proved the relationship between proliferation rates and clinical and pathological findings. Regarding the clinical and pathological findings, mean AgNORs value was higher in cases T3 and T4, in groups N1 and N2.

A high AgNOR counting characterizes tumors with increased metabolic activity, which is likely to have high DNA content or to be poorly differentiated and to proliferate rapidly. Therefore, it predicts a poor prognosis.[12]

Xie et al.[25] have introduced a method for AgNORs evaluation named pAgNOR, by which the percentage of nuclei with more than 1, 2, 3, 4 or more AgNORs is determined. They have shown that pAgNOR is useful in distinguishing between normal epithelium dysplasia and oral squamous cell carcinoma, being a good marker for prognosis.

The aim of this study was to establish the relationship between the total mean value and/or the percentage of cells with 1, 2, 3, 4 or more AgNORs/nucleus and the histopathological grading of Wahi[2] for tongue squamous cell carcinoma and evaluate the superiority of one of them.

MATERIAL AND METHODS

Eighteen cases of biopsy specimens taken from tongue squamous cell carcinoma were selected from files of the Pathology Service of Santa Maria University Hospital (HUSM). The sample was divided into three distinct groups according to the histopathological grading proposed by Wahi,[2] with six cases in each group. Such score was carried by Hematoxylin and Eosin (H/E) stained sections observing the following criteria:

- GRADE 1: numerous corneal pearls, important cell keratinization with intercellular bridges, less than 2 mitoses per field when observed under microscope with increased magnification, rare atypical mitoses, sparse multinucleated giant cells, reduced cellular and nuclear pleomorphism;

- GRADE 2: sparse or absent corneal pearls, cell keratinization and apparent intercellular bridges, 2 to 4 mitoses per field when observed under microscope with increased magnification, some atypical mitoses, moderate cellular and nuclear pleomorphism , sparse multinucleated giant cells;

- GRADE 3: rare corneal pearls, cell keratinization practically absent and lack of intercellular bridges, more than 4 mitoses by field when observed under increased magnification, frequent atypical mitoses, pronounced cellular and nuclear pleomorphism, frequent multinucleated giant cells.

Further, new cuts were made for each block (3µm thick), and then they were submitted to the AgNOR staining technique in line by Ploton et al.,[6] at the Oral Pathology Laboratory at the School of Dentistry of Rio Grande do Sul Federal University.

On H/E stained slides the most significant microscopic fields, which have shown all characteristics described by Wahi,[2] were classified according to their malignancy grading. Necrosis areas, pronounced inflammation and artifacts were excluded. AgNOR staining slides were marked with the same microscopic fields chosen on H/E staining slides, providing similarity between histologic grading and AgNOR staining.

AgNORs counting was carried out by analysis of 100 epithelial cells on each slide, in an optical microscope (OLYMPUS-CX40) under ´400 magnification. Two methods for AgNORs counting were used: 1) total AgNORs counting of nuclei of 100 epithelial cells and mean AgNOR/nucleus (mAgNOR) value; 2) percentage of nuclei with 1, 2, 3, 4 or more

AgNORs counted in 100 epithelial cells (pAgNOR).

Comparison between mean values of each histopathological grading was computed by an Analysis of Variance, considering it a totally casual experiment, followed by the Tukey test. The percentage of AgNORs (pAgNOR) at the different histopathological grades was submitted to the Pearson's correlation test. All statistical analysis was carried out using SPSS software.

The study was submitted and approved by the Ethics Committee of the School of Dentistry of the Federal University of Rio Grande do Sul.

RESULTS

Table 1 shows mean AgNOR's value/nucleus range according to the histopathologic grading. Statistically significant differences among the three mean values obtained were observed by Analysis of Variance (ANOVA).

Table 1 – Mean AgNORs/nucleus value in relation to histopathological grades based on Wahi[2] score

	Histopathological Grade		
	I	**II**	**III**
	(n=6)	(n=6)	(n=6)
AgNORs Mean Value (SD)	1.5950 (0.2243)	2.1583 (0.4155)	2.5850 (0.6033)

It was possible to note that the mean value increases as the histopathologic grading increases ($p<0.05$).

Results presented in the Table 2 and Figure 1 show a negative correlation among pAgNOR series (1, 2, 3, 4 or more) obtained on Grade I and Grade III lesions (r=-0.718). The pAgNOR graphic shows that in Grade I tumors the predominance of the percentage of cells with 1 and 2 AgNORs/nucleus, while in the Grade III shows the predominance of the percentage of 3 or more AgNORs/nucleus. Grade II lesions show intermediate values of correlation (Grade I and Grade II: r=0.285; Grade II and Grade III: r= 0.352), since its initial curve portion (1 and 2 AgNORs/nucleus) is similar to that observed in Grade III lesions, while the segment of the curve that represents frequency 3, 4 or more AgNORs/nucleus is similar to the corresponding segment of the Grade I lesions curve.

Table 2 – AgNORs percentage (pAgNOR) at different histopathological grades based on Wahi[2] score

	Number of AgNORs/Nucleus			
Histopathological Grade	**1**	**2**	**3**	**4 OR +**
GRADE I	35.24%	39.23%	20.33%	5.19%
GRADE II	14.26%	34.04%	36.31%	15.36%
GRADE III	8.60%	25.61%	30.47%	35.28%

DISCUSSION

In this study, the histopathological grading proposed by Wahi[2] has correlated tongue squamous cell carcinomas with the AgNORs/nucleus number. In order to verify this correlation two methods for AgNORs counting proposed by the literature were used: mean AgNORs/nucleus value (mAgNOR) and the percentage of AgNOR/nucleus (pAgNOR). The WAHI´s histopathological grading was used instead of the ANNEROTH grading since some factors of the latter, such as, the epithelial invasion grade, underlying tissue invasion and inflammatory infiltrate intensity usually are not clearly observed in partial biopsies of the mouth due to their small dimensions.

Another aspect to be emphasized is that histopathological grades such as those of

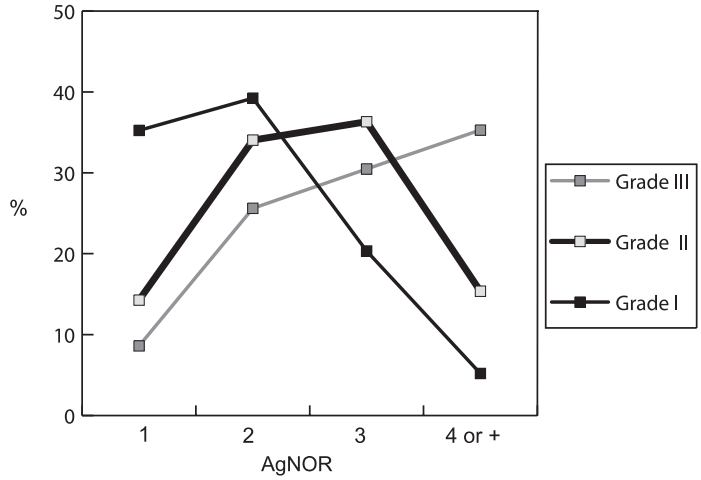

Figure 1 *– Percentage of AgNORs/nucleus according to their histopathological grade*

Anneroth and Wahi show some degree of subjectivity to the observer, making their reproducibility difficult. In this study we have looked for an association between morphological criteria and nuclear events in an attempt to minimize individual variability according to that reported in studies by Èemerikic—Martinovic et al.[5]

The AgNOR staining identifies those proliferating cells that are undergoing faster division, differently from most proliferation markers, that only indicate whether a cell is undergoing division.[21,22].

The most used method to count AgNORs is mAgNOR and several reports have shown the accuracy of this method in discriminating lesions with a good and a poor prognosis,[25] as a biological marker of tumor progression,[4,16,23,30] as a method to evaluate the aggressiveness of the lesion,[16] to evidence cell differentiation grade, [12,31] and to characterize cell proliferation rate of the lesions.[12,24,30] The results found in the present study for mAgNOR are in agreement that mean AgNORs value by nucleus has a clear relationship with tumor differentiation.

In recent years, the pAgNOR analysis was introduced. Some authors such as Xie et al.,[11] in a study on glottis carcinoma, have shown that pAgNOR is considerably a better method than mAgNOR on discriminating good or poor prognosis lesions. On the other hand, Ray et al.,[26] studying methods to aid in the diagnosis of epithelial dysplasia, have considered that mAgNOR is more appropriate than pAgNOR.

In Grade I and III, the morphological analysis was sufficient to define the extent of cell differentiation of such neoplasia, whereas in Grade II it seems that subjectivity is prevalent. It corroborates that histopathological grading is not precise, is subjective and shows variations among pathologists, especially when considering Grade II; which suggests that in oral squamous cell carcinomas scored as Grade II the use of AgNOR quantification is necessary to define the tumor aggressiveness.

We have observed that pAgNOR highlights better the tumor proliferation velocity. For example, cases morphologically classified as Grade II, which had most cells with 1 or 2 AgNORs/nucleus (pAgNOR=1, pAgNOR=2) had the same pattern as Grade I tongue squamous cell carcinomas. However, some cases classified as Grade II, which had most of the cells with 3 or more AgNORs/nucleus (pAgNOR>3), being the same as Grade III pattern of tongue squamous cell carcinomas. Such analyses (pAgNOR) may show the proliferative behavior of each lesion and are used as prognosis marker.

The results lead us to believe that pAgNOR is important to define the proliferation velocity of the Grade II tongue squamous cell carcinomas where some tumors present similar characteristics to Grade I (pAgNOR 1 and 2) while others have proliferative behavior similar to Grade III (pAgNOR above 3), thus

suggesting lesions with low differentiation or a more aggressive behavior. We believe that this method is useful for a more precise histopathological grading.

Finally, these results have confirmed that AgNORs (mAgNOR and / or pAgNOR) counting have a direct relationship with Wahi's[2] histopathologic grading, leading us to suggest that AgNOR technique can be used as a biological marker of tongue squamous cell carcinoma tumor progression. We believe that mAgNOR, and mainly the pAgNOR can be used as an adjunct method to Wahi's[2] histopathologic grading for evaluation of the proliferative behavior of tongue squamous cell carcinomas, especially in Grade II tumors.

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REFERENCES

1. Broders AC. The microscopic grading of cancer. Surg Clin North Am 1941; 21:947-61.
2. Wahi PN. Tipos histológicos de tumores orales y orofaringeos. Genebra: Organizacion Mundial de la Salud; 1971.
3. Martins Neto M. Sistemas de graduação histopatológica de malignidade (SGHM) do carcinoma espinocelular: revisão da literatura e sua importância dentro do contexto da estomatologia. Rev Odonto Ciência 1999; 28:97-106.
4. Elias JM. Cell proliferation indexes: a biomarker in solid tumors. Biotech Histochem 1996; 72:78-85.
5. Èemerikic-Martinovic V, Trpinac D, Recegovac M. Correlations between mitotic and apoptotic indices, number of interphase NORs and histologic grading in squamous cell lung cancer. Microsc Res Tech 1998; 40:408-17.
6. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region of the optical level. Histochem J 1986; 18:5-14.
7. Crocker J, Egan MJ. Correlation between NOR sizes and numbers in Non-Hodgking's limphomas. J Pathol 1988; 56:223–39.
8. Smith PJ, Skilbeck N, Harrison A, Crocker J. The effect of a series of fixatives on the AgNOR technique. J Pathol 1988; 155:109–12.
9. Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JCE. Silver-binding nucleolar organizer regions (AgNORs) in benign and malignant breast lesions: correlations with ploidy and growth phase by DNA flow cytometry. J Pathol 1989; 157:307-13.
10. Teixeira G, Antonangelo L, Kowalski L, Saldiva P, Ferraz A, Silva Filho G. Argyrophilic nucleolar organizer regions staining is useful in predicting recurrence-free interval in oral tongue and floor of mouth squamous cell carcinoma. Am J Surg 1996; 172:684-8.
11. Xie X, Clausen OPF, Sudbö J, Boysen M. Diagnostic and prognostic value of nucleolar organizer regions in normal epithelium, dysplasia and squamous cell carcinoma of the oral cavity. Cancer 1997; 79:2200-8.
12. Pich A. Prognostic relevance of AgNORs in thumor pathology. Micron 2000; 31:133–41.
13. Reeves BR, Csasey G, Harris H. Variations in the activity of nucleolar organizers in differents tissues, demonstrated by silver staining of human normal and leukemic cells. Cancer Genet Cytogenet 1982; 6:223-30.
14. Crocker J, Macartney JC, Smith PJ. Correlation between flow cytometric and nucleolar organizer region data in Non-Hodgking's lymphomas. J Pathol 1988; 154:151–6.
15. Crocker J, Boldy DAR, Egan MJ. How we should count AgNORs? Proposals for a standardized approach. J Pathol 1989; 158:185-8.
16. Sano K, Takahashi H, Fujita AS, Inokuchi T, Pe MB, Okabe H. et al. Prognostic implication of silver biding nucleolar organizer regions (AgNORs) in oral squamous cell carcinoma. J Oral Pathol Med 1991; 20:53-6.
17. Orrel JM, Evans AT, Grant A. A critical evaluation of AgNOR counting in benign Naevi and malignant Melanoma. J Pathol 1991; 163:239-44.
18. Oliveira MG, Sant´Ana Filho M, Rados PV, Lauxen IS. Quantificação de AgNORs e expressão do PCNA em ceratocisto odontogênico. Rev Faculdade Odontol Porto Alegre 2001; 43:51-6.
19. Egan MJ, Crocker J. Nucleolar organizer regions in cutaneous tumors. J Pathol 1988; 154:247-53.
20. Landini G. Nucleolar organizer regions (NORs) in pleomorfic adenomas of the salivary glands. J Oral Pathol Med 1990; 19:257-60.
21. Derenzini M, Trerë D, Pession A, Montanaro L, Sirri V, Ochs RL. Nuclear function and size in cancer cells. Am J Pathol 1988; 152:1291-7.
22. Chen M, Lee JCK, Shikyuen LOM, Shen J. Argyrophilic nuclear organizer regions in nasopharingeal carcinoma and paraneoplastic epithelia. Head Neck 2003; 25:395-9.
23. Pillai KR, Sujathan K, Kannan S, Abrahan EK, Mathew B, Amma NS. Argyrophilic nucleolar organizer regions in the evaluation of tumour progression in the oral mucosa: correlation with tissue pathology. J Cancer Res Clin Oncol 1994; 120:723-6.
24. Yue L, Iwai M, Furuta I. Evaluation of argyrophilic nucleolar organizer regions in tongue squamous cell carcinoma. Oral Oncol 1999; 35:70-6.
25. Xie X, Stenersen TC, Clausen OPF, Boysen M. Nucleolar organizer regions and prognosis in glotic squamous cell carcinoma. Head Neck 1997; 19:20-6.
26. Ray JG, Chattopadhyay A, Caplan DJ. Usefulness of AgNOR count in diagnosing epithelial dysplasia. J Oral Pathol Med 2003; 32:71-6.
27. Derenzine M. The AgNORs. Micron 2000; 31:117-20.
28. Derenzine M, Trerè D, Pession A, Govoni M, Sirri V, Chieco P. Nucleolar size indicates the rapidity of cell proliferation in cancer tissue. J Pathol 2000; 191:275-9.
29. Trerè D, Pession A, Derenzine M. The silver-stained proteins of interphasic nucleolar organizer regions as a parameter of cell duplication rate. Exp Cell Res 1989; 184:131-7.
30. Spolidoro LC, Neves KA, Soares CP, Spolidoro DMP, Basso MFM, Malavazzi I, et al. Evaluation of argyrophilic nucleolar organizer regions in oral tumor progression. Micron 2002; 33:605-8.
31. Migaldi M, Criscuolo M, Zunarelli E, Bianco LL, Martinelli AM, Barbolini G. p120 and AgNOR nucleolar protein expression: a comparison with nuclear proliferation markers in oral pathology. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998; 85:189-96.