Morphometric Analysis of Nucleus and Nucleolar Organizer Regions (NORs) in Tongue Squamous Cell Carcinoma (SCC)

Análisis Morfométrico de Núcleos y Regiones del Organizador Nucleolar (NORs) en Carcinomas de Células Escamosas de la Lengua (SCC)

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SUMMARY:The tongue is one of the most likely places for the primary squamous cell carcinoma (SCC) to arise in the western world. This work intends to do a morphometric evaluation of the cellular nucleus, as well as a morphometric analysis of the nucleolar organizer regions (NORs). The biopsy specimen was removed from patients with tongue SCC, staged T2N0M0, before they underwent treatment. They were, then, subject to hemiglossectomy and supraomohyoid neck dissection (SOHND) and presented different clinical evolution. According to that, they were subdivided in three groups: Group A, control group, formed by patients without tongue neoplasm (the histopathologic specimen was obtained from the Department of Pathology at Hospital de Base and Faculdade de Medicina de São José do Rio Preto - FAMERP-HB); Group B, T2N0M0+ hemiglossectomy + SOHND, survival of only 36 months; Group C, T2N0M0 + hemiglossectomy + SOHND, survival of 5 years. The morphometric analysis included the study of larger diameter, smaller diameter, medium diameter, the relation between larger/smaller diameters, perimeter, area, volume, the relation between volume/area, idiosyncrasy, shape coefficient and the nuclear contour index of the tumor cells from patients with tongue SCC, as well as the same measurements of the nucleolar organizer regions. The analysis was aleatory and double-blind. All the morphometric data were compiled and statistically evaluated by the Kruskal-Wallis test and by the ANOVA test, and a 5% alpha error rate was adopted.

KEY WORDS: Morphometric; Tongue, squamous cell carcinoma.

INTRODUCTION

The oral cavity is the preferable place, in the head and neck region, for primary malignant tumor to manifest. The tongue and the floor of the mouth are the most common places of origin of primary SCCs in the western world. The characteristics of the cell from oropharynx SCC, specially from the tongue, and their probable relation to the post operative prognosis, can be defined by analysing the images from the cell nucleus and nucleolus. From the nuclear morphometry, and the morphometric analysis of the nucleolar organizer areas, it is possible to optimize the prognosis for a better conduct orientation (Dantas *et al.*, 2003).

The term nucleolar organizer regions, or NORs, is used to describe the regions formed by chromosome fragments around which the nucleoli are formed at the final stage of mitosis. They correspond to loops of DNA that contain genes responsible for the transcription of ribosomal

RNA, of 18S and 28S, located at the nucleus of the cell. Since they have affinity with silver, they were named AgNORs (Herman *et al.*, 1984; Ooms *et al.*, 1983). In human beings, these regions correspond to secondary constrictions, and are localized at the short arms of acrocentric chromosomes 13, 14, 21 and 22. Since NORs are associated to acrocentric chromosomes, it was initially thought that they were related to ploidy. However, studies correlated to NORs with conventional histological variables (Hsu *et al.*, 1975), studies about the interphasic nucleus through cytogenetic methods (Derenzini *et al.*, 1989), cellular kinetics (Crocker *et al.*, 1988), Ki67 immunoreactivity (Hall *et al.*, 1988), and flow cytometry (Kramer & Epstein, 1994) suggest that AgNORs are mainly associated to cellular proliferation. Thus, through this technique it is possible to establish a new dimension to pathological anatomy, transforming its character from qualitative to quantitative (Baak *et al.*, 1985).

The aim of this work was to characterize morphometrically the alterations in the nucleus and in the nucleolar organizer regions (AgNORs) of tongue SCC in its different stages, to compare the morphometric parameters among each other, and to determine the relation of these findings to prognosis.

SUBJECT AND METHOD

After acquiescence from Research Ethics Committee of FAMERP, São José do Rio Preto, SP, Brazil, three groups of patients were studied. Those groups were divided according to the type of staging and responses to the treatment they were submitted to.

Group A: control group, formed by patients without tongue neoplasm (the histopathologic specimen was

obtained from SVO-FAMERP);

Group B: T2N0M0+ hemiglossectomy + SOHND, survival of only 36 months;

Group C: T2N0M0 + hemiglossectomy + SOHND, survival of 5 years.

The analysed material was a slide with the diagnosis of neoplasm obtained from the tongue biopsies done at the lesion's initial clinical features. The analysed specimens were stained with hematoxylin-eosin, and the same paraffin section was treated in order to stain the AgNORs. The slides were observed using a Olympus BX.60 microscope (magnification 1240 times) connected to Image-Pro-Plus 4.0 – Media Cybernetics (an automatic image analysis software system). To obtain the diameters, the larger and smaller axes of the nuclei and AgNORs images were measured. (Fig.1)

Fig. 1. Morphometry of the nuclei (D) and AgNORs (d) in slide. Magnitude of 1240X.

This way, each of the three groups presented the larger and smaller diameter sizes, grouped, summing up two hundred and fifty sizes for each group (fifty sizes from each patient). After determining the dimensions of two hundred and fifty nuclei of each group, the larger (D) and smaller (d) axes, and through the use of a computer programme (Dantas *et al.*; Ploton *et al.*, 1987; Graph, 2002), the following parameters were evaluated: larger diameter, smaller diameter, medium diameter, the relation between larger/smaller diameters, perimeter, area, volume, the relation between volume/area, idiosyncrasy, shape coefficient and the nuclear contour index of the tumor cells from patients with tongue SCC, as well as the same measurements of the AgNORs. The Kruskal-Wallis test was used to compare the morphometric parameters

obtained from the three groups. The test was applied with the assistance of the computer programme GraphPad In Stat®, 3.0 version for Windows 95, San Diego, California, USA, 1998 (Brodes, 1927). With this programme, we calculated the median values of the three groups that were compared among each other, through Dunn's Multiple Comparison Post Test. The P values were considered relevant when $P < 0,05$.

RESULTS

Tables I and II represent, respectively, the comparisons between nuclear calrimetry and AgNORs variables.

Table I. Nucleus

	AxB	AxC	BxC
Larger diameter	ns	$P < 0.001$	$P < 0.001$
Smaller diameter	$P < 0.001$	ns	$P < 0.001$
Medium diameter	$P < 0.001$	$P < 0.001$	$P < 0.001$
Volume	$P < 0.001$	$P < 0.001$	$P < 0.001$
Area	$P < 0.001$	$P < 0.001$	$P < 0.001$
Perimeter	$P < 0.01$	$P < 0.001$	$P < 0.001$
V/A	$P < 0,001$	$P < 0.001$	$P < 0.001$
Idiosyncrasy	$P < 0.001$	$P < 0.001$	ns
Shape	$P < 0.001$	$P < 0.001$	ns
Contour	$P < 0.001$	$P < 0.001$	ns
D/d	$P < 0.001$	$P < 0.001$	ns

Table II. AgNORs

	AxB	AxC	BxC
Larger diameter	ns	$P < 0.001$	$P < 0.001$
Smaller diameter	$P < 0.001$	ns	$P < 0.001$
Medium diameter	$P < 0.001$	$P < 0.001$	$P < 0.001$
Volume	$P < 0.001$	$P < 0.001$	$P < 0.001$
Area	$P < 0.001$	$P < 0.001$	$P < 0.001$
Perimeter	$P < 0.01$	$P < 0.001$	$P < 0.001$
V/A	$P < 0,001$	$P < 0.001$	$P < 0.001$
Idiosyncrasy	$P < 0.001$	$P < 0.001$	ns
Shape	$P < 0.001$	$P < 0.001$	ns
Contour	$P < 0.001$	$P < 0.001$	ns
D/d	$P < 0.001$	$P < 0.001$	ns

Group A = control ; Group B = survival of up to 36 months; Group C = survival of 5 years; $P < 0.05$.

DISCUSSION

The principles of morphometry have been described, for than a century, because the histological characteristics of normal and sick cells have been used as a measure of prognosis, and as a way of predicting the course of the disease. The size of the malignant cell nuclei superposing the size of the cells without neoplasm has already been observed and related in studies dating from 1930 (Dobros *et al.*, 1999).

From Chalkey's (1943) study, by using the principle of Delesse, morphometry has been established as a quantitative method, and has demonstrated changes in the activities of the sick cells when related to normal cells. Thus, solutions for problems in histology and pathology were highlighted. This study also made possible to obtain information about three dimensional structures from two dimensional structures, thus, it happened an extrapolation of the two dimensional to the three dimensional space (Baak *et al.*, Dobros *et al.* (2000), studied which specific nuclear parameters, and histoclinical factors in patients with advanced larynx cancer could be related to the presence of metastatic nodules. He established that the nuclear area $\geq 66\text{mm}$, the perimeter $\geq 32\text{ mm}$, the density, the long and short axis, frequently produced metastasis in lymphatic nodules. He determined that the short axis and the optical density were the most significant morphological parameters. Fernández-López *et al.* (1999), evaluated, in his study, the prognostic value of the colorectal cancer in ninety patients. It was noticed that patients with greater nuclear diameters also had a significant increase in their prognosis than the patients with cells of smaller diameters. This study asserted that the variations could be related to the survival of these patients. Thus, he concluded that the size and shape of the nucleus are useful to predict patients survival, and that large nucleus have worse prognosis when compared to smaller diameters. Dobros *et al.* (1999) by doing a morphometric analysis of nuclear shapes, studied, retrospectively, patients with advanced larynx cancer, and detected that there is no significance between the examined parameters: lymphatic nodules and tumoral differentiation. Reifen *et al.* (1992), studied the morphometry and the stereology in nasopharyngeal carcinomas. He studied two groups: those with carcinoma confined to the nasopharynx, and those that already presented cervical metastasis. The first group presented larger nuclei, nuclear area, perimeter and volume. However, there was a smaller nuclear and nucleolar rate in patients with cervical metastasis, and the diameters and nuclear shapes didn't present significant differences between the two groups. As a result, it was determined that the subjective analysis of the lesion cannot

supply data about the prognosis. Morphometric techniques were used because they are of easy reproduction, low cost, and, above all, because they establish a fast estimative of the studied object. Blomjous *et al.* (1989), found, in his morphometric analysis of the urinary bladder, greater nuclear areas presenting greater DNA nucleus index. The authors considered that the nuclear value of the shape will determine the quantity of DNA existent, and that it finally decides the tumor characteristics. These findings suggest that patients with larger nuclei should have a stricter follow-up to detect recurrences and/or metastasis of early disease. Hence, it is possible to predict if the patient shall receive a more aggressive treatment.

The morphometric study can quantify and objectively describe the characteristics of tongue carcinoma, predicting prognosis and guiding treatment. So, by doing a morphometric analysis of the tissues, a quantitative analysis of the changes in the studied cell is also being performed. This happens because the changes found in neoplastic cells - such as architecture, cellular grade, cellularity, extension of the invasion, nature and extension of the inflammatory reaction - exemplify the extension of qualitative characteristics.

Delides *et al.* (1981) and Ooms *et al.*, described high variability rates among pathologists in quantitative non-morphometric grading analysis in urinary bladder and mammary carcinomas. They exceeded in 30%, respectively, suggesting that morphometry diminished this variability among pathologists. In the last twenty-five years, many studies have published descriptions of the use of morphometry as a method to characterize both changes in tissues, and diagnosis of diseases (Herman *et al.*; Collan *et al.*, 1987; Bilbo *et al.*, 1981) Nevertheless, morphometry is a technique that hasn't been used very much among pathologists. This study considers the morphometric technique as a valuable tool for predicting the prognosis of patients with tongue SCC. The present work has, as its main focus, the analysis of tongue cellular structures among three observed groups. Through the two dimensional analysis of these cells, the larger and smaller diameters of the nuclei and of the AgNORs were gauged. From these diameters, it was established a derivation for the three dimensional analysis of the cellular structure, and, consequently, it was also established a comparison among groups 1, 2, and 3.

Morphometry produces a better quantitative scale instead of a non numeric characterization of the structure alteration grades. Grades are ordinary rates of changes of the tissues, in which numbers are typically pointed out. So, to evaluate changes in tissues, the morphometric

method, for being quantitative, is much better reproducible with an ordinal grading. It becomes a method sensitive enough to identify minimal changes of cellular characteristics, as described by Bilbo *et al.* The author analysed and compared cells from patients with intraepithelial neoplasms presenting abnormalities in quantity and distribution of cellular chromatin, to cells from patients without any intraepithelial neoplasm. Thus, morphometry made possible to stratify the process of the disease, and raised the number of categories that could be done over the visceral estimation, and the extension of the cellular change. This way, it was possible to raise the number of opinions about how to lead the treatment, such as Kramer *et al.* This author analysed prostatic cells through this quantitative method, and verified that cellular structures that exceeded in 1,6µm cellular diameter size were suggestive in prostatic carcinomas when compared to normal cells.

To calculate the size of the sample in this study, it was used the Williams (1977) methodology (Ruggiero, 2007). It basically consists in calculating the means of the event to evaluate the acceptable level of variation. This method preconizes that we initially count, by chance, the number of cells with carcinoma in 100 aleatory fields, that represent the whole histological section. So we could calculate the accumulated mean, the number of cells with carcinoma per field was summed up and then divided by the number of fields (division factor). By doing this, we got to the result that the number of 50 cases for each analysed patient was representative in the sample.

A consensual parameter to gradate malignancy that could be reproduced and applied to relevant lesions, and that could also be clinically valid to indicate prognosis and guide treatment is clearly necessary. This parameter will be able to lead different pathologists into an equal diagnosis system. The reproducibility of the assessment is, to many tongue SCC patients, frequently variable, for the carcinoma cells are very differentiated, and externally present dots of different grades, making the analysis more difficult to pathologists. In the last years, morphometry has proved to be more objective, and a more accurate technique for the grading of malignancy.

The present paper has demonstrated that the parameter larger diameter of the nucleus is not significant when groups A and B are compared. But there has been a significant difference in all groups in this aspect. We, then, conclude that the larger diameter has a prognostic value, after all, through the larger diameter we can differentiate these groups in prognosis. However, analysing the results of the comparison of the parameter smaller diameter, we

observed that there was a significant difference when collating group A to group B. Though, when we collated groups A and C we observed there wasn't a significant difference between the smaller diameters. We can conclude that the smaller diameter, for patients with tongue neoplasm classified as T2, has a prognostic value, since through the smaller diameter we can differentiate them in prognosis.

The interest in supplementing the traditional methods used to evaluate the prognosis of cancer has lead to the development of different kinds of tumor markers. The finding of these markers would permit to discriminate more clearly patients that would be benefited from surgery, from surgery related to chemotherapy, from more aggressive treatments or experimental protocols. Crocker *et al.*, suggested that the AgNOR technique would have practical utility in pathology to demonstrate the neoplastic potential, and to approach prognosis and aggressiveness of malignant neoplasms. According to Trevisan (1995), if the cellular cycle is accelerated, the staining by silver will present many black dots in the nucleus, indicating, indirectly, the places where the genes of ribosomal RNA are active. Considering this, the practice of quantifying AgNORs was introduced in histopathology as a marker of the cellular and nucleolar activities. The analysis of the AgNORs expression can be performed by pattern of distribution and by the number of black dots inside the nucleus in optical microscopy, and by the measurement of its area by image analysis or by the calculus of the indexes that relate these two criteria (Pardue & Gall 1969). In this study, the analysis was performed by measuring the AgNORs inside the nucleus, for these areas vary in size and shape – according to the nucleolar 2transcription – and are closely related to the cellular cycle, proliferation, and ploidy (Reifen *et al.*). We observed that the AgNORs presented themselves as dark brown intranuclear dots that could be big and isolated, small and numerous, and gathered. We avoided the analysis in regions with incomplete representations of the nucleus, and, also, whenever they were gathered and superposed making it difficult to visualize. By analysing the number of black dots inside the nucleus and determining the sizes of the smaller and larger diameters of these areas, we got to the results presented on Table II. This way, when we analyse this table - that is based on the sizes of larger and smaller diameters of intranuclear AgNORs from groups A, B, and C - we observe that groups B and C have significant differences between them. We, then, conclude that the qualitative analysis of AgNORs has a prognostic value in cellular differentiation. Still observing the parameters idiosyncrasy, shape coefficient and the nuclear contour index on Tables I and II, when we compare the neoplastic groups A, B and AC we notice that they present significant

differences, which means, they are tumors (B and C), and they differentiate from normal cells. When they are compared among each other, we observe that there wasn't a significant difference, for all of them are very similar in this parameter. This fact deserves attention because these parameters are basically bi dimensional, what explains, again, the necessity of differentiating the grades of cellularity.

Through morphometric evaluation of the nucleus and nucleolus (AgNORs), we can differentiate them, guiding to better conducts and optimizing the patient prognosis.

The smaller diameter of the nucleus, and the larger and smaller diameters of AgNORs, for patients with tongue SCC, T2N0M0, have prognostic values.

PADOVANI, J. A. JR.; MONTEIRO, R.; ZAN, T.; AZOUBEL, R.; DE SANTI, D.; TABOGA, S. & MARTINS, A. Análisis morfométrico de núcleos y regiones del organizador nucleolar (NORs) en carcinomas de células escamosas de la lengua (SCC). *Int. J. Morphol.*, 25(3):493-499, 2007.

RESUMEN: La lengua es probablemente, el lugar en donde más frecuentemente surge el carcinoma primario de células escamosas (SCC), en el mundo occidental. Este estudio propone realizar la evaluación morfométrica del núcleo celular, así como el análisis morfométrico de las regiones de los organizadores nucleolares (NORs). La muestra de biopsia fue removida de pacientes con SCC de lengua, etapificado en T2N0M0, antes de recibir tratamiento. Los pacientes fueron sometidos a una hemiglossectomía y una disección supraomohioidea del cuello (SOHND) y presentaron diferente evolución clínica. De acuerdo a esto, fueron subdivididos en tres grupos: Grupo A, grupo control formado por pacientes sin neoplasia de lengua (la muestra fue obtenida del Departamento de Patología del Hospital Base de la Facultad de Medicina de São José do Rio Preto - FAMERP-HB, Brasil); Grupo B, T2N0M0 + hemiglossectomía + SOHND, sobrevida de 36 meses; Grupo C, T2N0M0 + hemiglossectomía + SOHND, sobrevida 5 años. El estudio morfométrico incluyó los análisis del diámetro mayor, diámetro menor, diámetro medio, la relación entre el diámetro mayor/menor, perímetro, área, volumen, relación volumen/área, idiosincrasia, coeficiente de forma y el índice de contorno nuclear de células tumorales en pacientes con SCC de lengua, así también las mismas mediciones de las regiones nucleolares organizadas. El análisis fue aleatorio y doble ciego. Todos los datos morfométricos fueron recopilados y evaluados estadísticamente con el test de Kruskal-Wallis y el Test ANOVA, con un valor alfa de un 5%.

PALABRAS CLAVE: Morfometría; Lengua, Carcinoma de células escamosas.

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