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Nuclear Morphometry in Squamous Cell Carcinoma (SCC) of the Tongue

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In the histological grading of oral squamous cell carcinoma (OSCC) nuclear features are very important. Nevertheless evaluation is usually performed in a subjective and not highly reproducible way. The aim of this work was to investigate the relationship between nuclear shape and survival in 30 cases of carcinoma of the tongue. All the patients were divided into two groups: short-term survival and long-term survival. Twenty nuclei for each tumour were submitted to a morphometrical study by the shape analytical morphometry (SAM) software system. It was thus possible to evaluate not only nuclear dimensions but also nuclear contour irregularities and nuclear shape asymmetries. Multivariate discriminant analysis (MDA) of the quantitative parameters obtained by the morphometrical study distinguished the patients of the two groups with only a 10% error; moreover successful cluster analysis was performed by using Fourier parameters. Both these sets of results were achieved mainly owing to the parameters for contour irregularities. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

Prognostic evaluation of oral squamous cell carcinoma (OSCC) is mainly based on clinical pathological stage as well as on histological grading.

Although the TNM staging system has been found to be a reliable prognostic factor, many patients die despite the fact that their tumours had been considered to be at an early stage of OSCC [1].

Moreover, diagnosis is often performed on very small biopsies; in these cases histological features are of importance in the choice of therapy and in the estimation of expected survival. It is mainly in these cases that histological grading acquires a very great prognostic importance.

Since Broders [2, 3] first proposed a malignancy grading for OSCC, many other systems have been proposed over the past decades [1, 4–9] in an attempt to express prognostic evaluation in an ever-more objective way.

The problem inherent in the grading evaluation has always been that estimation is too subjective and consequently has a low grade of reproducibility [10]. In fact, generally in the histopathological field diagnostic and prognostic evaluation is entirely based on the experience of the observer [8, 11, 12] and it is not unusual for different pathologists to reach different conclusions on the same material.

In recent years, some authors have sought to remove this problem by using quantitative methods such as stereology [13], point counting [14], ploidy evaluation [15] or measuring nuclear dimensions [16, 17], applying them all to prognostic evaluation of oral cancer and to the problem of precancerous lesions.

Although these works express a particularly strong need for more objective methods of evaluation in histopathology, they nevertheless fail to reach unanimous results.

In this work, on the basis of the great importance given to nuclear pleomorphism in grading evaluation, we wished to study the relationship between prognosis and nuclear shape and dimensions in terms of patient survival time after surgery using analytical morphometry methods [18–20]. The final goal was to build a malignancy grading of OSCC based on quantitative and more objective parameters.

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PATIENTS AND METHODS

30 cases of oral squamous cell carcinoma of the tongue, which were diagnosed in the Institute of Pathological Ana-

tomy of the University of Bari in the period 1985–1990, were studied.

The end of the clinical observation period was December 1993 so all cases had a minimum observation time of 36 months.

The patients were 23 men and 7 women, and the mean age was 60 years (range 29–81 years).

Although for each case tumour size, depth of invasion and lymph node status were also known, in this phase of the work we wished to investigate the relationship between morphological features and tumour behaviour without taking into account the neoplasia stage.

For each patient, the length of follow-up after surgery was known. The minimum length of follow-up was 4 months, the maximum 109 months. 15 of the patients died of the disease: 9 died within 14 months of surgery; the other 6 survived longer (36–68 months). For this reason we subdivided the patients on the base of their survival time into two groups: the first (9 patients) with short-term survival, the second (21 patients) with long-term survival (over 36 months).

All the biopsies were formalin-fixed, wax-embedded and the histological sections (5 μm) were haematoxylin-eosin stained. For each case the mitotic count was performed simultaneously by two observers. The mitotic index was the total number of mitoses counted in 10 HPF (× 400) according to the criteria proposed by Baak *et al.* [21].

We considered the mitotic index as a reference variable to be used in understanding the morphological features of the nuclei on the basis of numerical descriptors for size and shape.

Morphometry

For each tumour, a histological section, 5 μm thick, H & E stained was photographed by a TV camera at × 1000 magnification and the photographs printed by a video printer (Fig. 1). At the same magnification a millimetric slide designed as a graphic scale was photographed and used for dimensional evaluations. Twenty nuclei for each tumour were submitted to morphometric study. All of them were selected in the deepest areas, or "advancing front" of the tumour. The only necessary condition in nuclei selection was that their entire contour was well evident. Prints of 600 nuclei were analysed with the shape analytical morphometry (SAM) software system [18–20], which has already been utilized in previous works on analytical morphometry [22, 23].

Each nucleus was digitised by a semiautomatic contour following procedure. The original contour (OC) (Fig. 2) was recorded as a series of points of known coordinates. Four dimensional nuclear parameters were assessed: area, perimeter, maximum diameter and roundness factor.

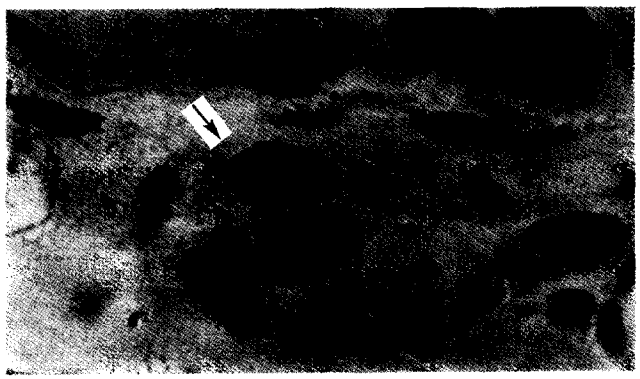

Fig. 1. Videoprint of oral squamous carcinoma nucleus (H&E × 1000).

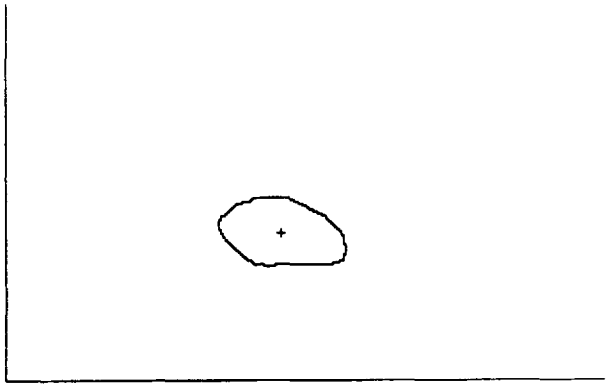

Fig. 2. Original contour of nucleus in Fig. 1.

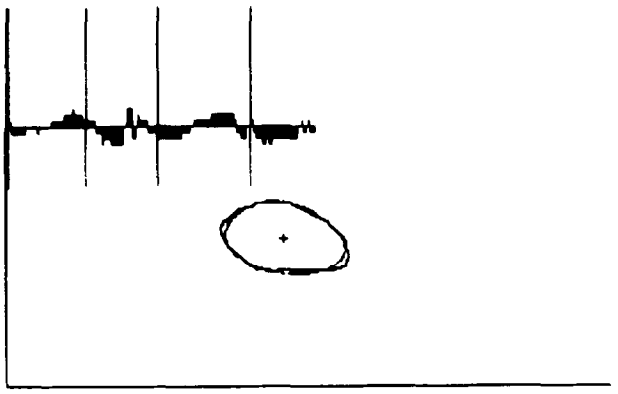

Fig. 3. Superimposition of OC and FC with graph of their differences.

Each nucleus area was then normalised to the smallest nucleus area value to evaluate nuclear shape independently of dimensions.

The analytical morphometrical procedure consists of three steps.

(1) For each OC the function curve (FC), which describes the basic shape of the OC without its irregularities, was calculated. The extraction of FC was performed by using two upper-degree polynomial equations with the least-squares method.

This equation is used separately for x and y values as dependent variables and positive integers from 1 to N, where N is the number of points into which the profile is subdivided as independent variables [18].

Using the match between OC and FC (Fig. 3) four parameters were considered: percentage of chained points, square root of mean square error on global divergence, mean of x/y difference ratio and x/y coinciding points ratio. They were related to little contour irregularities, to their entity, to their trend and to their distribution on the nuclear contour.

(2) The second step was the description of contour irregularities of each nucleus by Fourier harmonic analysis, which was performed using the differences between the OC and FC

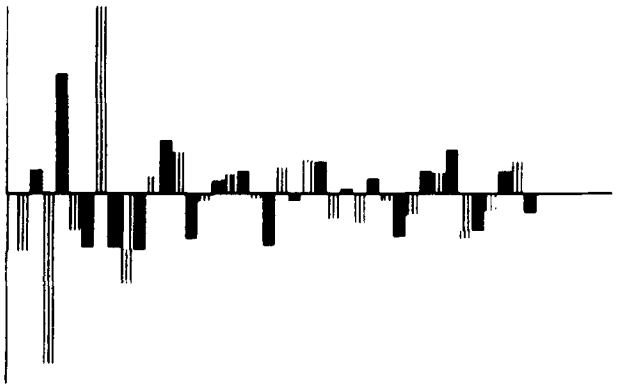

Fig. 4. Fourier spectrum of OC and FC of nucleus in Fig. 1.

after rectifying the FC considered as a zero reference line (Fig. 4). Four parameters were utilized by Fourier harmonic analysis: the sum of harmonic amplitudes and its mean value, the maximum amplitude value and its percentage.

(3) The analytical study of nuclear asymmetry was performed using the shape asymmetry evaluator (SAE). This is the ratio between the length of a parabola segment interpolating the FC points and a straight line joining its extremities for a progressive rotation by 10° steps up to 180°. The convexity of the parabola is oriented toward the prevalent part of the figure. The arc–chord ratio reaches its maximum value when, during a rotation it recognises the part of the nuclear contour that is prevalent in both length and point number. In the absence of asymmetry the arc–chord ratio equals 1 (Figs 5 and 6). The parameters utilized by the SAE procedure were: the minimum, maximum, mean and range values of the arc–chord ratio and, in percentage, the area defined by the parabola and the chord (allometric fraction).

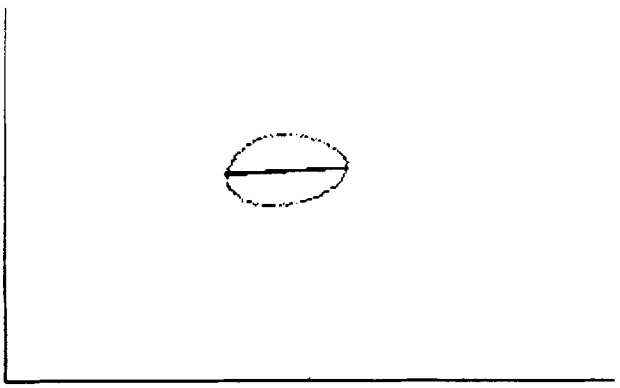

Fig. 5. Minimum value of SAE of nucleus in Fig. 1.

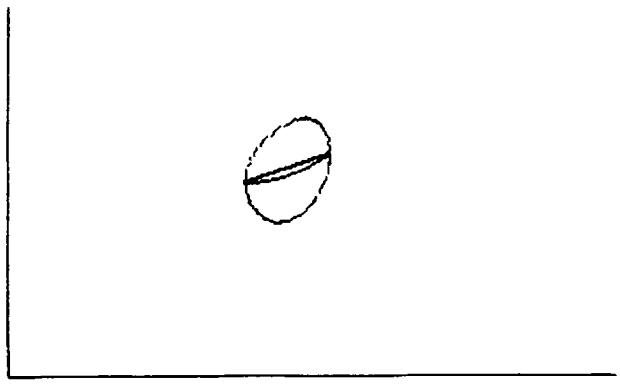

Fig. 6. Maximum value of SAE of nucleus in Fig. 1.

Statistics

Independent of the final outcome, all the patients were divided into two groups: short-term survival (9 patients) and long-term survival (21 patients) and statistically compared.

The mean value of 17 parameters obtained by the study of the two different nuclear populations related to tumours with long- and short-term survival were considered and compared using the Student *t*-test for univariate statistical analysis: the distribution of the values was normal.

All SAM parameters have a deterministic meaning and this creates a unique link between each numerical value and the corresponding morphological feature described by each parameter. Because of the logical and numerical independence of the parameters they are useful as variables in multivariate discriminant analysis (MDA).

MDA was carried out using the Hotelling test in which the discriminating value was expressed as the minimum percentage of error obtained by the various arrangement of different parameters.

Each time MDA was performed the number of parameters used was smaller than the number of cases in the smaller group (i.e. the short-term: 9 cases).

Moreover, cluster analysis was performed to verify which parameters allowed clustering of the two different groups (short-term and long-term survival). While in MDA the whole study-group was subdivided into two groups to evaluate in quantitative terms how much they differed from one another using dimensional and analytical parameters as independent variables; cluster analysis is based on the opposite concept: the whole study-group is considered as a single pool and, using dimensional and analytical parameters two at a time, we evaluated on a graph whether the pool is subdivided into two or more clusters or whether it is homogeneous.

Intra-observer and inter-observer data reproducibility were tested by considering one series of 35 nuclear contours that were recorded by two observers, one of whom recorded the series twice at different times. The three series of outlines were processed in the same way as all other nuclei and the observer variations and correlations were paired by *t*-test and the Pearson correlation coefficient.

RESULTS

On the basis of the different survival times we decided to compare the short- and long-term survival groups. The mean value of the mitotic number was 32 (S.D. 15.5) in the short-term survival group, and 18.4 (S.D. 16.6) in the long-term survival group. This difference proved highly significant ($P < 0.001$).

Table 1 shows the mean value and standard deviations of dimensions and shape-related parameters obtained by the analytical study of the nuclei of the two groups. Nuclei of cases with long-term survival showed significantly higher values of dimensional parameters, whereas parameters related to nuclear shape asymmetry (except the percentage of allometry)

Table 1. Mean, standard deviation (S.D.) and statistics (P) of nuclei dimensions and analytic parameters of carcinomas with short- and long-term survival

	Survival < 14 months (180 nuclei)		Survival > 36 months (420 nuclei)		Survival 14/36
	Mean	S.D.	Mean	S.D.	*t*-test
1. Maximum diameter (μm)	11.5	2.54	11.9	2.69	$P<0.001$
2. Perimeter (μm)	32.9	7.61	33.9	7.01	$P<0.01$
3. Area (μm²)	75.8	34.5	79.9	33.6	$P<0.1$
4. Roundness factor	0.83	0.055	0.84	0.057	$P<0.05$
5. Percentage of chained points	6.4	2.2	6.26	2.38	N.S.
6. Square root mean square error	1.5	0.5	1.44	0.499	N.S.
7. Mean of x/y difference ratio	0.95	0.073	0.95	0.07	N.S.
8. x/y coinciding points ratio	0.79	0.318	0.852	0.379	$P<0.001$
9. Sum of amplitudes	3921	1181	3830	1278	N.S.
10. Amplitude mean	196	58.7	191.5	64.3	N.S.
11. Harmonic max amplitude	793.5	409.3	744	371.8	$P<0.05$
12. Percentage harmonic max amplitude	19.5	5.67	19	5.26	N.S.
13. SAE minimum	6.59	8.52	5.5	7.16	$P<0.05$
14. SAE maximum	196	121.4	176	108.4	$P<0.001$
15. SAE mean	79.2	42.8	73.9	39.1	$P<0.05$
16. SAE range	189	118	171	105.6	$P<0.001$
17. Percentage of allometry	7.21	4.22	6.89	5.66	N.S.

Table 2. Multivariate discriminant analysis: Hotelling's test

	Parameters		
	Dimensional	Analytical	Dimensional and analytical
Short-term survival < 14 months / Long-term survival > 36 months	33% diameter area	13% Match OC/FC*: (parameter Nos 5, 6, 7) Fourier analysis: (parameter Nos 9, 10, 12)	10% diameter, roundness factor Match OC/FC*: (parameter Nos 5, 6, 7) Fourier analysis: (parameter Nos 9, 10, 12)

*OC, original curve; FC, function curve.

were significantly higher in nuclei of short-term survival. Only two parameters obtained by the study of contour irregularities were significant: the maximum amplitude of Fourier harmonic which is greater in the group with short-term survival and the "x/y coinciding points ratio" which is higher in the long-term survival group. This shows that the contour irregularities of the OC are prevalent on the abscissa axis more than on the ordinate in nuclei of the long-term survival group.

Multivariate analysis (Table 2) performed using the Hotelling test showed that by considering only dimensional parameters a minimum percentage of error (33%) was obtained with a subset of two parameters: diameter and area. Comparing the two groups on the basis of analytical parameters alone gave rise to a great decrease in error; in fact it dropped to 13% by using a subset of six parameters: three obtained by the match between OC and FC (square root mean square error, mean of x and y differences, % of coinciding points on FC) and three from Fourier harmonic analysis (sum of amplitude, mean value of amplitude and percentage value). All these parameters were related to nuclear contour irregularities. Finally, using a subset composed by the same analytical parameters as in the previous analysis, together with diameter and roundness factor it was possible to obtain the lowestpercentage of error (10%) in discriminating the two groups. Three cases were then erroneously classified by this analysis: 1 case was a poorly differentiated carcinoma with absent keratinisation and a very high cellularity. The nuclei of this tumour were large and hyperchromatic with many nucleoli but their shape tended to be round with a smooth nuclear contour. This patient died 13 months after surgery. However, MDA failed to classify it in the short-term survival group. The other two cases on the contrary had an evident keratinisation of neoplastic sheets and they were histologically classified as well-differentiated carcinomas. Nevertheless, their nuclei were clear with evident nucleoli and a very irregular contour. Of these 2 patients one died 49 months after surgery, the other was alive and well at the time of the end of the observation follow-up. MDA failed to recognise them as belonging to the long-term survival group.

The discriminant function giving the minimum error was the following:

$$f(x) = -0.18\,(x^1) - 201.8\,(x^3) - 0.779\,(x^4) - 19.3\,(x^5) + 152.8\,(x^6) + 0.014\,(x^7) - 0.21\,(x^8) + 0.61\,(x^9).$$

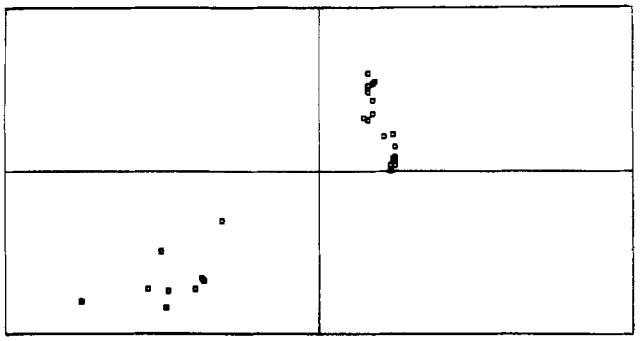

Fig. 7. Cluster analysis. Cluster of short- and long-term survival cases. Components: sum of harmonic amplitudes and harmonic amplitude mean value of Fourier analysis.

Cluster analysis confirmed the subdivision of the whole study-group into two clusters according to survival (short-term or long-term) using only Fourier analysis parameters used two at a time (Fig. 7).

With reference to the reproducibility test, in all the cases the t-test was not significant; the minimum value of the correlation coefficient ($r=0.897$) was found in the inter-observer comparison of maximum diameter. This coefficient was very highly significant ($P<0.001$).

DISCUSSION

The only quantitative parameter in malignancy gradings is represented by the mitotic index, even though many authors [1, 9, 13, 24] believe that nuclear pleomorphism is one of the best prognosis predictors.

The problem in histopathological evaluation of grading is the subjective nature of the observations and the low inter-observer agreement [8, 11, 12]. In recent years some authors have tried to reduce this level of subjectivity by turning to quantitative methods.

According to Bryne *et al.* [6, 13, 24] the low reproducibility of Anneroth's modified histological system was not improved by considering a grading based on the expression of a tumour-associated antigen [6]. Moreover, Bryne *et al.* [13] showed that patients with a low grade of pleomorphism had a significantly better chance of survival than patients with a high grade and, in the search for an objective evaluation of this parameter, carried out stereological assessment of nuclear volume. This, however, showed no prognostic value. In their work Bryne *et al.* underlined the close relationship between size and volume and the independence of shape from volume [13].

When pathologists observe a histological sample they perform evaluations on a two-dimensional image. For this reason we believe that it is in this type of image that we should seek quantitative and reproducible parameters for diagnostic or prognostic purposes.

Kramer *et al.* [11, 25, 26] in their studies on oral keratoses and precancerous lesions used a computer-aided discriminant and cluster analysis. In these studies they obtained a good discrimination of lesions, by combining the potential of the computer with the ability of pathologists; in fact in their study the quality of the results is strictly dependent on the subjective recognition and graduation of between 37 and 45 histological features.

Other studies based on two-dimensional histological images [14, 16, 17, 27, 28] showed a progressive increase in mean value from benign lesions to *in situ* carcinoma, but no quantitative studies exist in the literature to relate nuclear dimensions or shape to prognosis in oral cancer. Even DNA content only adds a little additional prognostic information when associated with histological grade and tumour size of oral carcinoma [15].

In this work the mean value of the mitotic count proved to be very important in discriminating between the two groups (long-term and short-term survival) of patients ($P<0.001$).

This result further justifies the group subdivision and supports the hypothesis that any significant morphological differences have to be found in the comparison between these two groups.

The division of the patients into two groups was, first, because of the different survival times. As demonstrated by some authors [1, 5, 7] most of the patients who expire from their oral cancer die within the first 2 or 3 years. In our cases a time-gap survival was evident between the two groups of patients we compared.

Comparison of the mean values of nuclear parameters by univariate analysis showed that nuclei of short-term survival patients are characterised by smaller dimensions and by higher values of contour irregularities and asymmetries. On the other hand, the mitotic activity index was significantly higher ($P<0.001$) in this group of tumours.

It could be inferred that the greater value of cell kinetics is related to smaller nuclei whereas in better prognosis tumours, when the mitotic number is lower, cell dimensions can become larger and their shape more regular.

With MDA using different dimensional and analytical parameter subsets we tried to achieve the best result.

The role of shape-related parameters is particularly stressed by MDA; in fact the percentage of discriminating error, which was 33%, using only dimensions, was remarkably reduced (20%) when only analytical parameters were utilized. These were all related to contour irregularities.

The most effective subset in lowering discriminating error in short–long survival groups was made up of the same six parameters obtained by the analytical study of nuclear contour, together with diameter and roundness factor. Therefore, tumour aggressivity appears to be related to nuclear shape and size and to mitotic index. This result backs up the above-mentioned hypothesis based on the mitotic count; because it allows for a very good discrimination between long- and short-term survival groups.

Secondly, it confirms on a quantitative basis the great importance attributed by many authors to nuclear pleomorphism, and finally, it stresses the particular importance of nuclear contour in defining "pleomorphism".

In fact, the use of six analytical parameters related to contour irregularities together with two dimensional parameters correctly identified 90% of cases with regard to survival time.

Nevertheless, the 3 misclassified cases show that even though nuclear shape is a very strong prognostic indicator as affirmed by Anneroth *et al.* [1], other factors such as hyperchromatism, cellularity mitoses, number of nucleoli, etc., have a great importance in the final diagnostic synthesis. When pathologists observe a squamous cell tumour they mentally evaluate all these parameters and express a synthetic evaluation of the tumour cell aggressivity.

The importance of this work is not only in showing the great significance of nuclear shape but in showing that it could be possible to express prognostic evaluation not by a discontinuous series of values (for example, grade 1, 2, 3) but by a

continuous series of values in which it will be important to find a cut-off point.

Cluster analysis further confirmed the great importance of the contour irregularities of nuclei, in fact Fourier parameters allowed us to obtain two different clusters according to survival time.

Even though the number of cases can still not be considered large enough to deduce general rules from their study, the results of this work encourage us to extend the morphometric analytical study to a larger number of cases in order to find a threshold value to distinguish between good prognosis and bad prognosis cases of tongue carcinoma.

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