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Morphometric Analysis of Nuclear Features and Volume-Corrected Mitotic Index in the Prognosis of Oral Squamous Cell Carcinoma

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Abstract: In the present study, an objective and reproducible evaluation of mitotic activity and nuclear morphometric factors was attempted in 30 patients of oral squamous cell carcinoma with a view to predicting local relapse and survival. Various nuclear parameters and volume-corrected mitotic index were calculated and compared with the recurrence and death of the study group (n = 30). Volume-corrected mitotic index (M/V index) was the single best prognosticator for recurrence of oral squamous cell carcinoma ($p = 0.008$ for recurrence; $p = 0.015$ for death). The combination of M/V and SD of nuclear area (forward stepwise regression) was a more efficient and better predictor of survival (Log rank test, Kaplan-Meier's survival analysis; $\chi^2 = 17.46$, $p = 0.00001$). The present study proved the effectiveness of the M/V index in predicting the biological behavior and the outcome of oral squamous cell carcinoma patients.

Key words: Mitotic index, squamous cell carcinoma, Nuclear morphometry

Introduction

Cancer of the oral cavity is one of the most common cancers in the world and is a major cause of morbidity and mortality. Developing countries have the world's highest reported incidence of oral cancer. Even with the advancement of medical technologies and prognostic aids, oral cancer in the Indian subcontinent accounts for approximately 40% of all malignant tumors, with an incidence of about 56,000 cases per year.

Identification of high risk groups with respect to recurrence or rapid development of disease is important in the development of therapies for oral cancer. Assessment of the size of the primary lesion, the number of regional lymph nodes showing metastasis and the presence of distant metastasis are widely used to define the extent of tumor load and determine treatment options for patients with intra-oral squamous cell carcinoma. The TNM staging system is the conventional method for making treatment decisions. However, one of the criticisms of the TNM staging is that it ignores individual histological characteristics of tumors, and it has been proposed that a combined assessment of histological grading as well as clinical staging might provide a more precise measure for predicting the outcome of neoplasms and deciding the optimal treatment for each patient. Although there are several different approaches for predicting the prognosis of patients with oral cancer, none of them are completely satisfactory[1, 2].

The assessment of nuclear features is very

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important in the histological grading of oral squamous cell carcinoma. The various morphometric parameters studied for epithelial tumors are nuclear area, nuclear perimeter, nuclear shape factor, smallest and longest axes, nuclear size along with nuclear-cytoplasmic ratio and nucleolar-nuclear ratio[3]. The morphometry of epithelial tumor cells has been studied to examine its correlation with the prognosis of various tumors of the breast, cervix, ovary and bladder[4, 5].

Haapasalo *et al.*[6] developed the volume-corrected mitotic index which was shown to be superior to the clinical stage and histologic grade in predicting the outcome of ovarian cancer. The efficiency of this index was tested in oral cancer by Imai *et al.*[1]

This study was undertaken to assess the prognostic correlation of the various morphometric nuclear parameters—nuclear-cytoplasmic ratio (N/C ratio), nuclear-nucleolar ratio, mitotic activity index (MAI) and volume-corrected mitotic activity (M/V index)—in oral squamous cell carcinoma. An attempt was made to identify the single best prognostic indicator as well as a combination of morphometric parameters which can efficiently predict the outcome of the disease.

Materials and methods

1. Selection of patients

The study group consisted of 30 patients with oral squamous cell carcinoma who were diagnosed, treated and followed from 1999 to 2004 at the Department of Oral and Maxillofacial Surgery, Manipal College of Dental Sciences, Mangalore. The clinical features are summarized in Table 1.

Table 1 Clinical features, staging and clinical outcome of the patients with primary oral squamous cell carcinoma

S No	Clinical Parameters		Number of Cases	Percentage
1	Age	<60	15	50
		≥ 60	15	50
2	Gender	Male	22	73.3
		Female	8	26.7
3	Location	Lip	1	3.3
		Palate	2	6.7
		Alveolus	8	26.7
		Tongue	9	30.0
		Buccal Mucosa	10	33.3
4	T Stage	T2	8	26.7
		T3	9	30.0
		T4	13	43.3
	N Stage	N0	6	20.0
		N1	13	43.3
		N2	11	36.7
	Distant Metastasis	M0	29	96.7
		M1	1	3.3
5	TNM Stage	II	3	10.0
		III	13	43.3
		IV	14	46.7
6	Recurrence	Recurred	15	50.0
		Not Recurred	15	50.0
7	Clinical Outcome	Death	7	23.3
		Alive	23	76.7

Disease-free survival, overall survival, time of recurrence (if any), and mortality due to disease (if any) were chosen as prognosticators for the study. Disease-free survival is defined as the time elapsed between the initial treatment of the tumor and the reappearance of a tumor of the same histological type. Overall survival is defined as the time elapsed between initial diagnosis and death attributable to carcinoma. Approval from the institutional ethical committee was obtained prior to the study. The patients enrolled in the study satisfied the criteria of having undergone surgery as their initial and only mode of treatment; with histologically clear excisional margins and a follow-up for a minimum of 36 months or death, whichever was earlier.

2. Histomorphometric Parameters

For the morphometric studies hematoxylin and eosin (H&E) stained paraffin-embedded sections (of five μm thickness) from the core of the tumor were used. The histological malignancy grading and mode of invasion were judged according to the method proposed by Bryne *et al.*[7]

Parameter measurement

1. Mitotic activity index (MAI)[4]

Mitotic activity index is expressed as the total number of sharply defined mitoses per 10 high power fields at the invasive front.

Accurate definition of mitotic figures was essential to the study. Mitoses were counted at × 400 magnification with a 40 × objective (numerical aperture 0.65, field diameter 450 μm). Once focused, no further adjustments were made and structures that could be interpreted differently (such as artifacts) were not counted. The existence of hairy protrusions or the presence of amphophillic cytoplasm was regarded as signs in favor of mitotic figures. In contrast, the presence of a fire cone figure, a dark line parallel with the margin or the presence of large, dark round spots were not regarded as mitotic figures. If all these criteria did not allow a definite assignment, the figure under discussion was eliminated from the measurement.

2. Volume-corrected mitotic index (M/V index)[6]

The M/V index (mitosis/volume index) was determined as described by Haapasalo *et al.*[6] as M/V index = $\sum MI / \sum V\nu$, where MI = number of mitotic figures in a randomly selected microscopic field of neoplastic epithelium from the invasive front and $V\nu$ = volume fraction percent of neoplastic epithelium assessed by the point-counting technique with a 1000-point square grid overlaid on the tumor image in each field of vision. In each field, after counting the number of mitoses, points overlying the stroma and epithelium were counted and the percentage of points overlying the epithelium was taken to be the volume percent of epithelium.

3. Nuclear features[4]

The nuclear features were measured using an eye-piece reticule, at 1000 × magnification (numerical aperture 1.25, oil immersion). From 25 nuclei, the largest and the smallest diameters were measured (D = largest diameter of nucleus, d = smallest diameter of nucleus). Using these dimensions the area [$\frac{Dd}{4}\pi$], perimeter [$\frac{D+d}{4}\pi$], shape factor [$\frac{4\pi(Area)}{perimeter^2}$] and nuclear size [$2\sqrt{area/\pi}$] were assessed. The shape factor is thus dimensionless, its value equaling 1.0 in round nuclei and being less than 1.0 in elliptical nuclei. The mean and standard deviations were calculated for each of these variables and accordingly 12 nuclear parameters were used in the study.

4. Nuclear cytoplasmic ratio (N/C ratio)

Usually, cytoplasmic boundaries are clearly visible in the sections and cytoplasmic area can be measured accurately. If distinction was not possible, the cytoplasm of adjacent cells with the nuclei in focus was divided between the cells. Thus, the mean nuclear-cytoplasmic (N/C) value per case could be reproduced with satisfactory accuracy.

5. Nucleolar-nuclear ratio

Nucleolar area was measured in 25 nuclei at 1000 × magnification. If more than one nucleolus was present in a nucleus, the largest was selected

for measurement. The ratio was obtained by dividing the measured nucleolar and nuclear areas.

Statistical analysis

Four methods were used to study the prognostic value of observed and measured features using Statistical Package for Social Sciences (SPSS) version 11.0. First, the various morphometric parameters were compared with the recurrence and mortality parameters by means of an independent Student's t test. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of each significant parameter were evaluated to identify the single best prognosticator. Cut-off values were arrived at for each parameter based on the efficiency (Tables 2 and 3).

Second, a multivariate survival analysis was performed using Cox's regression model. This survival analysis took into account the duration of disease-free survival from the time of surgical treatment to either recurrence or death.

Thirdly, a discriminant analysis using stepwise logistic regression was performed to derive coefficients for each of the most important prognosticators and to apply the parameters in combination in the regression model.

Final analysis of survival was performed by Kaplan-Meier curves for each parameter separately using log rank statistics.

Definitions of efficiency, sensitivity and specificity were taken from Galen and Gambino as follows:[4]

$$\text{Efficiency} = \frac{TP + TN}{TP + TN + FP + FN} \times 100$$

$$\text{Sensitivity} = \frac{TP}{TP + FN} \times 100$$

$$\text{Specificity} = \frac{TN}{TN + FP} \times 100$$

where,

TP = true positives, *i.e.*, number of patients who died or had recurrence and are correctly predicted by a test,

TN = true negatives, *i.e.*, number of patients who survived and are correctly predicted by a test,

FP = false positives, *i.e.*, number of patients who survived and are incorrectly predicted by a test, and

FN = false negatives, *i.e.*, number of patients who died or developed recurrence and are incorrectly predicted by a test.

Results

The study revealed that both nuclear and histologic grades are significant prognosticators of recurrence / death. Of all the parameters associated with recurrence of the disease, only three parameters—invasive front grading ($p = 0.008$), mitotic activity index ($p = 0.001$) and volume-corrected mitotic index ($p = 0.008$)—were significant according to Student's t test (Table 2). Of these three parameters, the M/V index showed the highest sensitivity, negative predictive value and efficiency (Table 2).

The cut-off values of morphometric features demonstrated that a mitotic activity index exceeding 15 mitoses per 10 high power fields was an ominous sign. If the mean nuclear area exceeded 80 μm^2 and the standard deviation of nuclear area exceeded 30 μm^2 the prognosis was considerably worse. The M/V values ranged from 0.23 to 4.52 mitotic figures / mm^2 with mean $\pm$ SD of 1.53 $\pm$ 1.18. Keeping a cut-off of 1.5 for M/V, significant differences between the groups was observed with a p value of 0.015 (Table 3).

Table 4 lists the parameters showing significant differences for the event of death due to disease. Since some of these parameters contained redundant information, a reduction to fewer parameters was desirable. Thus, the ten significant parameters shown in Table 4 were used in the multivariate survival analysis. Both Cox regression analysis and forward stepwise regression yielded coefficients of two parameters: volume-corrected mitotic index and standard deviation of nuclear area. The coefficients of Cox regression analysis are given in Table 5, using which the formula for prognostic indicator of death (PI_{death}) was derived:

$$PI_{death} = 0.01816(\text{SD nuclear area}) + 0.147(M/V) - 0.537$$

Kaplan-Meier curves were plotted for the above three parameters (M/V index, SD nuclear area and PI_{death}). Figure 1 gives the two Kaplan-Meier

Table 2 Statistically significant parameters predicting recurrence of tumor

Factor	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)
Invasive front grading (p = 0.008)	10.0	80.0	66.6	70.6	76.9	73.33
Mitotic activity index (p = 0.001)	7.5	93.3	73.3	77.8	91.6	83.33
Volume-corrected mitotic index (p = 0.008)	1.0	100.0	73.3	91.6	100.0	86.87

Table 3 Comparison of different significant parameters associated with prediction of death

Factor	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)
Volume-corrected mitotic index (M/V)	1.5	71.4	73.9	45.5	89.5	73.33
Nuclear area (μm^2)	80.0	85.7	52.2	35.3	92.3	60.00
SD Nuclear area	30.0	71.4	65.2	38.5	88.2	66.67
Perimeter (μm)	438.0	85.7	60.9	40.0	93.3	66.67
SD perimeter	150.0	71.4	69.6	41.7	88.9	70.00
Circularity	0.65	85.7	69.6	46.2	94.1	70.00
Nuclear size	10.0	85.7	52.2	35.3	92.3	60.00
SD Nuclear size	1.7	71.4	60.9	35.7	87.5	63.33
Larger diameter (μm)	12.8	71.4	69.6	41.7	88.9	70.00
SD smaller diameter (μm)	2.0	85.7	69.6	46.2	94.1	73.33

PPV: Positive Predictive Value
NPV: Negative Predictive Value
SD: Standard Deviation

curves for the low PI_{death} index (< 0.4, n = 21; 3-year survival 95.324%), and high PI_{death} index patients (> 0.4, n = 9; 3-year survival 33.33%). Survival analysis done for all parameters (deemed significant by Student's t test) yielded significant values for 7 of the 11 parameters (Table 6). The highest χ^2 value for a single parameter was 7.44 for circular rate (shape factor), followed by SD of smaller diameter (χ^2 value = 6.94). In comparison with all the parameters, PI_{death} had the highest χ^2 value of 17.46 accounting for the highest significance (Table 6). The volume-corrected mitotic index was the single best predictor of prognosis (χ^2 value of 4.46, p = 0.0347) (Table 6 and Figures 2 and 3).

Discussion

Predicting the prognosis of oral squamous cell carcinoma (OSCC) greatly helps decide the treatment modality. Although there are various guides such as tumor size, lymph node metastasis, mitotic activity, mode of invasion and histological malignancy grading, their efficiency in predicting the disease outcome is not completely satisfactory. This is attributed to the variability and subjectivity present in the techniques of assessment. Quantitative methods are objective and reproducible, and their prognostic predictive value has been emphasized by various studies.

Mitotic activity has been assessed by various techniques and has proven to be an efficient prognostic indicator of squamous cell carcinoma of var-

Table 4 Independent t test demonstrating the significance of various parameters for the event of death to occur

	Died	N	Mean	t	Sig. (2-tailed)
1. Invasive front grading (IFG)	Dead	7	12.086	1.054	0.301
	Alive	23	10.983		
2. Tumor size	Dead	7	3.571	1.498	0.145
	Alive	23	3.044		
3. Node	Dead	7	1.571	1.690	0.102
	Alive	23	1.044		
4. Metastasis	Dead	7	0.000	−0.545	0.590
	Alive	23	0.044		
5. Mitotic activity index (MAI)	Dead	7	12.286	1.738	0.093
	Alive	23	7.739		
6. Volume-corrected mitotic index (M/V)	Dead	7	2.462	2.605	0.015
	Alive	23	1.250		
7. Nuclear area (μm^2)	Dead	7	113.307	2.341	0.027
	Alive	23	84.201		
8. SD Nuclear area (μm^2)	Dead	7	40.483	3.177	0.004
	Alive	23	26.814		
9. Perimeter (μm)	Dead	7	569.270	2.601	0.015
	Alive	23	423.461		
10. SD Perimeter(μm)	Dead	7	203.408	2.756	0.010
	Alive	23	135.339		
11. Shape factor	Dead	7	0.530	−2.491	0.019
	Alive	23	0.719		
12. SD Shape factor	Dead	7	0.204	−1.911	0.066
	Alive	23	0.317		
13. Nuclear size	Dead	7	11.712	2.297	0.029
	Alive	23	10.106		
14. SD Nuclear size	Dead	7	2.053	2.395	0.024
	Alive	23	1.632		
15. Larger diameter (μm)	Dead	7	14.383	2.555	0.016
	Alive	23	12.426		
16. SD Larger diameter (μm)	Dead	7	2.688	1.550	0.132
	Alive	23	2.251		
17. Smaller diameter (μm)	Dead	7	9.703	1.718	0.097
	Alive	23	8.421		
18. SD Smaller diameter (μm)	Dead	7	2.297	2.704	0.012
	Alive	23	1.877		
19. Nuclear cytoplasmic ratio	Dead	7	0.910	−0.221	0.826
	Alive	23	0.972		
20. SD Nuclear cytoplasmic ratio	Dead	7	0.896	−0.311	0.758
	Alive	23	1.380		
21. Nucleolar nuclear ratio	Dead	7	0.105	−0.577	0.569
	Alive	23	0.111		
22. SD Nucleolar nuclear ratio	Dead	7	0.059	−0.128	0.899
	Alive	23	0.061		

SD: Standard Deviation

Table 5 Coefficients of Cox's regression analysis of the most important prognosticators (forward stepwise regression)

Feature	Coefficient	Standard error
Volume-corrected mitotic index (M/V)	0.147	0.053
Standard deviation of nuclear area (SDNA)	1.816×10^{-2}	0.006
(constant)	−(0.537)	0.189

Table 6 Three-year survival rate with respect to different parameters based on univariate analysis

Parameter	Cut-off	Number (n)	Mean survival time (in months)	Log rank test	
				χ^2	P value
1. Volume-corrected mitotic index (M/V)	≤ 1.5	19	63	4.46	0.0347
	> 1.5	11	23		
2. Nuclear area	<80	13	66	3.33	0.0679
	>80	17	33		
3. SD Nuclear area	<30	17	63	3.39	0.0657
	>30	13	26		
4. Perimeter	<438	15	66	5.02	0.025
	>438	15	31		
5. SD Perimeter	<150	18	64	4.38	0.0363
	>150	12	25		
6. Shape factor	>0.65	17	67	7.44	0.0064
	<0.65	13	28		
7. Nuclear size	<10	17	66	3.33	0.0679
	>10	13	33		
8. SD Nuclear size	<1.7	16	44	2.59	0.1077
	>1.7	14	46		
9. Larger diameter	<12.8	18	64	4.38	0.0363
	>12.8	12	25		
10. SD Smaller diameter	<2.0	17	66	6.94	0.0084
	>2.0	13	22		
11. PI_{death}	<0.4	21	67	17.46	0.00001
	≥0.4	9	16		

SD: Standard Deviation

ious sites, but the studies cannot be compared due to various methodological and biological factors. Haapasalo *et al.* (1989)[6] introduced the volume-corrected mitotic index (M/V index) to reduce this variability, and the technique has been reported to be a valuable tool in carcinoma of the ovaries, bladder, pancreas, breast and colon. Our present study showed that the M/V index is a better predictive tool for recurrence than mitotic activity index with an efficiency of 86.67%. Compared to other standard prognostic tools, the M/V index was proved by discriminant analysis to be superior (Table 5).

The rationale behind this significantly better performance of the M/V index may be as follows.

1. The M/V index calculates the mitotic activity in the tumor proper, thus giving an accurate mitotic load of the tumor. The actual tumor load is calculated by assessing the volume fraction of the tumor based on Delesse's principle which

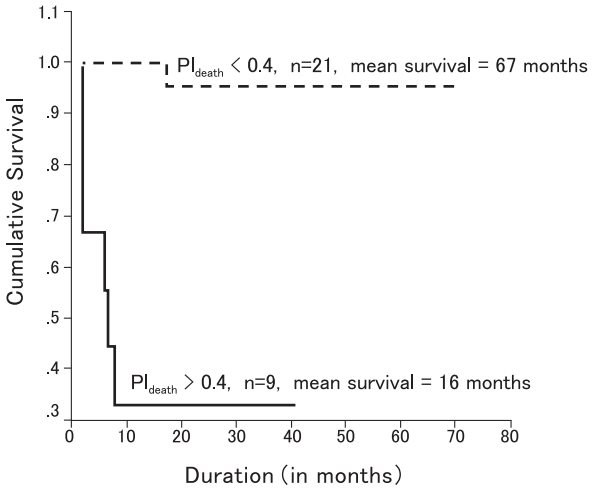

Fig. 1 Survival functions (Kaplan-Meier curves) of prognostic indicator of death with a cut-off of 0.4. Survival is significantly increased in individuals with values below 0.4.

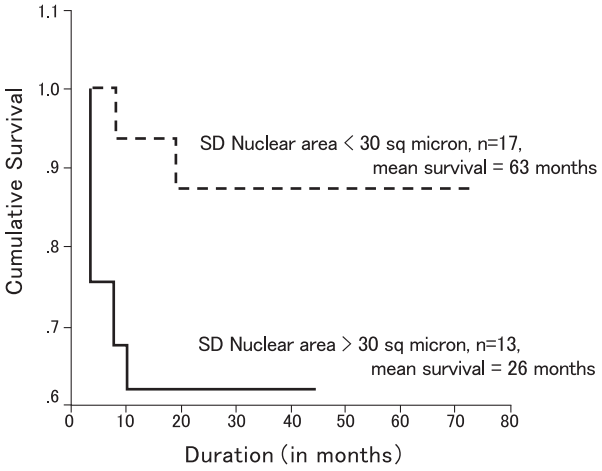

Fig. 3 Survival functions (Kaplan-Meier curves) of standard deviation of nuclear area with a cut-off of 30 square microns. Survival is significantly increased in individuals with values below 30 square microns.

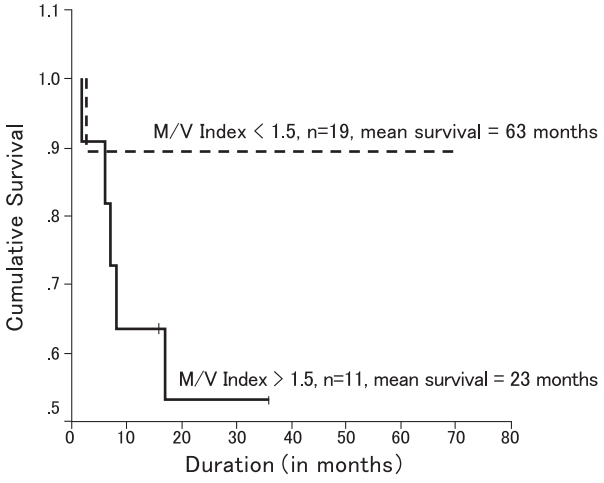

Fig. 2 Survival functions (Kaplan-Meier curves) of volume-corrected mitotic index with a cut-off of 1.5. Survival is significantly increased in individuals with values below 1.5.

states that "in a rock composed of a number of minerals the area occupied by any given mineral on a surface of a section of the rock is proportional to the volume of the mineral in the rock." This means that the volume of mineral can be assessed by calculating the area fraction in a cross-section. Weibel (1980)[8] provided the mathematical proof of Delesse's principle in histological sections. Thus, the area fraction of the tumor is given as a set of random fields correlated with the true tumor load.

2. The M/V index accounts for the tumor pattern. The value of the index increases when a tumor is invading in smaller groups and vice versa, whereas the MAI remains constant as it does not include the fraction of neoplastic tissue.

3. The use of a 1000-point grid eliminates the variability of size of the field area for all microscopes (regardless of the manufacturer and model), thus making the index standardized and comparable across various study groups.

4. Assessment of M/V index at the invasive tumor front (ITF). Most tumors consist of a heterogeneous cell population with variable biological behavior. Tumor behavior is dependent on complex interrelationships between tumor and host. Evidence suggests that cells present at the ITF of a carcinoma have different molecular characteristics from those in superficial areas of tumor[9]. Thus, the M/V index assessed at ITF makes the index a superior prognostic tool.

Bryne *et al.* (1989)[7] first described a multiple histologic grading system for the ITF in head and neck tumors, based on the pattern of invasion, degree of keratinization, nuclear pleomorphism and host response. They reported a strong correlation between total malignancy grade and prognosis of glottic carcinoma. Kurokawa *et al.* (2005)[10] observed a strong correlation between invasive

tumor front grading and prognosis of tongue squamous cell carcinoma. The present study also analyzed the prognostic value of the histological grade of malignancy in the deep invasive tumor front of oral squamous cell carcinoma and it was seen that values of < 10 significantly ($p = 0.008$) showed a reduced rate of local relapse (Table 2).

Nuclear polymorphisms, which refer to variations in nuclear size, shape and hyperchromatism due to an increased DNA content and chromatin condensation in interphase nuclei, are a widely-used criterion to recognize dysplastic and neoplastic changes of mucosal epithelium. Nuclear size corresponds to its DNA content and the amount of histone proteins, organic and inorganic material and water. Numerous studies have shown a progressive increase in nuclear area in the process of transformation from normal or metaplastic mucosa to dysplasia to carcinoma[3, 11].

In the present study, the prognostic relevance of these nuclear alterations was substantiated and quantified. The current data shows that discrimination between short-term and long-term survival is possible with morphometry and that the application of this technique adds to the qualitative evaluation. Nuclear area, perimeter, nuclear size and larger diameter were significantly larger in the short-term survivors (Table 4).

In long-term survivors the neoplastic nuclei are significantly more circular *i.e.* less ellipsoid/spindled than those in short-term survivors. Nuclear shape factor and perimeter, indicators of regularity of nuclear boundary, were powerful discriminators. The nuclei in the group with a favorable prognosis had regular profiles (Table 4). Our study supports the findings of Sutton *et al.* (2003)[12] who have shown that tumor cells with greater diameter tend to exhibit more aggressive patterns of invasion.

The standard deviations of various stereological parameters are indicative of variation from the mean value and thus may be an objective reflection of pleomorphism. In our study, the standard deviations of nuclear area, perimeter, shape factor and smaller diameter of nucleus were significantly different among the two groups of subjects *i.e.*, those with favorable prognosis and those with poor prognosis (Table 4). These parameters indicate that the tumor cells have already reached a stage in their evolution where they exhibit molecular events that permit metastasis, such as the expression of cellular receptors and production of enzymes to facilitate their access to lymphatic vessels. All these factors collectively may account for the reduced survival rate[13]. The nuclear morphometry of malignant tumors can thus reveal features that may otherwise escape the subjective analysis of cellular morphology. Nuclear morphometry can aid in the accurate diagnosis and grading of oral squamous cell carcinoma. The combination of morphometric parameters may shed light on the hidden implications of numerical values obtained by discriminant analysis, where the standard deviation of nuclear area and M/V index were combined (PI_{death}; $p = 0.00001$ and $\chi^2 = 17.46$). This could be histopathologically correlated with the variation of nuclear area (SD nuclear area) and proliferative load (M/V index) being the best prognosticators.

In the present study, clinically, the size of tumor (T) and regional lymph node (N) did not significantly correlate with M/V index (Table 4). In early cancer cases (T_2) the recurrence rate was higher in patients with higher M/V index. Similarly, in patients with N0 cancer, those with a high M/V index had a higher recurrence rate than those with a low M/V index. Imai *et al.*[1] in their study found that M/V index and DNA ploidy showed similar prognostic results for predicting the outcome of oral cancer. Thus, the M/V index appears to be a better independent prognostic factor. Tumor control is difficult even in the early stages in patients with a high M/V index. Thus, patients with high M/V values should be recalled for clinical assessment more frequently. The results of the present study emphasize the applicability of the M/V index as a prognostic indicator of oral squamous cell carcinoma. Additionally, since measurement of M/V index was performed without difficulty in routine hematoxylin and eosin sections and took approximately 15 minutes per case, we strongly advocate the inclusion of M/V index as a prognostic indicator in oral cancer. Aggressive treatment protocols should be followed up in the initial and fol-

low-up treatment of oral cancer patients with high M/V index values.

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