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NUCLEAR MORPHOMETRIC ANALYSIS OF ORAL SQUAMOUS CELL CARCINOMA USING DIGITAL IMAGE ANALYSIS AS A PROGNOSTIC TOOL FOR OSCC

Oral Pathology

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ABSTRACT

Purpose: Oral squamous cell cancer (OSCC) ranks sixth among cancer related mortality worldwide. Various malignancy-grading systems have been used as predictors for its prognosis but cervical lymph node metastasis is proved out to be the most reliable factor. So, an attempt was made to evaluate the possible role of nuclear morphometry as a prognostic tool in oral squamous cell carcinoma by comparing nuclear morphometry of tumor cell with that of normal epithelium and also by comparing nuclear morphometry between metastatic and non-metastatic oral squamous cell carcinoma. **Methodology:** Grading systems based on nuclear morphometry using image analysis was used in this study to predict the prognosis in oral squamous cell carcinoma. **Results And Conclusion:** The results showed that nuclear grading can be used as an adjuvant to clinical staging that can assist the clinician to determine the most appropriate treatment modality for the patients with oral squamous cell carcinoma for better prognosis.

KEYWORDS

OSCC (Oral squamous cell carcinoma), Nuclear Morphometry, Digital Image analysis, Nuclear size and Nuclear shape

INTRODUCTION

Genetic and epigenetic alterations play a main role in uncontrolled proliferation of damaged cells in cancer and are characterized by a relatively poor prognosis with an increasing rate of mortality.[1,2] The presence of cervical lymph node metastasis is universally accepted as the major factor influencing survival rate among patients with oral and oropharyngeal carcinomas.[3] Several histopathological features with respect to the primary tumor have been studied for predicting nodal metastases and most of the features are qualitative like histopathological grade and lymph-vascular invasion hence are subjective, so a more objective means of evaluation is needed.

Digital image analysis is one such quantitative research tool which augments histopathological tissue analysis. Morphometric image analysis not only limits interobserver variability but also is more reproducible, more sensitive and allows better assessment of quality control with less human effort. [4]

Epicenter in the process of carcinogenesis is nucleus and cancer cells contain many structural abnormalities in their nuclei which play an important role in development and progression of cancer. [5,6] Historically, nuclear features have been an integral part of tumor grading and results have shown that an increase in nuclear size was more detectable in carcinomas than in benign tumors. Additionally, several studies have reported that the increasing abnormalities of nuclear morphometric features parallels tumor progression. [7] However, only few quantitative studies exist detailing the relationship between the morphology of nucleus in oral squamous cell carcinoma (OSCC) and cervical lymph node metastasis. [8]

Thus, the present study was aimed to evaluate the possible role of nuclear morphometry through computer assisted image analysis as a prognostic tool for OSCC and the objectives were to assess and compare nuclear morphometry of tumor cell with that of normal epithelium and also to compare nuclear morphometry between metastatic and non-metastatic OSCC.

MATERIALS & METHODS

Sample consisted of 20 cases of metastatic and non-metastatic OSCC respectively and 5 cases of normal oral mucosa from gingiva and buccal mucosa during minor oral surgical procedures was taken as control. The samples consisted of fresh and archival tissue blocks received and retrieved from dept of oral pathology and general pathology of two elite institutes of the city and the sampling was done under strict inclusion and exclusion criteria. (Table 1 and 2)

The tissue sections and the archival blocks were routinely processed and stained with H & E stain and the diagnosis of OSCC was reconfirmed by three histopathologists (blind diagnosis) and was categorized under well differentiated carcinoma (WDSCC), moderate differentiated carcinoma (MDSCC) & poorly differentiated carcinoma (PDSCC) as given in the (Table 3) and the most representative invasive front region was chosen for morphometric analysis. The methodology included the following steps -

A. Acquiring Digital Images:

Images were then captured using a "Magnus MIPS UPS 070" attached to a "trinocular research microscope" with a 100X oil immersion objective and the final image with magnification of 1000 X obtained was classified by three independent histopathologists and stored. Microscopic fields were selected randomly, commencing with first representative field on the left-hand side of the section, then moving the stage to the next field and then continuing the selection to include a minimum of 7-10 representative fields from each section and each selected field included cells with distinct cellular and nuclear outlines. The 5-7 largest cells and nuclei were selected on the assumption that the plane of section would have passed through the center of the cell or nuclei being measured and would more closely represent the actual size.

B. Nuclear Morphometry:

The measurements were done using image analysis V-3 software (Windows 95/NT/98 Media Cybernetics, USA) after accurate calibration using a stage micrometer. The complete outline of nuclei was traced on the screen using a mouse pointer. The nuclear measurements were carried out using measurement tool bars of the software which was in microns (1 micron – pixel). All the measurements were saved in Microsoft excel for further statistical analysis.

Morphometric Parameters Studied

Nuclear profile was studied under two headings i.e. size and shape of nucleus which are as follows:

1. Size of Nucleus
2. Shape of Nucleus

Size of Nucleus – It is determined by 3 values -

i. Nuclear Perimeter (NP): The nuclear outline was traced using mouse cursor and software automatically calculated nuclear perimeter in microns (μ).
ii. Nuclear Area (NA): It was measured similar to nuclear perimeter and was in square microns (μ2).
iii. Nuclear Diameter (ND) or L/S ratio: It was calculated by tracing the distance between the two farthest and nearest points on the nuclear outline. The L/S ratio was calculated by taking the ratio of the two values in μ.

Shape of Nucleus- It is determined by 3 factors
i. Form factor (FF): is the deviation from circularity. Circular rate and L/S ratio denote the variation in shape, the values of which correspond to 1 in case of a round circle. If circular rate < 1 and L/S ratio > 1 then the object is elliptical.

Formula for FF = {4 x 3.14xarea/ (perimeter x perimeter)}

ii. Contour Index (CI): this measures the regularity of the particle surface. Formula for CI = NP/ √NA

If the value is 3.54 then it corresponds to a circle and if the value is > 3.54 it indicates indentations or convolutions of the outline.

iii. Coefficient of variation of nuclear area (NACV): Formula for NACV = 100 x SD of NA/MNA. (Mean Nuclear Area)

Then one-way ANOVA (analysis of variance) was used for comparing the parameter for multiple groups. Student's t-test was applied for assessing possible significant difference between two groups. P-value of 0.05 or less was considered for statistical significance.

Table 1 – Inclusion And Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
Carcinomas involving buccal mucosa and gingiva only were taken into consideration. Only cases with detailed case history with TNM staging were included. Only histologically proven metastatic and non-metastatic tumors were taken. Cases in which invasive tumor front was evident in tissue sections.	Cases in which presence of metastasis was based only on clinical examination and cases which were treated non-surgically.

Table 2 - Sample Size Of Study Groups

STUDY GROUPS	NUMBER OF CASES
Group I – Normal Mucosa	05
Group II Non-Metastatic oral squamous cell carcinoma	20
Group III Metastatic oral squamous cell carcinoma	20

Table 3 – Sample Size Of Subgroups In Each Study Group

SUBGROUPS	Non-Metastatic group II	Metastatic group III
Well differentiated squamous cell carcinoma	11	10
Moderately differentiated squamous cell carcinoma	08	09
Poorly differentiated squamous cell carcinoma	02	01

RESULTS:

Nuclear Profile And Groups:

The results have shown that the parameters of nuclear size (NA, NP and ND) have shown a statistical significance of variance between normal, metastatic and non-metastatic cases. The present result was obtained by statistically analyzing the profiles from the data stored by three independent histopathologists. When the comparison was done for the nuclear shape factors, FF and CI showed significant results between group I and II and between group I and III, but not between group II and III. However, coefficient of variation between the groups showed a consistent variation from normal to non-metastasis to metastases whereas L/S ratio did not show any statically significance between any groups. (Table 4 and 5) (Fig 1a, b: Fig 2a, b: Fig 3a, b)

Table 4 – Results And Observation Of Nuclear Profile (size) In Various Study Groups And Subgroups

Parameters	t-value	Difference between groups	Significance
Largest Diameter	44.98 16.57 7.89	Group I Vs Group II Group I Vs Group III Group II Vs Group III	P <0.05
Shortest Diameter	9.92 11.79 3.67	Group I Vs Group II Group I Vs Group III Group II Vs Group III	P <0.05
Perimeter	19.79 21.79 7.19	Group I Vs Group II Group I Vs Group III Group II Vs Group III	P <0.05
Area	20.97 19.80 6.44	Group I Vs Group II Group I Vs Group III Group II Vs Group III	P <0.05

P values <0.05 and <0.01are statistically significant and P value <0.0001 is highly significant.

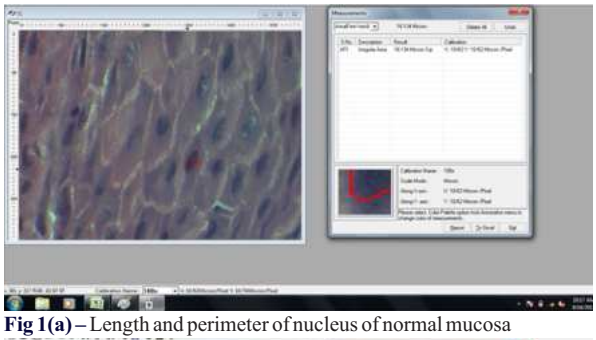

Fig 1(a) – Length and perimeter of nucleus of normal mucosa

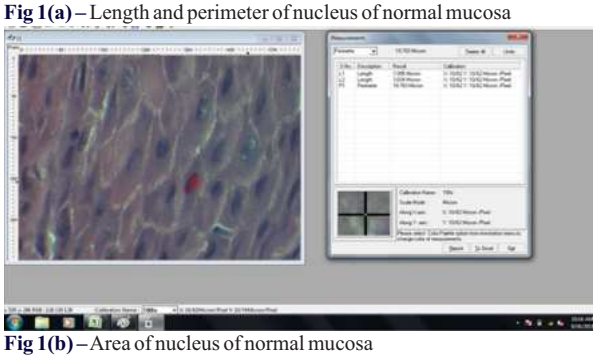

Fig 1(b) – Area of nucleus of normal mucosa

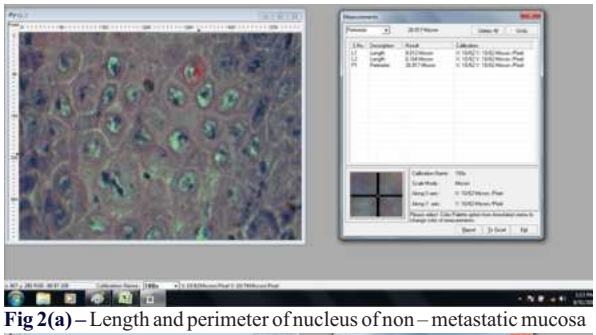

Fig 2(a) – Length and perimeter of nucleus of non – metastatic mucosa

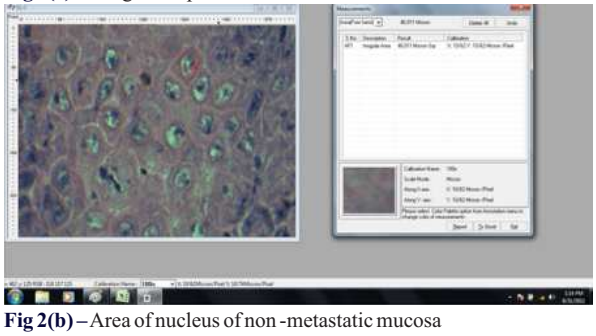

Fig 2(b) – Area of nucleus of non -metastatic mucosa

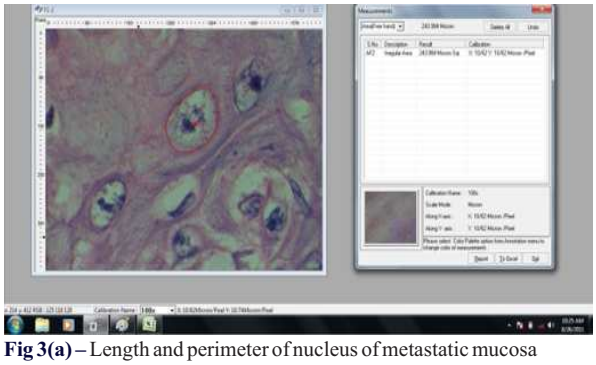

Fig 3(a) – Length and perimeter of nucleus of metastatic mucosa

Nuclear Profile And Grades Of OSCC

Comparison was made between the histopathological grades of non-metastatic and metastatic study groups. WDSCC of group II & III showed statistical significant correlation for all the parameters whereas for MDSCC only FF and CI hold statistical significance. (Table 5)

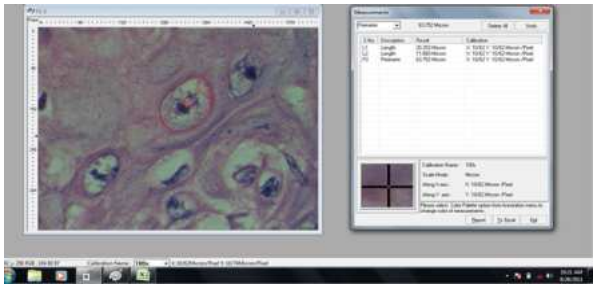

Fig 3(b) – Area of nucleus of metastatic mucosa

Table 5 – Results And Observation Of Nuclear Profile (shape) In Various Study Groups And Subgroups

Parameters	t-value	Difference between groups	Significance
L/S Ratio	1.20	Group I Vs Group II	Non-Significant
	1.78	Group I Vs Group III	Non-Significant
	0.82	Group II Vs Group III	Non-Significant
Form Factor	18.42	Group I Vs Group II	Significant
	18.47	Group I Vs Group III	Significant
	01.86	Group II Vs Group III	Non-Significant
Contour Index	20.24	Group I Vs Group II	Significant
	19.47	Group I Vs Group III	Significant
	01.15	Group II Vs Group III	Non-Significant

*P values <0.05 and <0.01are statistically significant and P value <0.0001 is highly significant.

DISCUSSION & CONCLUSION

Oral squamous cell carcinoma is a result of genetic changes occurring largely under the influence of various risk factors. The number of acquired genetic alterations progressively and sequentially increases from squamous hyperplasia through graded dysplasia to invasive carcinoma. The chromosomal loss and gain preferentially target critical components of key genetic pathways regulating cell growth, motility, and stromal interactions. These genetic damages often precede many microscopic changes. Among the observed morphological changes in tumor cells are alterations of nuclear structure. [9]

Nucleus is one of the most prominent cellular organelles because of its morphological and functional heterogeneity. The morphology of nucleus is the sum of contribution of chromatin, nuclear membrane and nuclear lamina proteins. Among the observed morphological changes observed in tumor cells are alterations of nuclear structure (shape and size) which result in chromatin reorganization thereby affecting gene expression. [5]

Structurally the metastasis to lymph nodes is manifested as specific cellular and nuclear changes which results because of distinctive clone of tumor cells. The identification of such tumor clones at an early stage will greatly aid in reducing the morbidity of oral cancer.

Morphometry is the quantification of changes in the "objects" of tissues, i.e., cells and organelles, and their organization, using quantitative evaluation tools. Modern techniques including computer systems and software with an enhanced and user-friendly approach has made the measurement of these anomaly changes much easier. The principles of morphometry illustrate the analysis of area, number, shape and size. Moreover, as the other reference studies state, the morphometric assessment of nuclear parameters including shape, size, perimeter and area can be used as additional aids not only in diagnosis but also in prognostication. [4]

An image analysis system involves a microscope, camera and a computer. Image from the microscope is captured by the camera, digitized and processed by a software program. The software can then perform measurements on this digital image through automatic and semi-automatic interactive procedures and allows not only linear measurements but also computation of area, number, texture, orientation and crowding.

Nuclear Size:

Nuclear Diameter:

Two types of measurements, largest and shortest diameter were obtained. The mean of shortest and largest diameter was lowest in the non-metastatic group whereas in the metastatic group it was highest. However, the results were not statistically significant between any of the groups (Table 4) Size of the nucleus is a result of the DNA content, the chromosomal rearrangements. [10,11] and is also closely linked with cellular volume. [12] Sutton et al (2003) reported that tumor cells with greater nuclear diameter showed more aggressive pattern of invasion. Natarajan S et al (2009) concurred with this view in their study of morphometric analysis of nuclei in prognosis of OSCC. They reported significant differences in largest and shortest diameter between oral cancer patients who were long term survivors and short-term survivors. Padovani JA (2007) reported similar differences in largest and shortest nuclear diameter in metastasized and non-metastasized tongue SCC. [13,14,15] The findings of the present study along with the previous reports seem to underline the value of nuclear diameter as an independent variable in assessment of tumor aggressiveness and have to be explored further.

Nuclear Area and Nuclear Perimeter:

Most studies have assessed nuclear size in the form of nuclear area and perimeter. The SD of these is a measure of variation of the nuclear size and consequently, the pleomorphism. In the present study, NA and NP showed a gradient increase in the values from control to non-metastatic and metastatic tumors. (Table 4). The obtained results reveal statistically significant results between all the three groups for both NA and NP. The results of the present study suggest that malignant and metastatic nuclei seem larger and that tumor cells with larger size show a more aggressive pattern of invasion and higher incidence of nodal metastases and this change is probably due to proliferation and aneuploidy which in turn is caused by non-disjunction during mitosis. [16,17,18,19,20,21,22]

Nuclear shape:

The nuclei of most cells are round to oval. Altered nuclear shape is important for cell function and is also associated with aging and many disease processes. Favored by two hypotheses

1. The first hypothesis posits that changes in nuclear shape alter the rigidity of the nucleus that could be beneficial for cells to squeeze through tight spaces but could be deleterious to cells that are under mechanical stress.
2. The second hypothesis proposes that changes in nuclear shape results in chromatin reorganization and thereby affect gene expression.

Both scenarios are applicable to cancer cells. Tumor cell nucleus is rendered irregular in shape because of:

- Abnormally short cross nuclear bands of chromatin or nuclear matrix/scaffold could cause internal angulations of nuclear membrane.
- Aggregated foci of abnormal constituents of the nuclear membrane could bind chromatin and /or nuclear matrix/scaffold and draw foci of nuclear membrane inwards.
- Abnormal perinuclear structures such as intermediate filaments or abnormal residual (post mitotic) spindle proteins could form shortened structuring bands around tumor cell nuclei. [21]

The most persistent parameter taken for assessment of nuclear shape are form factor, contour index and NACV. Smitha et al found a significant correlation for contour index and form factor between normal buccal mucosa and leukoplakia and between normal buccal mucosa and SCC however there was no statistical significance found between leukoplakia and squamous cell carcinoma. Nandini et al and Sunitha et al found a significant correlation between normal mucosa and squamous cell carcinoma but non-significant correlation between the histological grades of tumor (i.e. between moderately and well differentiated SCC) however Giradiana showed significant correlation for this parameter in histological grades. Natrajan et al suggested that this shape parameter is also a reproducible method of evaluating of biological behavior and outcome of oral SCC. Joji Sekine et al (2003) showed these two parameters were significantly higher in cases with metastasized lymph nodes when compared to node negative cases. [8,14,17,18,19,20]

Form factor showed statistical significance between group I and group II and also between group I and III however non-significant between group II and group III and contour index also showed statistical significant results between group I and group II and also between group I and III however non-significant between group II and group III which suggests that variation in nuclear shapes does exists in

carcinomas compared to normal but fails to predict the lymph node metastasis. (Table 5)

The shape parameters of nucleus can thus be an indication of anaplastic progression of the diseases however it being an important predictor of metastases in OSCC cannot be justified. But the overall results of morphometric analysis of shape and size parameters of nuclei can be considered as a more objective for quantitating the malignant potential of cancer cells and is claimed to be a good prognostic guide.

Nuclear morphometry in histological grades of tumor

Nuclear morphometry has proved to yield highly significant and independent prognostic information, so furthermore its putative value in different histological grades of tumor was also assessed. The results showed significant correlation for all the nuclear parameters in well differentiated SCC of both the groups however as the differentiation ceases no significant correlation was evident in the nuclear size but only nuclear shape factors like contour index and form factor showed significant results. (Table 4 and 5) An association between the biological behavior of tumors and chromatin pattern has been reported which exhibit a denser and condensed pattern of nucleoli and chromatin clumps in grade I tumors. [16] However, as the differentiation decreases and the aggressiveness of the tumor increases, condensation of chromatin gets altered which is not depicted in change of nuclear size but rather depicted as change in nuclear shape on visual examination.

Our study showed that nuclear morphology reflects cell's biological potential and functional activity. No single structural change in the nucleus is diagnostic by itself and hence, a combination of several nuclear characteristics provides a better indication of the tumor aggressiveness and behavior. However, when histological grades are considered under each group form factor and contour index seems to be better indicators in higher grade tumors which suggests that change in the shape is more pronounced as chromatin clumping is disturbed with loss of differentiation in high grade tumors and so is morphologically evident as pleomorphic.

So, the result here suggests that nuclear grading is an objective marking system for grading OSCC, so it can be established that, if nuclear grading is used in incisional biopsies it can serve as an adjuvant to clinical staging that can assist the clinician to determine the most appropriate treatment modality for the patients with OSCC for better prognosis. So, a large series of patients and application of this morphometry on incisional biopsies and truly prospective studies are required to determine the practical use and validity of nuclear morphometry.

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Abbreviation

OSCC = Oral squamous cell carcinoma, NP =Nuclear perimeter, NA = Nuclear area, ND = Nuclear diameter, FF = Form factor, CI = Contour Index, NACV = Coefficient of variation of nuclear area.

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