



# Evaluation of Nuclear Morphometry in Oral Squamous Cell Carcinoma: A Retrospective Study

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## ABSTRACT

**Aim:** To evaluate and compare various nuclear morphometric parameters in different histopathological grades of oral squamous cell carcinoma (OSCC) by using computerized image analysis and also to correlate it with regional lymph node metastasis.

**Materials and methods:** This retrospective study was conducted on paraffin blocks of 40 tissue specimens of OSCC cases treated with neck dissection, which were retrieved from the archives of the Department of Oral Pathology and Microbiology, Mahatma Gandhi Mission's Dental College and Hospital, Navi Mumbai, India. All cases were histopathologically graded as well, moderately, and poorly differentiated OSCC. Further, they were also categorized based on pathological lymph node status as with or without lymph node metastasis. Sections from tumor proper were subjected to Feulgen nuclear staining technique. Images of 10 microscopic fields at the deepest invading part of tumor were captured randomly and 100 nuclei of tumor cells with clear, complete, nonoverlapping outlines were selected in each case. Nuclear morphometric parameters, such as large diameter, small diameter, nuclear area, and nuclear perimeter were measured for each of the 100 cells.

**Results:** An increase in mean nuclear area coefficient of variation (NACV) was observed in OSCC cases with lymph node metastasis pN(+) than in those without lymph node metastasis pN(-), ( $p = 0.67$ ). A significant increase in nuclear area and perimeter was observed in pN(+) cases ( $p < 0.01$ ). A significant decrease in circular rate and increase in largest to smallest nuclear diameter (L/S) ratio ( $p < 0.01$ ) was observed in pN(+) cases. On comparing the nuclear morphometric parameters with different histopathological grades of OSCC, we found an increase in mean NACV values from well-differentiated OSCC to moderately differentiated and poorly differentiated OSCC ( $p = 0.612$ ). An increase in mean nuclear area and perimeter was noted as grades of OSCC advanced ( $p > 0.01$ ). The mean circular rate was found to be lowest in poorly differentiated OSCC ( $p = 0.362$ ). A significant increase in mean L/S ratio was observed within different histopathological grades of OSCC ( $p = 0.044$ ), which when further confirmed using least

significant difference (LSD) *post hoc* test, indicated a difference only between well-differentiated and poorly differentiated cases of OSCC ( $p = 0.132$ ).

**Conclusion:** Our observations reveal that tumor cells with greater nuclear dimension and more elliptical shape tend to show increased incidence of nodal metastasis. Also, a positive inclination was observed in nuclear size and shape with increasing histopathological grades of OSCC. However, our data warrant a large-scale study to establish nuclear morphometry as a quantitative objective parameter and also for the rational application of the same.

**Keywords:** Image analysis, Lymph node, Nuclear morphometry, Oral squamous cell carcinoma.

**How to cite this article:** Narayanan N, Pathak J, Patel S, Swain N. Evaluation of Nuclear Morphometry in Oral Squamous Cell Carcinoma: A Retrospective Study. J Contemp Dent 2017;7(2):107-113.

**Source of support:** Nil

**Conflict of interest:** None

## INTRODUCTION

Oral cancer is one of the most dreaded and aggressively combated diseases of this era. Worldwide, oral cancer accounts for 2 to 4% of all cancer cases.<sup>1</sup> The annual estimated incidence for oral cancer is around 275,000.<sup>2</sup> The prevalence of oral cancer is estimated to be around 45% in India.<sup>3,4</sup> Oral and pharyngeal cancer, grouped together, is the sixth most common cancer in the world.<sup>5</sup> Of all malignancies in the head and neck region, OSCC constitutes a majority of 90 percent.<sup>6</sup>

Despite the advances of therapeutic approaches, percentages of morbidity and mortality of OSCC have not improved significantly since the last 30 years.<sup>1</sup> This could be attributed to delay in diagnosis, the rapid advancement of the disease, and inaccessibility to tumor due to their metastasis.

Locoregional lymph node metastasis occurs frequently in patients with head and neck cancer.<sup>7</sup> In OSCC, tumor metastases in the regional lymph nodes at presentation is the most significant adverse prognostic factor and a major determinant of poor survival.<sup>8</sup> The process of metastasis in regional lymph nodes begins with invasive growth of tumor cells and its detachment from the primary lesion. This is followed by lymphogenous transport of cancer cells that results in lodgment

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and proliferation of cancer cells in and around lymph nodes.<sup>9</sup> The metastasis to lymph nodes manifests when specific cellular and nuclear changes occur in tumor cells, which represent a distinctive clone. The identification of this clone of tumor cells at an early stage will aid in reducing the morbidity of OSCC cases by predicting lymph node metastasis.<sup>8</sup>

Hence, specialized diagnostic and prognostic factors to predict regional lymph node metastasis is essential, as evaluation of clinical staging and conventional histopathological grading is too subjective and consequently has low-grade reproducibility.<sup>10,11</sup> Abnormal nuclear morphometry seems to provide an accurate indication of chances of regional lymph node metastasis.

Abnormal nuclear morphology is a hallmark of neoplasia. Altered nuclear morphology of transformed cells is known to associate with nuclear matrix changes. The matrix provides three-dimensional (3D) shapes to nucleus and also aids in deoxyribonucleic acid (DNA) organization. The nuclear changes taking place in neoplasia include the genetic content, the transcription factors, nongenetic content, and nuclear membrane changes. Manipulation of nuclear shape and size has been shown to affect DNA synthesis and can be well correlated with the quantity of DNA and histones.<sup>8</sup> A combination of several nuclear variables, such as nuclear dimension, which includes the area and perimeter, the shape of nuclei; and chromatin organization seems to provide a more accurate indication of tumor aggressiveness and their metastatic potential.

Digital image analyses have proved to be a useful tool in morphometric analysis of neoplastic cells. They are used to extract information contained in images by detecting, diagnosing, and describing the texture and geometry of the images.<sup>12</sup> The nuclei, being the vital structure in image analysis, mandate the use of certain special nuclear stains to enhance their study. Feulgen is a special stain that binds stoichiometrically with DNA molecule and, hence, becomes indispensable in the study.<sup>13</sup>

The purpose of this study was to evaluate and compare various nuclear morphometric parameters in different histopathological grades of OSCC using computerized image analysis software and correlate these findings with regional lymph node metastasis. This may help in evaluating the role of nuclear morphometry as a predictive marker, hence predicting the metastatic potential of OSCC.

## MATERIALS AND METHODS

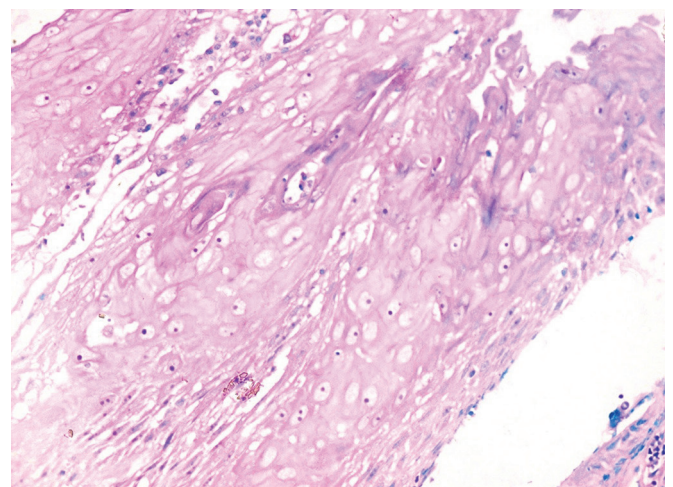
The study was carried out on tissue sections obtained from 40 diagnosed cases of OSCC retrieved from the archives of the Department of Oral and Maxillofacial

Pathology of which 20 were with pathologic lymph node involvement and other 20 without pathologic lymph node involvement. All cases were histopathologically graded as well, moderately, and poorly differentiated OSCC. Further, they were also categorized based on pathological lymph node status as with or without lymph node metastasis in accordance with the descriptive criteria for OSCC provided by the Royal College of Pathologists and World Health Organization (WHO).<sup>14,15</sup> The sections from tumor proper were subjected to Feulgen nuclear staining technique (Fig. 1). Images of 10 microscopic fields at the deepest invading part of the tumor were captured randomly and nuclei of 100 tumor cells with clear, complete, nonoverlapping outlines were selected in each case. The images were transferred to a computer, and various nuclear morphometric parameters for evaluating nuclear size (large diameter, small diameter NACV, nuclear area, and nuclear perimeter), and nuclear shape (LS ratio and circular rate) were measured with Magnus pro image analysis software for each of the 100 cells. The mean values of every parameter were thus evaluated (Figs 2 to 4). The NACV was calculated by using the formula  $NACV = 100 \times SD \text{ of } NA \div \text{mean } NA$ . The LS ratio and circular rate were computed using the measurement formulae  $D/d$  and  $(4\pi \times NA \div \pi NP^2)$  respectively.

Descriptive statistical analysis was carried out. Independent t-test was used to compare the nuclear morphometric parameters with the lymph node status and one-way analysis of variance was performed to compare the morphometric parameters with different histopathological grades of OSCC.

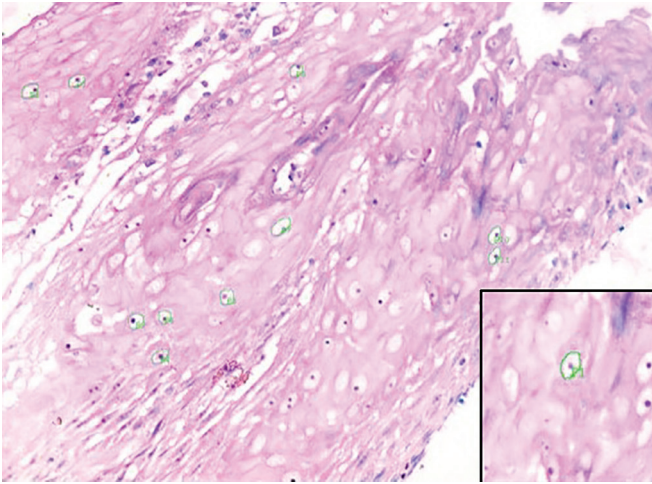
## RESULTS

Of the 40 cases of OSCC, 11 cases were well-differentiated squamous cell carcinomas ( $n = 11$ ), 17 moderately differentiated squamous cell carcinomas ( $n = 17$ ), and 12 were

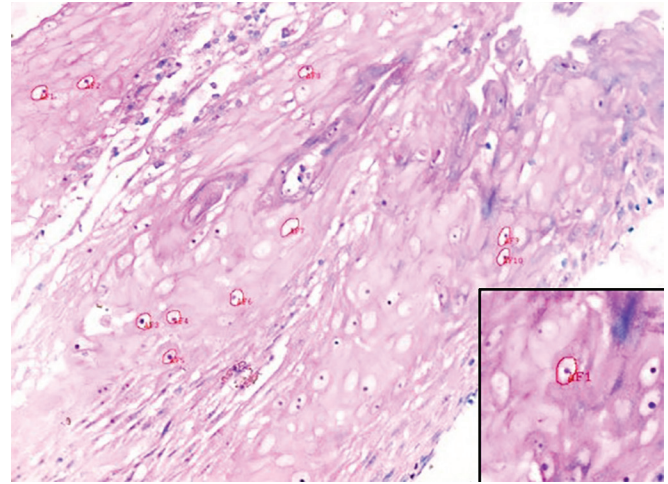


**Fig. 1:** Photomicrograph of Feulgen-stained soft tissue section in OSCC (400×)

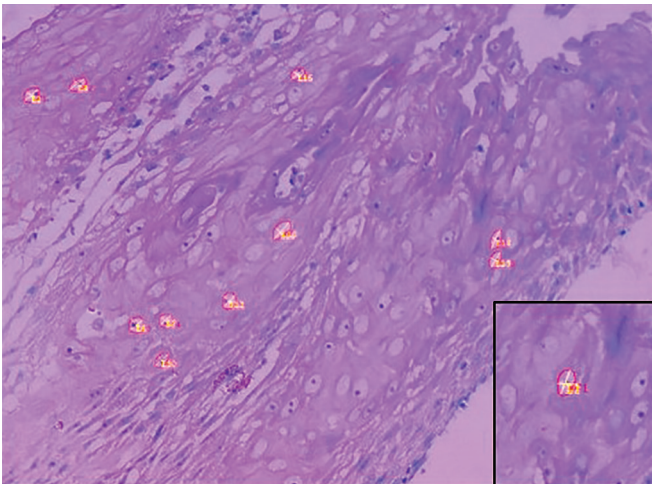




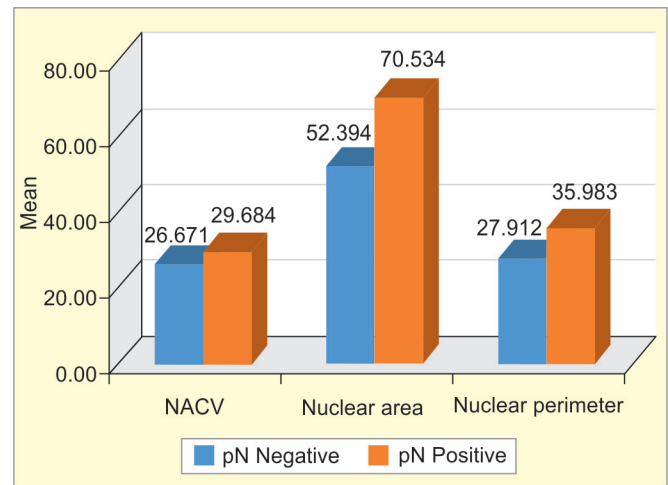
**Fig. 2:** Photomicrograph showing morphometric measurements (nuclear perimeter) using image analysis software (Feulegn, 400×)



**Fig. 3:** Photomicrograph showing morphometric measurements (nuclear area) using image analysis software (Feulegn, 400×)



**Fig. 4:** Photomicrograph showing morphometric measurements (nuclear area [red] and small and large diameter [yellow]) using image analysis software (Feulegn, 400×)



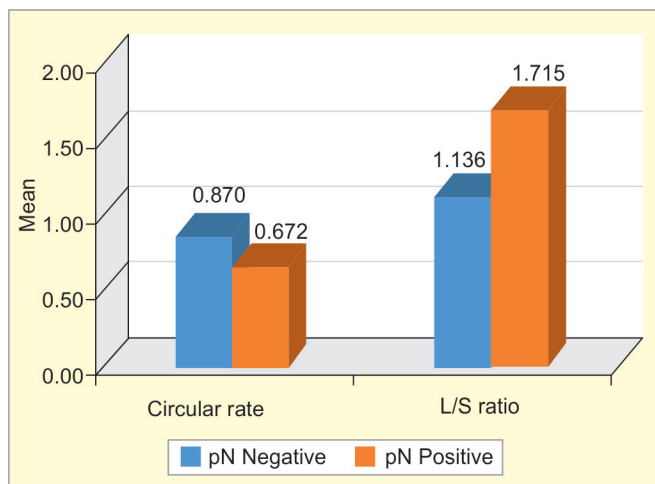
**Graph 1:** Comparison of the mean of NACV, nuclear area, and nuclear perimeter with nodal status. An increase in all three nuclear parameters is observed in lymph node positive cases pN(+) of OSCC

poorly differentiated ( $n = 12$ ) as graded in accordance with the descriptive criteria for OSCC provided by the Royal College of Pathologists and WHO.<sup>14,15</sup> On comparing the nuclear morphometric parameters with pathologic lymph node status, we observed that the mean NACV in cases with pN(−) ( $n = 20$ ) was 26.671% (standard deviation [SD] = 4.565), whereas in cases with pN(+) ( $n = 20$ ), it was 29.684% (SD = 5.513). Though an increase in mean NACV was observed from pN(−) to pN(+), it was not statistically significant ( $t = -1.882$ ,  $df = 38$ ,  $p = 0.67$ ). The mean nuclear area in cases with pN(−) ( $n = 20$ ) was found to be 52.394% (SD = 8.218), whereas in cases with pN(+) ( $n = 20$ ), it was 70.534% (SD = 16.547). An increase in mean nuclear area was observed in pN+ than in pN−, which proved statistically significant ( $t = -4.391$ ,  $df = 38$ ,  $p < 0.01$ ) (Graph 1).

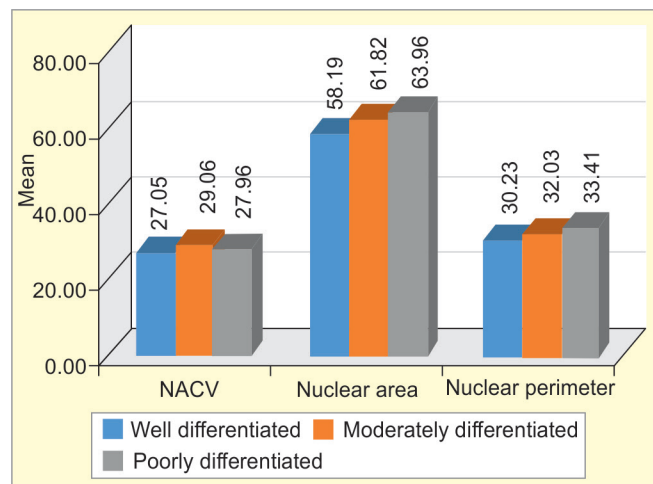
The mean nuclear perimeter in cases with pN(−) ( $n = 20$ ) was 27.912% (SD = 3.537), whereas in cases with pN(+) ( $n = 20$ ), it was 35.983% (SD = 3.830). Statistical test

indicated a significant difference in the mean nuclear perimeter ( $t = -6.924$ ,  $df = 38$ ,  $p < 0.01$ ). In our study, we found the mean circular rate in cases with pN(−) ( $n = 20$ ) to be higher, 0.870% (SD = 0.172), than in cases with pN(+) ( $n = 20$ ) where it was 0.672% (SD = 0.041) with an established p-value  $< 0.01$  ( $t = 4.994$ ,  $df = 38$ ). The mean L/S ratio in our study was found to be 1.136% (SD = 0.060) in pN(−) ( $n = 20$ ), whereas in cases with pN(+) ( $n = 20$ ), it was 1.715% (SD = 0.223). Statistical analysis indicated a significant difference in the mean L/S ratio ( $t = -11.197$ ,  $df = 38$ ,  $p < 0.01$ ) (Graph 2).

On statistically evaluating the nuclear morphometric parameters with different histopathological grades of OSCC, we found the mean NACV in cases with well-differentiated OSCC ( $n = 11$ ) was 27.048% (SD = 4.958), in moderately differentiated OSCC ( $n = 17$ ) was 29.061% (SD = 6.226), and in poorly differentiated OSCC was 27.961% (SD = 3.937). However, no statistical significance was



**Graph 2:** Comparison of the mean values of circular rate and L/S ratio with nodal status. A decrease in circular rate and an increase in L/S ratio is seen in lymph node positive cases pN(+) of OSCC

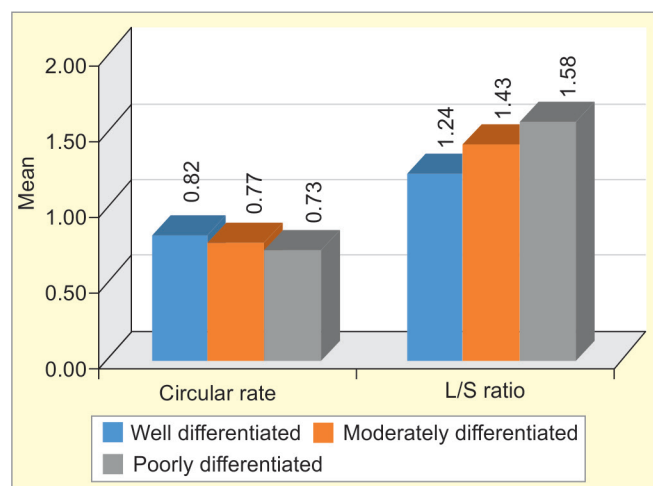


**Graph 3:** Comparison of mean values of NACV, Nuclear area & Nuclear perimeter in different histopathological grades of OSCC. An increase in NACV is noted in moderately differentiated OSCC whereas Nuclear area and Nuclear perimeter is increased in poorly differentiated OSCC

established ( $p = 0.612$ ). The mean nuclear area in cases with well-differentiated OSCC ( $n = 11$ ) was 58.194% (SD = 11.464), in moderately differentiated OSCC ( $n = 17$ ) was 61.820% (SD = 18.293), and in poorly differentiated OSCC was 63.958% (SD = 16.274). The mean nuclear perimeter in cases with well-differentiated OSCC ( $n = 11$ ) was 30.225% (SD = 4.689), in moderately differentiated OSCC ( $n = 17$ ) was 32.026% (SD = 6.091), and in poorly differentiated OSCC was 33.414% (SD = 5.187). An increase in mean nuclear area and perimeter was noted as grades of OSCC advanced, though the difference was not statistically significant ( $p > 0.01$ ) (Graph 3). The mean circular rate in cases with well-differentiated OSCC ( $n = 11$ ) was 0.822% (SD = 0.181), in moderately differentiated OSCC ( $n = 17$ ) was 0.769% (SD = 0.159) and in poorly differentiated OSCC was 0.726% (SD = 0.136,  $p = 0.362$ ). The mean L/S ratio in cases with well-differentiated OSCC ( $n = 11$ ) was 1.241% (SD = 0.268), in moderately differentiated OSCC ( $n = 17$ ) was 1.433% (SD = 0.317), and in poorly differentiated OSCC was 1.584% (SD = 0.352). A significant increase in mean L/S ratio was observed within different histopathological grades of OSCC ( $p = 0.044$ ), which when further confirmed using LSD *post hoc* test indicated a difference only between well-differentiated and poorly differentiated cases of OSCC ( $p = 0.132$ ) (Graph 4).

## DISCUSSION

Oral cancer is a multifactorial disease with a remarkable incidence worldwide, yet with a fairly burdensome prognosis. No significant improvement has been established in the morbidity and mortality rates of OSCC. The mortality, secondary to metastasis, has been estimated to be from 20 to 60%.<sup>16</sup> This could be attributed to aggressive local invasion and inaccessibility to tumor due to



**Graph 4:** Comparison of mean values of circular rate and L/S Ratio in different histopathological grades of OSCC. A decrease in mean circular rate is noted from well to moderately to poorly differentiated OSCC. An increase in L/S ratio is seen as the grade of OSCC increases

their metastasis. The management of the neck in early squamous cell carcinomas of the oral cavity has been the subject of debate for many years, since metastasis to the cervical lymph nodes is associated with a significant decrease in survival.<sup>16</sup>

Oral squamous cell carcinoma has a great predisposition to produce metastasis in cervical lymph nodes.<sup>17</sup> The metastasis to lymph nodes manifests when specific cellular and nuclear changes occur in tumor cells, which represent a distinctive clone. The identification of this clone of tumor cells at an early stage will aid in predicting lymph node metastasis.<sup>8</sup> Thus, a vigilant approach to morphologic factors is essential, which may prove valuable in predicting the likelihood of regional lymph node metastasis in OSCC. These morphological variations could

be best assessed by observing the alteration in size and shape of cell, more precisely the nucleus. The cellular and nuclear morphology, normally, are within limits reflecting normal cell division and maturation. However, neoplastic cells show significant variation in cellular and nuclear size and shape (pleomorphism and anisonucleosis).<sup>18</sup>

Altered cell structure and abnormal nuclear morphology are hallmarks of neoplasia, reflecting fundamental alterations in DNA organization and nuclear skeleton. Thus, abnormal nuclear morphometry seems to provide an accurate indication of chances of regional lymph node metastasis. Though several parameters and biomarkers are offered as prognostic indicators that could help identify metastasis, nuclear morphometry is a predictive and objective biomarker, which could tap the earliest changes occurring in primary tumor leading to metastasis. Quantitation of altered nuclear morphometrical parameters, such as nuclear dimension, which includes the nuclear area and perimeter and the shape of nuclei, seem to provide a more accurate indication of tumor aggressiveness and their metastatic potential. Computerized image analysis systems allow objective analysis of the nuclear morphologic features that can also be compared with the molecular genetic alterations in the same tumors.<sup>19</sup>

In our study, we observed a higher nuclear area and nuclear perimeter in OSCC cases with lymph node metastasis than those without metastasis, thereby indicating an alteration in the nuclear size as the tumor cells underwent metastasis. Our result was in accordance to studies conducted by various authors.<sup>8,20-25</sup> The metastasis to lymph nodes manifests when specific cellular and nuclear changes occur in tumor cells, which represent a distinctive clone. The tumor cells undergo certain changes phenotypically that aids in transportation of these cells to the lymphatic stream. Nuclear morphology of the cells seems to be determined in large part by the nuclear matrix that contains structural elements of pore complexes, lamina, an internal network of filaments, and nucleoli. Nuclear matrix not only imparts a 3D shape to the nucleus, but also serves as an important tool in DNA organization. The DNA loop domains are found attached at their bases to nuclear matrix, and this organization is maintained throughout both interphase and metaphase.<sup>26,27</sup> The nuclear matrix also plays an important role in DNA replication, as the matrix contains fixed sites for DNA synthesis located at the base of the loops. Various types of DNA organization including gene amplification, sister chromatid exchange, and a series of deletions and rearrangements have been proposed to be involved from initiation of carcinogenesis to progression and metastasis. A change in order of arrangement of DNA sequence would further alter the cell morphology. The nuclear content and, therefore, the content of DNA are directly proportional

to the nuclear size (diameter, area, and perimeter).<sup>8,28</sup> Tumor cells with greater nuclear dimension tend to show increased incidence of nodal metastasis thus, indicating that size of the nucleus of tumor cells could prove valuable in predicting the metastatic potential.

We also observed a significant variation in shape of the nucleus of tumor cells. This was in accordance with Natarajan et al.<sup>8</sup> and Sekine et al.<sup>25</sup> The nuclear matrix not only imparts a 3D structure to nucleus, but also plays an important role in DNA organization. A change in order of arrangement of DNA sequence would alter cell morphology.<sup>8</sup> Nuclear pleomorphism is a characteristic feature defining neoplastic cells.<sup>7</sup> The measurement of spindling of cells was effectively done by circular rate and L/S ratio, which signified less spherical (elliptical) nuclei in metastasized tumors. Thus, a variation in shape, indicative of pleomorphism, may also have a predictive value in detecting metastatic clones in primary tumor.

The only quantitative parameter used presently in malignancy grading is mitotic index, though it is proved that nuclear pleomorphism is one of the best prognostic indicators. A major drawback of conventional histopathological grading is its subjective nature of observation and consequently low-grade reproducibility.<sup>10</sup> Hence, a quantitative method of grading is essential to reduce this level of subjectivity. In our study, we have found an increase in nuclear dimensions (area and perimeter) with increasing grades of OSCC. This was in accordance to Nandini and Subramanyam,<sup>29</sup> who in their study, observed a gradual increase in mean nuclear area and perimeter with increasing grades of carcinoma ( $p < 0.001$ ). Nuclear size corresponds to its DNA content and the amount of histone proteins, organic and inorganic material, and water.<sup>8</sup> Alterations in nuclear DNA content and chromosomal aberrations are basic characteristics of tumor cells. A change in order of arrangement of DNA sequence resulting in genetic instability would further alter the cell morphology.<sup>8,30</sup>

Variation in nuclear shape is a distinctive feature of malignancy. In our study, we found the mean circular rate was found to be lowest in poorly differentiated OSCC when compared with well- and moderately differentiated. This is in accordance to a study conducted by Nandini and Subramanyam,<sup>29</sup> who also found a gradual decrease in value of form factor/circular shape with increasing grades of carcinoma ( $p < 0.001$ ). The L/S ratio was found to gradually increase with increasing grades of OSCC, attaining a highest value in poorly differentiated OSCC cases. Statistical test revealed a significant difference in the mean L/S ratio within different histopathological grades of OSCC, which was further confirmed using LSD *post hoc* test. The test indicated a statistically significant mean difference only in L/S ratio between well-differentiated



and poorly differentiated cases of OSCC. No study has been executed hitherto comparing L/S ratio with different histopathological grades of OSCC. Lowest levels of malignancy are characterized by more spherical nuclei of approximately equal size, whereas highest grade tumors are characterized by profound anisonucleosis, thus resulting in a more elliptical shape as the grade of OSCC increases.<sup>31</sup> Thus, from our study, we infer that change in nuclear shape may be a more reliable quantitative, objective parameter than nuclear size in assessing different histopathological grades of OSCC since the results were statistically significant.

## CONCLUSION

The results of our study showed an increase in nuclear size (area and perimeter) with alteration in nuclear shape (elliptical nuclei) in tumor cells of OSCC cases with lymph node metastasis. Thus, it can be inferred from our study that altered nuclear morphometry can be used as a reliable objective marker in predicting lymph node metastasis. Our results also showed a positive inclination in nuclear size (area and perimeter) and shape (LS ratio) with increasing histopathological grades of OSCC. The nuclear size increased with increasing histopathological grades of OSCC, although the results were not statistically significant due to insufficient sample size. The shape became progressively elliptical as determined by L/S ratio. Now, having determined the significance of altered nuclear size and shape in OSCC, a clinical application could be encouraged by employing these morphometric changes in incisional biopsy. However, no baseline values are known to be documented, describing morphometric parameters of normal oral mucosa in histopathologic tissue sections. Hence, the urge for further studies authenticating the morphometric parameters of normal oral mucosal tissue specimens, centered on which the alterations could be assessed. Thus, obtaining a precise objective parameter in OSCC could aid in better understanding of advancement of this disease, consequently augmenting the role of nuclear morphometry in modern era oncopathology.

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