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Research Article

MORPHOMETRIC ANALYSIS OF NUCLEAR FEATURES IN ORAL SQUAMOUS CELL CARCINOMA AND ORAL LEUKOPLAKIA BY USING FEULGEN NUCLEAR STAIN

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A B S T R A C T

Background: Oral Squamous Cell Carcinoma (OSCC) is the sixth most common cancer worldwide. OSCC will develop from dysplastic oral mucosal lesions if an early diagnosis has not been made and treatment rendered. Feulgen stain is a reliable and specific histochemical method for nuclear DNA. Morphometric analysis is simple, fast and reliable when using the computer assisted area measurement and it is felt that this technique might be an adjunct to the diagnostic possibilities for determining tumor behavior.

Aim & Objective:
To study and compare the nuclear changes in epithelial cells of Oral Leukoplakia (OL) and OSCC with normal buccal mucosa (NBM) using computer aided image analysis in tissue section.

Materials & Method:
The Study group consisted of 60 cases;30 of OL and 30 cases of OSCC. The control group consisted of 10 cases of NBM. The specimen was subjected to Feulgen Stain and H&E stain. The images were captured using (Olympus E331 SLR) Digital Camera by attaching to Olympus research microscope (BX-51). All measurements were done using the measuring tools of the image analyzer software.

Statistical Analysis: Using Chi-Square Test and ANOVA and Correlation were assessed using Pearson's Correlation test.

Results: Our results were significant for the morphometric parameter. The values of nuclear perimeter and are increased gradually from NBM to OL to SCC.

INTRODUCTION

OSCC is defined as a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges.[1] OSCC is recognized as the most common malignant epithelial neoplasm of the oral cavity, resulting from genetic damage, leading to uncontrolled cell proliferation of damaged cells. In the course of its progression, visible changes are taking place at the cellular level (atypia) and at the resultant tissue level(epithelial dysplasia). The sum total of these physical and morphological alterations is of diagnostic and prognostic relevance and is designated as precancerous changes.[2]

From the vast literature on molecular markers in oral cancer and oral precancer, no reliable prognostic markers for risk assessment in putatively premalignant lesions have emerged. However, histopathologically assessed severity of oral epithelial dysplasia is considered as the "gold standard" for the prediction of malignant transformation of precancerous lesions.[2] In the assessment of dysplastic and neoplastic lesions great emphasis is placed on the changes in nuclear parameters. Among these parameters, changes in nuclear perimeter, nuclear diameter, nuclear area and nuclear cytoplasmic ratio are considered to be significant.[3]

Prognostic evaluation and treatment planning for OSCC is mainly based on clinical staging and histological grading, which, in turn is based on subjective evaluation of parameters and is therefore often not sufficiently reproducible. Since nucleus is the main center controlling cell metabolism and function, nuclear analysis for predicting biological behavior of diseases has gained importance in the recent years. Computer-assisted morphometry reduces the problems of inter observation variation.[4]

In the current study, an attempt has been made to evaluate the morphometric changes in Nuclear area (NA), Nuclear

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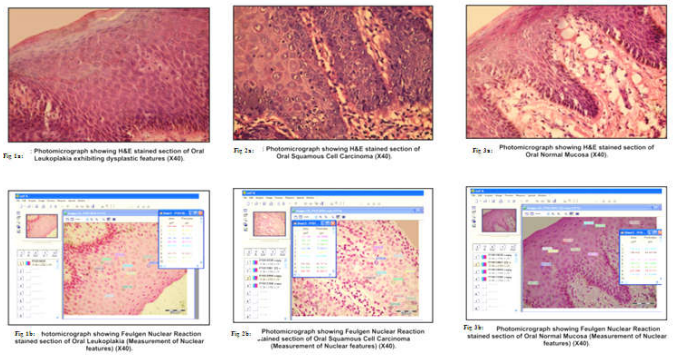
perimeter (NP) and Nuclear form factor(NF) in dysplastic epithelial cells of OL and OSCC by using feulgen stain and compare it with epithelial cells of normal oral mucosa.

MATERIALS AND METHODS

The current study was a prospective case-control study. Institutional ethical and informed consent from the subjects was duly obtained. A total of 70 subjects participated in the study. Cases were randomly selected from the Out Patient Department of our institution. The subjects were divided into three groups: Group A consisted of 30 clinically and histopathologically confirmed cases of Oral Leukoplakia (with dysplasia); Group B comprised of 30 cases of clinically and histopathologically confirmed cases of Oral Squamous cell carcinoma and Group C includes 10 samples of Normal Oral Mucosa from healthy subjects taken as control.

Tissue sections of 4µm thickness were cut using soft tissue microtome from formalin- fixed, paraffin- embedded tissue blocks. The section obtained was stained with Hematoxylin and Eosin and Feulgen Nuclear Reaction.[5] Diagnosis of the lesions were made after observation of sections using Olympus pentahead research microscope (BX 51).

All the tissue stained sections were evaluated under a research light microscope. For nuclear morphometric analysis the images were captured using Olympus E331 SLR Digital Camera by attaching to Olympus pentahead research microscope (BX-51). The actual measurements were done using the windows based image analyzer software. (Image analysis software) The final image captured on the monitor had a magnification of X40. Later the images were stored for our study. (Fig. 1a, 1b, 2a, 2b, 3a &3b)



All measurements were done using the measuring tools of the image analyzer software. 50 cells with well-defined borders were selected randomly, commencing with first representative field on left hand side and then moving the stage to the next field and continuing the selection to include 5 fields from each section. Stage readings were noted for reassessment. Outline of cells were traced with the pointer using the cursor controlled by a mouse. The software automatically calculated nuclear area while nuclear form factor is calculated by using following formula[6]:

Form factor = 4π area/ perimeter^2

The data was expressed as the mean±standard deviation (S.D.). For statistical analysis, Chi-square tests along with one-way ANOVA tests were used. Correlation was done using Pearson correlation test and linear regression was used for regression analysis.

OBSERVATION AND RESULTS

A total of 70 age and gender matched subjects were recruited. Of total, 10 were normal healthy (14.3%), 30 were with OL (42.9%) and 30 were with OSCC (42.9%). The basic characteristics (age, sex, habits, site and grade) of three groups (Control, OL and OSCC) of subject's are summarized in Table 1.

Table 1 Basic characteristics of three groups

Characteristics	Control (n=10) (%)	OL (n=30) (%)	OSCC (n=30) (%)	F/χ^2 value	P value
Age (yrs): Mean ± SD Range	48.50 ± 4.28 45-59	48.23 ± 4.51 40-57	49.47 ± 3.40 45-56	0.73	0.484
Sex: Females Males	1 (10.0%) 9 (90.0%)	8 (26.7%) 22 (73.3%)	8 (26.7%) 22 (73.3%)	1.30	0.523

Nuclear area

The Nuclear area of three groups are summarized in Table 2 and also shown graphically in Fig. 8. The Nuclear area of control, OL and OSCC groups ranged from 154.74-189.43 µm^2, 239.55-258.79 µm^2 and 300.00-324.08 µm^2, respectively with mean (± SD) 163.74 ± 10.45 µm^2, 247.10 ± 5.24 µm^2 and 312.16 ± 6.57 µm^2, respectively. The mean nuclear area of both OL and OSCC groups were comparatively higher than Control group.

Table 2 Nuclear area (Mean ± SD) of three groups

Control (n=10)	OL (n=30)	OSCC (n=30)	F value (2,67 DF)	P value
163.74 ± 10.45 (154.74-189.43)	247.10 ± 5.24 (239.55-258.79)	312.16 ± 6.57 (300.00-324.08)	1972.00	<0.001

Numbers in parenthesis indicates the range (min to max)

Comparing the mean nuclear perimeter of three groups together (Table 3), ANOVA revealed significantly different nuclear perimeter among the groups (F=586.40, P<0.001). Further, comparing the mean Nuclear perimeter between the groups (Table 4), Tukey test revealed significantly different and higher Nuclear perimeter of both OL (19.7%) (48.50 ± 4.12 vs 60.41 ± 1.29, mean diff.=11.91, q=25.42; P<0.001) and OSCC (30.9%) (48.50 ± 4.12 vs 70.20 ± 0.82, mean difference=21.71, q=46.33; P<0.001) groups as compared to Control group (Fig. 8). Furthermore, the mean Nuclear perimeter of OSCC group (14.0%) was also found to be significantly different and higher as compared to OL group (60.41 ± 1.29 vs 70.20 ± 0.82, mean difference=9.80, q=29.57; P<0.001).

Table 3 Comparison of mean Nuclear perimeter of three groups by ANOVA

Source of variation (SV)	Sum of squares (SS)	Degrees of freedom (DF)	Mean square (MS)	F Value	P Value
Groups	3861.00	2	1930.00	586.40	<0.001
Residual	220.60	67	3.29		
Total	4081.00	69	1933.29		

Table 4 Comparison (p value) of mean Nuclear perimeter between the groups by Tukey HSD test

Comparisons	Mean Diff.	Tukey q value	P Value	95% CI of diff.
Control vs. OL	11.91	25.42	P < 0.001	-13.50 to -10.32
Control vs. OSCC	21.71	46.33	P < 0.001	-23.30 to -20.12
OL vs. OSCC	9.80	29.57	P < 0.001	-10.92 to -8.671

Nuclear form factor

The Nuclear form factors of three groups are summarized in Table 5. The Nuclear form factor of control, OL and OSCC groups ranged from 0.85-0.97, 0.72-0.89 and 0.73-0.84, respectively with mean (± SD) 0.92 ± 0.04, 0.85 ± 0.03 and 0.80 ± 0.02, respectively. The mean nuclear form factor of control group was the highest followed by OL group and OSCC group, the least.

Table 5 Nuclear form factor (Mean ± SD) of three groups

Control (n=10)	OL (n=30))	OSCC (n=30)	F value (2,67 DF)	P value
0.92 ± 0.04 (0.85-0.97)	0.85 ± 0.03 (0.72-0.89)	0.80 ± 0.02 (0.73-0.84)	83.41	<0.001

Numbers in parenthesis indicates the range (min to max)

Comparing the mean nuclear form factor of three groups together (Table 6), ANOVA revealed significantly different nuclear form factor among the groups (F=83.41, P<0.001). Further, comparing the mean nuclear form factor between the groups (Table 7), Tukey test revealed significantly different and lower nuclear form factor of both OL (7.3%) (0.92 ± 0.04 vs. 0.85 ± 0.03, mean diff.=0.07, q=9.66; P<0.001) and OSCC (13.1%) (0.92 ± 0.04 vs. 0.80 ± 0.02, mean diff.=0.12, q=17.50; P<0.001) groups as compared to Control group. Furthermore, the mean Nuclear form factor of OSCC group (6.3%) was also found to be significantly different and higher as compared to OL group (0.85 ± 0.03vs. 0.80 ± 0.02, mean difference=0.05, q=11.09; P<0.001).

Table 6 Comparison of mean nuclear form factor of three groups by ANOVA

Source of variation (SV)	Sumof squares (SS)	Degrees of freedom (DF)	Mean square (MS)	F Value	P Value
Groups	0.12	2	0.06	83.41	<0.001
Residual	0.05	67	0.00		
Total	0.17	69	0.06		

Table 7 Comparison (p value) of mean nuclear form factor between the groups by Tukey HSD test

Comparisons	Mean Diff.	Tukey q value	P Value	95% CI of diff.
Control vs. OL	0.07	9.66	P < 0.001	0.04 to 0.09
Control vs. OSCC	0.12	17.50	P < 0.001	0.10 to 0.14
OL vs. OSCC	0.05	11.09	P < 0.001	0.04 o 0.07

Correlation and regression

To see the association (correlation) between Nuclear parameters (Nuclear area, Nuclear perimeter and Nuclear form factor), a similar correlation and regression was done further between the nuclear parameters and summarized in Table 8 and also depicted graphically in Fig. 4 to Fig.6, respectively.

Correlation analysis revealed a significant and positive (direct) correlation between nuclear area and nuclear perimeter (r=0.97, P<0.001). However, nuclear form factor showed significant and negative (inverse) correlation with both nuclear area (r=-0.82, P<0.001) and Nuclear perimeter (r=-0.90, p<0.001)

Further, regression analysis showed that the nuclear perimeter and nuclear form feed both are estimated significantly by nuclear area with high coefficient of determination (R^2) 93.0% (Fig.4) and 67.0% (Fig. 5), respectively. Similarly, the nuclear form feed can also be estimated significantly by nuclear perimeter with 81.0% (Fig. 6) coefficient of determination. The estimated best-fit regression equations follow as below:

Nuclear perimeter = 0.1447 nuclear area + 24.832 …. (i)

Nuclear form factor= -0.0008 nuclear area + 1.0424 …. (ii)

Nuclear form factor= -0.0057 nuclear perimeter + 1.1975 … (iii)

Table 8 Correlation (n=70) between Nuclear parameters

Parameters	Nuclear area	Nuclear perimeter	Nuclear form factor
Nuclear area	1.00		
Nuclear perimeter	0.97***	1.00	
Nuclear form factor	-0.82***	-0.90***	1.00

***P<0.001

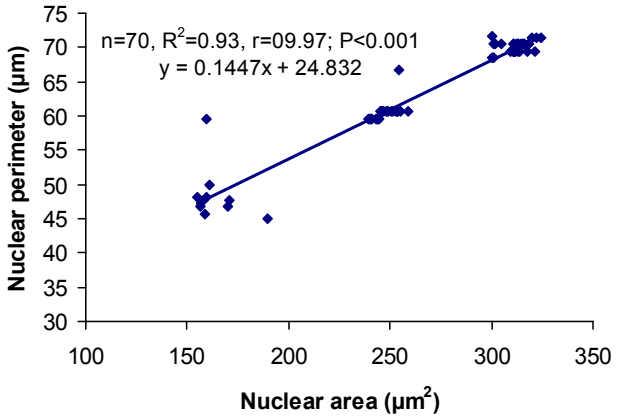


Fig 4 Correlation and best fit regression between Nuclear area and Nuclear perimeter

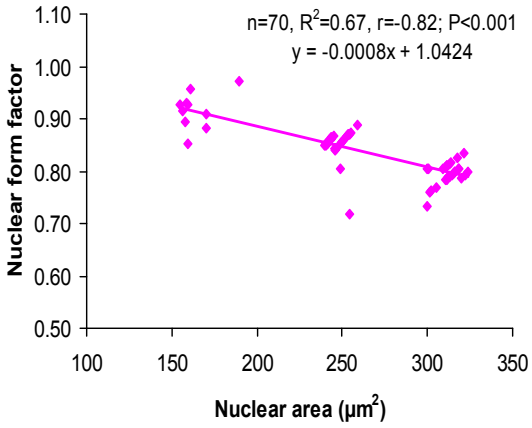


Fig 5 Correlation and best fit regression between Nuclear area and Nuclear form factor

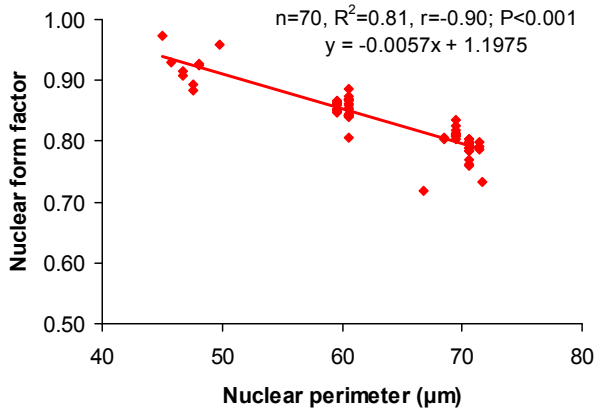


Fig 6 Correlation and best fit regression between Nuclear perimeter and Nuclear form factor

DISCUSSION

OSCC is the sixth most common cancer worldwide. More than 90% of all oral cancers are squamous cell carcinoma. The highest incidence and prevalence of OSCC is found in the Indian subcontinent where the risk of developing OSCC is increased by the very prevalent habits of chewing tobacco, betel quid and areca-nut.[7] Males are affected twice as frequently as females and the majority of patients are more than 45 years old.[8]

More than 75% of oral cancers are reported to occur in a preexisting Leukoplakia and the prevalence of Leukoplakia in the Indian population is reported to range from 0.7 to 5.0% in various regions in India and the global prevalence of Leukoplakia is 2.6%[9] Many OL regress or stay quiescent, but many progress and about 3-6% turn into OSCC in future.[10,11]

It is believed that identification and monitoring of these potentially malignant lesions and conditions allow clinician to detect and treat early intraepithelial stages of oral cancer, like epithelial dysplasia or carcinoma *in situ*, which usually precede the development of invasive OSCC. Alteration of cellular morphology and altered tissue architecture as observed from histopathology contributes in the determination and confirmation of pathological states. However visual estimation lacks reproducibility and may mislead diagnostic procedures.[12]

Computer assisted image analysis morphometry provides a new powerful tool for high precision measurement of several variables characterizing the size and shape of cancer cell nuclei in conventional tissue sections. Several of these nuclear profiles seem to be useful prognostic predictors in various human malignancies.[13]

In the assessment of dysplastic and neoplastic lesions great emphasis is placed on the change in nuclear and cellular size and shape.[14] Computer assisted image analysis can reduce the tedium of large number of measurements and calculations and hence reduced the time and increase the accuracy.[15] Morphometry has also reduced the problems of inter observer variation and reproducibility of grading systems.[16]

There were several studies done on morphometry by using routine histological and special stains. Doyle and Manfold first employed Feulgen reaction along with micro-spectrophotometry for the evaluation of oral cancer in 1975 for predicting the transformation of OL to OSCC.[16]

Feulgen stain contains fuschin or magenta I, a decolorized dye that has a strong affinity for DNA, producing a red color, the feulgen reaction is specific for deoxy-ribonucleoprotein thus nuclear chromatin and chromosomes are stained.[17] Nuclear DNA content is one parameter that can be reproduced repeatedly and is known to correlate well with the malignant potential of a tumor. Feulgen reaction is a reliable and specific histochemical method for nuclear DNA.[5]In the assessment of dysplastic and neoplastic lesions great emphasis is placed on the changes in nuclear parameters. Among these parameters, changes in nuclear area, nuclear perimeter and nuclear form factor are considered to be significant. [15]

In the present study for the nuclear area of normal mucosa, OL and OSCC, the mean values were 163.74 ± 10.45 µm^2, 247.10 ± 5.24 µm^2 and 312.16 ± 6.57 µm^2, respectively. Thus the morphometric findings demonstrated in this study revealed that the mean nuclear area of both OL and OSCC groups were comparatively higher than control groups. Comparing the mean nuclear area of three groups together (Table 4), ANOVA revealed significantly different nuclear area among the groups (F=1972.00, P<0.001).

Further, comparing the mean nuclear area between the groups (Table 5), Tukey test revealed significantly different and higher nuclear area of both OL (33.7%) and OSCC (47.5%) groups as compared to control group (Fig. 7).

Furthermore, the mean nuclear area of OSCC group (20.8%) was also found to be significantly different and higher as compared to OL group (247.10 ± 5.24 vs. 312.16 ± 6.57, mean diff. =65.04, q=52.95; P<0.001).

The findings in our study was in accordance with the study conducted by Nandini *et al*[4], Sunitha *et al*[18] and Saku and Sato[19] The result in the present study was also similar to the study done by Smitha *et al* [2] in their study showed that the nuclear area significantly increased steadily from normal buccal mucosa to Leukoplakia and reached the highest value in well differentiated OSCC.

Shabana *et al*[14] found that progressive increase in the dimensions of the nuclei from normal mucosa through traumatic keratosis, lichen planus, leukoplakia, and the risk group to carcinoma with considerable differences. The nucleus in OSCC was twice as large as in normal mucosa. In another study on cases of laryngeal cancer Truelson JM[20] noted that nuclear content and nuclear area were good indicators of the biologic aggressiveness of cancer in laryngeal cancer.

The increase in nuclear area in OL may be caused by an increase in chromosomes related to abnormal cell division. Studies have shown that the increase in nuclear area in the premalignant lesions and carcinoma may be a reflection of the increase in DNA synthesis.[20] Our results also showed a significant increase in nuclear area in study groups compare to control group.

In the present study the mean values of nuclear perimeter of Normal Mucosa, OL and OSCC were 48.50 ± 4.12µm, 60.41 ± 1.29 µm and 70.74 ± 0.82 µm, respectively. Thus the morphometric findings demonstrated that the mean nuclear perimeter of OSCC groups was highest followed by Leukoplakia group and control group the least. Comparing the mean nuclear area of three groups together, ANOVA revealed significantly different nuclear perimeter among the groups (F=586.40, P<0.001).

Further, comparing the mean nuclear perimeter between the groups, Tukey test revealed significantly different and higher nuclear perimeter of both OL (19.7%) and OSCC (30.9%) groups as compared to Control group.

Our study was in the accordance with study done by Natarajan *et al*[21] Their data showed that discrimination between short term and long-term survival is possible with morphometry. Perimeter, nuclear size and larger diameter were significantly larger in short-term survivors.

The result in the present study was similar to the study conducted by Smitha *et al*[2],Nandini *et al*[4]. Pektas *et al*[22] obtained a statistically significant difference in various nuclear parameters including perimeter, area, and diameter equivalent to circle, minimum and maximum ferret, intensity, DNA content and DNA index values between study and control groups for both malignant and nonmalignant lesions.

Our study was also in accordance with the study done by Shabana *et al*[14] in which they showed that the nuclear perimeter is increased from the normal epithelium through Traumatic Keratosis, Lichen planus, Leukoplakia and the highest value in OSCC**.**

Increased NP observed could be due to rapid and abnormal growth of neoplastic cells. Nuclei in carcinoma group showed increased values of mean NP when compared to the nuclei in Normal mucosa. Our results also show a significant increase in nuclear perimeter in study groups compare to control group.

In the present study the mean Nuclear form factor of Normal mucosa, OL and OSCC, the mean were 0.92 ± 0.04, 0.85 ± 0.03 and 0.80 ± 0.02, respectively. The mean nuclear form factor of control group was the highest followed by Leukoplakia group and OSCC group, the least.

Comparing the mean nuclear form factor of three groups together ANOVA revealed significantly different nuclear form factor among the groups ($F=83.41$, $P<0.001$). Further, comparing the mean nuclear form factor between the groups, Tukey test revealed significantly different and lower nuclear form factor of both OL (7.3%) (0.92 ± 0.04 vs. 0.85 ± 0.03, mean diff. =0.07, $q=9.66$; $P<0.001$) and OSCC (13.1%) (0.92 ± 0.04 vs. 0.80 ± 0.02, mean diff. =0.12, $q=17.50$; $P<0.001$) groups as compared to control group (Fig. 9).

Our study was in accordance with the study done by Nandini *et al*[4], Natarajan *et al* [21]and Smitha *et al*[2] Nuclear shape factor indicators of regularity of nuclear boundary were powerful discriminators. The nuclei in the group with a favorable prognosis had regular profiles. In our study we also found regular nuclei in the control group.

A perfect circle has a form factor 1.0 and elliptical structures deviate from unity towards zero as their degree of circularity becomes less perfect. The shape factor is thus dimensionless, its value equaling 1.0 in round nuclei and being less than 1.0 in elliptical nuclei. NF was found to be less in carcinoma groups than normal mucosa, suggesting that irregular or elliptical nuclei predominate in the carcinoma groups. Our results also show a significant decrease in nuclear form factor in study groups compare to control group.

Our study shows that nucleus reflects cell's biological potential and general activity. The increase in nuclear size in OL has shown it to be more prone for malignant change and therefore their measurements may provide an objective means for the assessment of epithelial dysplasia and to predict their malignant potential.

Techniques of image analysis offer an opportunity to quantify the nuclear changes associated with malignancy and provide an objective basis for grading dysplasia and tumors. Overall these results have shown that the quantitative histomorphometric techniques can detect features that may be overlooked by routine histological examination.

CONCLUSION

On the basis of our study, we conclude that nucleus reflects cells biological potential. A combination of several nuclear variables provides a more accurate indication of tumor aggressiveness and behavior rather than a single parameter. The dysplastic epithelium shows a greater variation in nuclear dimensions than normal epithelium. The present study has included only few nuclear parameters. Further studies using morphometry have to be conducted with larger sample size and with other nuclear features to assess the nuclear changes in premalignant and malignant lesions.

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