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Quantitating the Nuclear Discrepancies between Orthokeratotic and Parakeratotic Epithelial Dysplasia Using Computer Aided Diagnostics

(OKD Harbour Greater Nuclear Abnormalities: A Morphometric Study)

Dr. Suhani S. Khundia
Post graduate student
Government Dental College and Hospital
Aurangabad, India

Dr. Jyoti D. Bhavthankar
MDS
Government Dental College and Hospital
Aurangabad, India

Dr. Mandakini S. Mandale
MDS
Government Dental College and Hospital
Aurangabad, India

Dr. J.G. Humbe
MDS
Government Dental College and Hospital
Aurangabad, India

Abstract: - By using the assistance of digital imaging and specialized computer software nuclear morphometry provides an objective measurement of nuclear abnormalities which are reproducible. Pattern of abnormalities vary from simple balanced rearrangements to complex abnormalities between malignancies. Nuclear changes in the oral potentially malignant disorders (OPMDs) may help in prediction of the malignant transformation rate. Hence nuclear features being one of the criteria in distinguishing them. Oral leukoplakia (OLP) is a potentially malignant disorder with a malignant potential ranging from 0.13-17.5%. Recently a new histological variant of leukoplakia i.e. leukoplakia with Orthokeratotic dysplasia (OKD) has been identified which is often found to be associated with carcinoma-in-situ (CIS) and oral squamous cell carcinoma (OSCC). In the esophagus similar lesions termed esophageal epidermoid metaplasia were encountered and are poorly described within the literature. Dysplasias with orthokeratosis (OKD) showed greater recurrence rate and malignant potential compared to parakeratotic dysplasia (PKD). Studies have shown OKD to harbour greater chromosomal and mitotic abnormalities. Nuclear morphometry is useful in aiding diagnosis and thereby helpful in predicting prognosis in a variety of cancers. It has also been shown to provide accurate measurements.

- ***Aims:***
  To assess and compare nuclear morphometric parameters in oral leukoplakias with parakeratotic and orthokeratotic epithelial dysplasia and oral squamous cell carcinoma with regard to their malignant potential.

- ***Settings And Design:***
  Cross sectional descriptive

- ***Methods And Material:***
  The hematoxylin and eosin stained sections of 30 OLP, 15 with PKD; 15 with OKD and 15 OSCC were included in the study. For morphometric analysis, microscopic images were captured at 40X randomly and nuclear morphologic features of the groups were computed and compared using ImageProPremier software.

- ***Statistical Analysis Used:***
  One-way ANNOVA test (openepi Version 3.01).

- ***Results:***
  Nuclear morphometry results were statistically analyzed using ANNOVA test (openepi Version 3.01).

- ***Conclusions:***
  The nuclear changes associated with malignancy can easily be quantified with the help of Image analysis techniques. It was also concluded that the nuclear area, the nuclear perimeter and nuclear fractal dimensions increased from PKD to OKD and were maximum in OSCC.

Keywords:- *Parakeratotic Dysplasia (PKD), Orthokeratotic Dysplasia (OKD), Oral Squamous Cell Carcinoma (OSCC), CIS (Carcinoma In Situ), Oral Leukoplakia (OLK).*

I. INTRODUCTION

Oral cancer accounts for 2%–4% of all cancer cases worldwide. In India the prevalence of oral cancer is comparatively higher, around 45% [1,2]. It is strongly correlated with risk factors like tobacco and alcohol use which causes genetic damage. This leads to uncontrolled proliferation of cells resulting in dysplasia presenting as precancer and cancer [3,4].In the oral cavity leukoplakia stands as the most common potentially malignant disorder with a prevalence rate ranging from 0.2 to 4.9%.The overall malignant transformation rate for dysplastic lesions in leukoplakia ranges from 0.13 to 17.5 %

depending on the type, site and length of follow-up [5]. Recently a new histological variant of oral leukoplakia i.e. leukoplakia with orthokeratotic dysplasia (OKD) has been identified which is often found to be associated with multifocal and relapse group of carcinoma-in-situ (CIS) and oral squamous cell carcinoma (OSCC). Lesions with OKD have been found to have greater recurrence rate and malignant potential as compared to parakeratotic. Histological studies done on esophageal leukoplakia shows presence of superficial hyperorthokeratosis, a prominent granular cell layer, acanthosis seen in the midzone, epithelial hyperplasia, and a thickened basal cell layer. Paramount to the success of treatments is the improvement in survival rate of patients along with subsequent morbidity and mortality.

Conventionally oral epithelial dysplasias (OEDs) have been studied extensively based on the subjective evaluation of various nuclear and cytoplasmic features within the lesional tissue at the histological level. The prime interest of the cytopathologists is to study morphology of the cancer nuclei. Any morphological alteration in the cancer nuclei can easily be demonstrated by light microscopy on routine staining. The deranged cellular functions of cancer cell are associated with morphological changes which can be analysed visually [6]. The progressive changes in the nuclear structure of leukoplakia may herald its malignant transformation. The "gold standard" for the prediction of malignant transformation in precancerous lesions is still considered to be histopathological assessment by assessing severity of oral epithelial dysplasia (OED) [7]. It has not been shown to add significantly to the prognosis of the disease, as interobserver and intraobserver variability still exists. Recently with the recognition of factors linked to neoplastic cells as well as invention of newer biologic markers tissue morphology of cancer cells can be easily studied through computer-aided image analysis. It also depicts the prognosis and survival of the cancer patient with minimal subjectivity.

Computer aided image analysis provides a more objective, reliable and reproducible result. Many characteristic light microscopic changes such as the alteration of nuclear shape, size, margin, chromatin pattern, nucleoli, and perinucleolar space were studied. One such approach also includes assessment of fractal geometry, a mathematical technique first described by Benoit Mandelbrot (1982). Fractal geometry aids in precise assessment of architecture of natural objects including histopathological specimens as well as pictures captured from any other imaging methods [8,9,10]. A computer-based morphometry system may yield a more quantitative and accurate assessment of nuclear shape descriptors and can add to the prognostic information provided by the conventional [11] histopathology grading of oral epithelial dysplasias. The morphometric data for a given neoplasm can be used for providing insight into the role of specific nuclear proteins in tumorigenesis and for prognostication of tumor biology [12,13]. The presence of increasingly abnormal values of nuclear DNA content, size and shape variability, nucleolar organizer size and number are most frequently observed in morphometry [14].

The purpose of this work was to study the nuclear morphometric parameters in oral leukoplakias- Parakeratotic Vs Orthokeratotic oral epithelial dysplasia and to compare it with oral squamous cell carcinoma so as to evaluate their malignant potential.

II. MATERIAL AND METHOD

The cases included in the study were retrieved from the archives of Department of Oral Pathology and Microbiology. Study was conducted on 30 cases of oral leukoplakias with parakeratotic and orthokeratotic epithelial dysplasia and 15 cases of oral squamous cell carcinoma. The study groups comprised of clinically diagnosed cases of leukoplakia irrespective of age and sex, confirmed histopathologically as epithelial dysplasia based on World Health Organization (WHO) grading of epithelial dysplasia 2005.

The study groups were: -

- Group-I (n = 15): Leukoplakia with parakeratotic oral epithelial dysplasia
- Group-II (n = 15): Leukoplakia with orthokeratotic oral epithelial dysplasia
- Group-III (n = 15):Oral squamous cell carcinomas

Paraffin embedded tissue sections three to five mm in thickness were obtained from all the groups and were stained with Hematoxylin and Eosin.

➢ *Assessment of the Nuclear Morphometric Parameters: -*

After observing the stained sections under light microscope using 40X objective .10 microscopic fields were selected randomly for each case, commencing with the first representative field on the left hand side. Images were captured using micropublisher 3.3 RTV camera .Ten images per slide were taken. Nuclear morphologic parameters of the group were assessed and compared using Image analysis software (Image Pro Plus, Version 4.1.0.0 for Windows 95/NT/98, Media Cybernetics, Bethesda, USA), accurately calibrated using a stage micrometer where 1 micron was equal to 3.260 pixels). The nuclear parameters studied were nuclear area $(\mu m)^2$, perimeter (μm), circular rate = 4π x nuclear area / perimeter, Aspect Ratio = largest diameter/smallest diameter and nuclear fractal dimension.

100 nuclei in parabasal and spinous cell layers were analysed. Care was taken to only include randomly selected nuclei from the lower third of the epithelium, which showed complete, non-overlapping outline. Morphometry for each nucleus was done after opening the image in software and each nuclear outline was traced with the cursor without lifting the pointer once started till circle completed for computing the nuclear area (NA), nuclear perimeter (NP), circular rate and aspect

ratio. For nuclear fractal dimension on selecting the region of interest (ROI) the nuclei were automatically traced by the software and NFD were computed for the particular ROI. Number of nuclei were noted accordingly in a separate excel sheet for each cell [Table 1, Figures 1-5].

III. STATISTICAL ANALYSIS

The obtained data was statistically analyzed by computing descriptive statistics, viz., mean, and standard deviation. The differences in the various
groups for different parameters were compared by means of one-way analysis of variance (ANOVA). P value of <0.05 was considered as statistically significant.

IV. RESULTS

Statistical analysis was done using ANNOVA test (openepi version 3.01). The mean nuclear area (NA) in PKD was 2615 μm^2 , in OKD 3035.75 μm^2 and in OSCC 3396.77 μm^2 and the difference in them was statistically significant with p value =0.0004. Mean nuclear perimeter (NP) in PKD was 198 μm in OKD 212.51 µm and in OSCC 224.94 µm and the difference in them was statistically significant with p value =0.00025. Similarly mean nuclear fractal dimension (NFD) in PKD was 1.0243 in OKD was 1.0438 and in OSCC 1.1335 for which the p-value was statistically significant 0.0001. [Table 1]

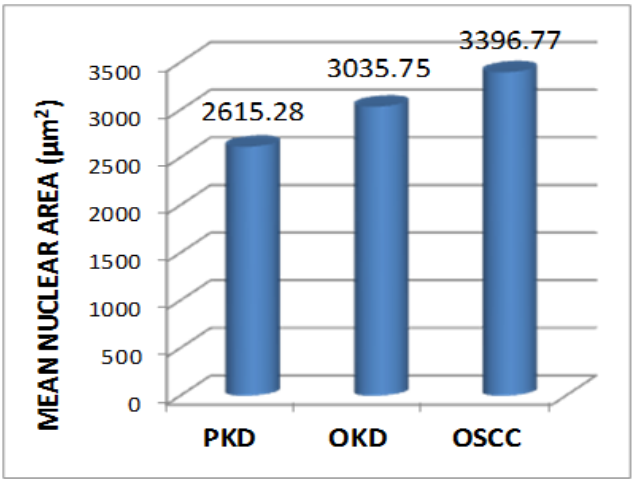

Fig 1:- Comparison of Mean Nuclear Area in PKD, OKD and OSCC

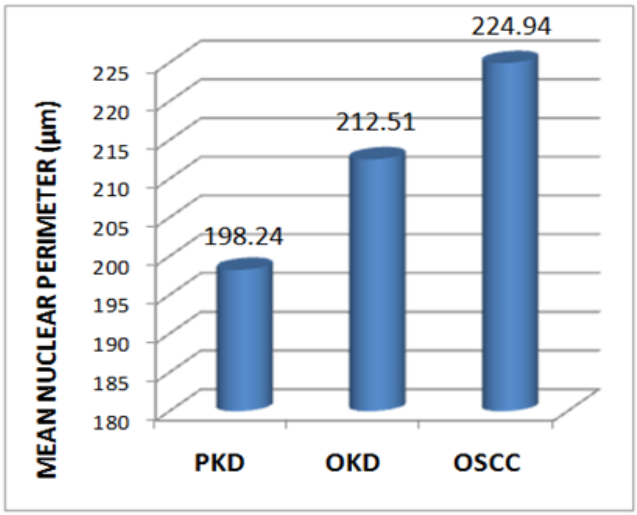

Fig 2:- Comparison of Mean Nuclear Perimeter in PKD, OKD and OSCC

The mean Circular rate (CR) in PKD, OKD & OSCC was 0.5977, 0.5984 and 0.5864 respectively. P-value for the mean circular rate was <0.005 (p = 0.009) and hence was statistically insignificant. Similar observation was made for the aspect ratio where in the p-value was calculated to be 0.060. The mean Circular rate (CR) in PKD, OKD & OSCC was 0.5977, 0.5984 and 0.5864 respectively. P-value for the mean circular rate was P-value for the mean circular rate was <0.005 (p = 0.009) and hence was statistically insignificant. Similar observation was made for the aspect ratio where in the p-value was calculated to be 0.060.

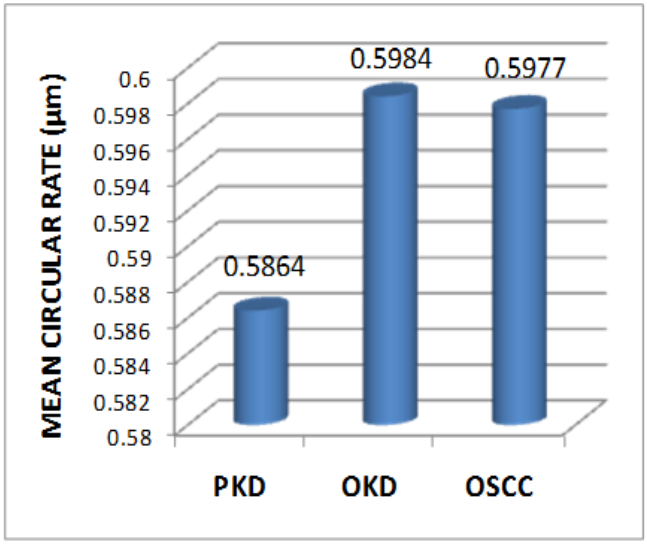

Fig 3:- Comparison of Mean Circular Rate in PKD, OKD and OSCC

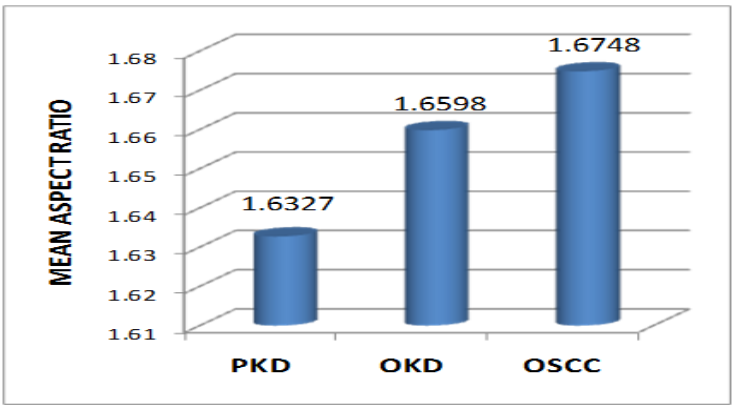

Fig 4:- Comparison of Mean Aspect Ratio in PKD, OKD and OSCC

Overall on adjusting the threshold it was observed that the nuclei in PKD were round shaped and had regular membrane outlines, whereas they increasingly became oval and irregular as it progressed from OKD to OSCC.

Nuclear parameter	PKD (Mean)	OKD (Mean)	OSCC (Mean)	p value
1) Nuclear area	2615.28	3035.75	3396.77	p=0.0004 (significant)
2) Nuclear perimeter	198.24	212.51	224.94	p=0.00025 (significant)
3) Circular rate	0.5864	0.5984	0.5977	p=0.09075(not significant)
4) Aspect ratio	1.6327	1.6589	1.6748	p=0.05962(not significant)
5) Fractal dimension	1.0243	1.0438	1.1335	p=0.00056 (significant)

Table 1:- Comparison of Nuclear Parameters in PKD, OKD and OSCC (One – Way ANNOVA)

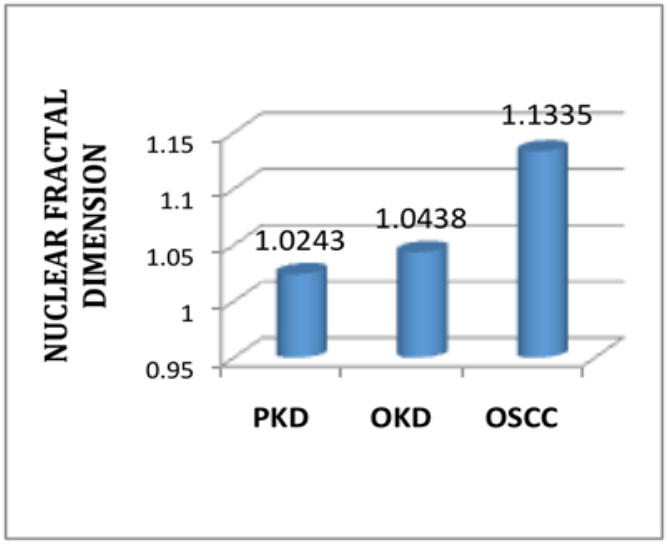

Fig 5:- Comparison of Mean Fractal Dimension in PKD, OKD and OSCC

V. DISCUSSION

By using nuclear and cytoplasmic morphometry investigators have evaluated the histological grading and prognosis of patients with esophageal, laryngeal and oral cancers. Study conducted by Vedam etal. assessed the prognosis in OSCC using histopathological parameters in 32 patients with a minimum follow-up of 5 years. Computerized image analysis software (Image J) was used to correlate and quantify the nuclear morphological parameters (nuclear area, perimeter, diameter, and circularity) with the survival of patients and were found to be the ideal prognosticators in OSCC.Vedam etal concluded that combined assessment are better prognosticators than individual parameters .The parameters included in combined assessment are clinical TNM staging, histopathological grading system, and nuclear morphometry at the invasive front of the tumor . This combination proved to be a more accurate predictive factor and was capable of eliciting varied molecular characteristics of tumor heterogeneity [15].

In addition to the nuclear morphometric parameters studied previously another nuclear parameter was added i.e. nuclear fractal dimension (NFD). Mincione et al.[16] compared NFD in their study between OSCC group vs. controls and revealed statistically significant differences ($p < 0.001$) similar finding was noted in our study. Also, in present study the nuclear irregularity and complexity increased as it progressed from PKD to OKD reaching maximum value in OSCC. In addition to these Mincione et al. also found a statistically significant difference ($P = 0.003$) with progressive increase in NFD between different stages of OSCC i.e. stage I and II lesions and stage III and IV lesions.

A relationship between patient's survival and FD was also detected with lower FD values associated to longer survival time and higher FD values with shorter survival time (P = 0.034). These data showed that during OSCC progression FD values significantly increased. Another similar study conducted by Phulari et al.[17] observed significant difference (P ≤ 0.001) with a progressive increase in mean NFD from normal mucosa to poorly differentiated SCC.

Cheng Lu studied digitized H&E slides of oral squamous cell carcinomas for histomorphometric features of the nucleus which was quantitatively assessed to predict the patient's survival [18]. Kumar M etal.[19] and DB Nandini [20] concluded highly significant difference seen between OSCC and controls with respect to cellular area (CA), NP and NA. Highly significant difference was also seen in N: C ratio between the means of cases and control groups. They observed NA and NP significantly increased in OSCC (P < 0.001) when compared with the control group. These values increased in correlation with increasing grades of OSCC.

Natarajan S etal. [21] with a view to predicting local relapse and survival studied 30 patients of OSCC by objective and reproducible evaluation of mitotic activity and nuclear morphometric factors. The combination of mitotic index by volume (M/V) and SD of nuclear area was found to be more efficient and better predictor of survival. Narayanan N etal. [22] observations revealed that tumor cells with greater nuclear dimension and tendency to acquire an elliptical shape show increased incidence of nodal metastasis. A positive inclination was observed in nuclear size and shape with increased histopathological grading.

In the study conducted by T.Smitha etal.[9] results were significant for the morphometric parameter, nuclear size. The values increased gradually from the normal buccal mucosa to leukoplakia, reaching the highest value in OSCC for nuclear perimeter and area, cellular perimeter and area .The morphometric parameter, N: C ratio, in this study results showed an increase in leukoplakia and OSCC compared to normal buccal mucosa. But the difference was not significant between leukoplakia and OSCC.There was a statistically significant difference in the nuclear and cellular areas to differentiate between leukoplakia and squamous cell carcinoma. Two variables used to study the nuclear shape, "form perimeter (PE)" and "contour index (CI)", showed significant difference between normal buccal mucosa and leukoplakia and between normal buccal mucosa and OSCC.Present study also showed similar result with increase in NA and NP from PKD to OKD reaching maximum value in OSCC.

In study conducted by Yang et al.[23] nuclear morphometric analysis also revealed significant differences in DNA content amount, DNA index, nuclear area, nuclear radius, nuclear intensity, sphericity, entropy, and fractal dimension (all P < .01) between low-grade and high-grade dysplasia .It also revealed DNA content analysis identified in 34 patients with OL (48.6%) with DNA content abnormality. Nonhomogeneous lesion (P ¼ .018) and high-grade dysplasia (P ¼ .008) were significantly associated with abnormal DNA content. The degree of oral dysplasia and DNA content status was significant (P ¼ .004, correlation coefficient ¼ 0.342). Importantly, their was a positive correlation between them. At invasive tumor front (ITF) and tumor proper (TP) of oral squamous cell carcinoma (test cases) in comparison to normal buccal mucosa (control cases) higher mean nuclear FD was observed when compared using independent sample t-test (p=<0.001) in study done by Yinti etal.[8] In our study the NFD increased from PKD to OKD reaching maximum value as we progressed towards OSCC.

The mean nuclear area (NA) and nuclear diameter (ND) was found to increase from normal mucosa to dysplasia and also within grades of dysplasia in a study conducted by Gadiwan etal [7]. It was found that most of the nuclei in normal mucosa were round shaped and had regular membrane outlines, whereas it increasingly became oval and irregular as the grade of dysplasia increased except in case of moderate dysplasia. In present study we investigated oral leukoplakia with characteristic hyperorthokeratosis which were identified as a distinct histopathological entity and termed OKD.

Based on the results of the studies done previously the present study was planned considering that OKD, a new histological variant known to harbour more nuclear abnormalities and is said to undergo a greater malignant transformation than PKD we did intragroup comparison. Along with this in addition to the previously studied nuclear morphometric parameter a new dimension that is the nuclear fractal dimension (NFD) has been added and is said to have greater accuracy. Classical morphometry does not reveal the structural complexity characterized by fractals. Fractals even though viewed at different magnifications exhibit similar features, in a scale-independent manner. They are complex structures with self-similarity. In future, nuclear fractal dimension might help us classify tumour as a low or high-grade lesion by deriving an appropriate cutoff. This can also help us in enabling neoadjuant treatment requirements. Following this we found the difference between OLK and OSCC was found to be significant which is in accordance with the study conducted by Gadiwan etal [7]. The difference was also significant between PKD and OKD followed by their comparison with OSCC.The nuclear area, nuclear perimeter and nuclear fractal dimension were found to increase from PKD to OKD and as we progressed towards OSCC. Whereas circular rate and aspect ratio showed insignificant results.

VI. CONCLUSION

Nuclear abnormality showed significant increase from Oral Leukoplakia to OSCC.Also when we did intragroup comparison in Oral leukoplakia i.e. between PKD and OKD, OKD showed greater nuclear abnormalities compared to PKD .In addition to other nuclear parameter, NFD can be greater predictor for analyzing nuclear abnormality. Hence leukoplakia with OKD seems to have greater malignant transformation which warrants its close follow up .So it is important to recognize its existence.

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