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RESEARCH ARTICLE

HISTOMORPHOMETRIC IMAGE ANALYSIS OF LEUKOPLAKIA WITH DIFFERENT GRADES OF DYSPLASIA AND ORAL SUBMUCOUS FIBROSIS IN PARABASAL AND SPINOUS LAYERS OF ORAL EPITHELIUM

Keerthi Rao. J[1], Santhosh Kumar S Hiremath[2]., Govind Raj K Nalabolu[3]., Alapati Naga Supriya[4]., Sreenivasa Bharath t[5] and Swetha P[6]

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ABSTRACT

Introduction: Histopathological assessment as well as grading of Oral submucous fibrosis and Leukoplakia with dysplasia is rather subjective and insufficiently reproducible. One of the best tool, to avoid subjectivity in these lesions is the use of computer assisted image analyzer. Scientific literature based on histomorphometry of these potentially malignant disorders are few. Hence present study was under taken to assess OSMF and leukoplakia with dysplasia histomorphometrically utilizing normal oral mucosal tissue as control group.

Material and Methods: Study included Hematoxylin and Eosin sections of normal buccal mucosa (n=5), OSMF (n=15) and Dysplasia (n=15). Morphometric evaluation of these sections were performed in parabasal and spinous cell compartment separately by utilizing 5 different parameters (Cell area, Nuclear area, Cell diameter, Nuclear diameter and Nuclear cytoplasmic ratio)

Results: Cellular and nuclear parameters were greater in spinous cell compartment than parabasal cell compartment in all the three study groups. Morphometric parameters were exponentially increased from normal epithelium, OSMF to dysplasia.

Conclusion: All the three study groups showed site wise differentiation of morphometric parameters in parabasal and spinous cell compartments. Dysplasia showed higher nuclear and cellular parameters indicating the aggressiveness and possible malignant transformation.

INTRODUCTION

According to WHO 2005, leukoplakia (precancerous lesion) and Oral submucous fibrosis [OSMF (precancerous condition)] were considered potentially malignant disorders (PMDs) commonly evident in Indian sub population which showed high propensity for malignant transformation.[1] Many published literature emphasized the transformation of leukoplakia to oral squamous cell carcinoma (OSCC). Evidence of atypical features in the leukoplakia is an important aspect in predicting the malignant potential and studies have documented 17-25% of dysplasia in leukoplakic epithelium.[2,3] The rate of malignant transformation in oral leukoplakia ranges between 3-6%.[4] OSMF is an irreversible precancerous condition of upper aerodigestive tract[5] which typically affects the buccal mucosa, lips, retromolar area, soft palate, oropharynx and rarely larynx. Incidence of OSMF has increased mainly among the younger generations of Indian population. Areca nut is the chief causative agent. Malignant transformation of OSMF is expected to be 6%.[6,7]

Histopathological assessment of leukoplakia and OSMF was rather subjective and insufficiently reproducible. Interpretation of these lesions varies among different pathologists leading to inter and intra observer's variability. To overcome the subjectivity, computerized morphometric evaluation can be implemented which can provide a quantitative approach in assessing the structural changes of normal and lesional epithelium.[2]

Morphometric studies on leukoplakia and OSMF were documented separately and there is a paucity of literature relating to these PMDs in assessing their biological behavior. Hence present study is undertaken to compare and correlate leukoplakia and OSMF by histomorphometry. In this regard cellular and nuclear changes are paramount in assessing the grade and severity among these two lesions. To assess these changes, five different parameters were included which are cell diameter (CD), nuclear diameter (ND), cell area (CA), nuclear area (NA) and nuclear cytoplasmic ratio

(N: C). The aim of the present study was to assess the cellular and nuclear parameters in parabasal and spinous layers of epithelium in different grades of leukoplakia with dysplasia and OSMF, considering normal epithelium as a control.

MATERIALS AND METHODS

In the present retrospective study, 35 cases were selected from archives of the Department of Oral Pathology, which included 15 cases of leukoplakia with different grades of dysplasia, 15 OSMF cases and 5 normal oral mucosal tissues as a control group.

In all the study groups, lesions were strictly considered from the buccal mucosa to eliminate site wise differentiation. Control group was considered from the archives having histologically confirmed cases of benign lesions. Wax blocks of histopathologically confirmed cases of leukoplakia with different grades of dysplasia and OSMF were retrieved from the archives and 4 microns sections were prepared.

Sections were stained with routine hematoxylin and eosin. Leukoplakia and OSMF cases were histopathologically graded by three different oral pathologists.

To avoid observer's variability, Leukoplakia was graded according to Burkhart and Maerker[8, 9] and OSMF as per Pindborg and Sirsat.[10, 11] for the convenience in counting of OSMF, very early and early stages were categorized as one i.e early OSMF and moderately advanced and advanced stages were considered as it is. Cases were selected, based on the consensus of three different pathologists and in case of disagreement, that particular case was excluded from the study.

The stained sections were analyzed for histomorphometry, using Image proplus software version 6.0.0 with a research microscope using CCD camera. 5 random fields are selected in each of the parabasal and spinous layers.

Images were captured at the magnification of 40x objective. Basal cells were excluded from counting as they have indistinct cellular/nuclear outline. Also, non-keratinocytes, inflammatory cells and degenerative cells were exempted. The images were checked for appropriateness and saved in a computer for further analysis.

In each field, 5 representative cells with clear cellular and nuclear outlines were selected. Using the software cells were analyzed for parameters such as cell area (CA), nuclear area (NA), Cell diameter (CD), nuclear diameter (ND). Finally the nuclear cytoplasmic ratio was calculated by formula N/C = NA/CA. For the purpose of measurement, a cursor was used to outline the cellular and nuclear confines.

First parameter i.e., CA was measured by tracing the cell outline using the cursor; software automatically calculates CA in square microns. Similarly, the second parameter, NA was analyzed by tracing the nuclear perimeter.

The same cells were used for measuring the third and fourth parameter i.e., CD and ND which were calculated by taking the average of maximum and minimum diameter of both cells and nucleus respectively.

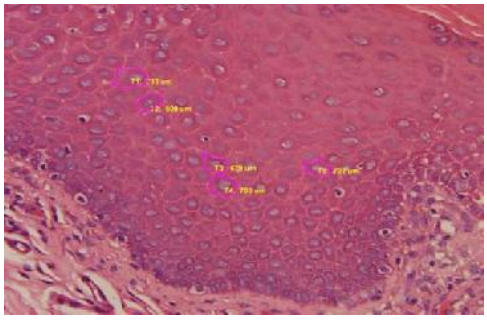

Figure 1 Photomicrograph showing morphometric measurements of Cell area (H&E x40)

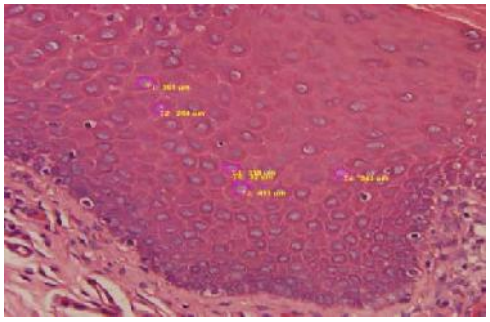

Figure 2 Photomicrograph showing morphometric measurements of Nuclear area (H&E x40)

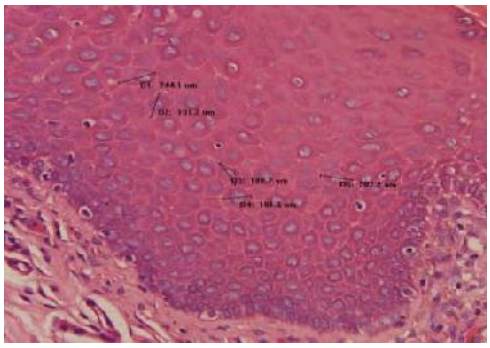

Figure 3 Photomicrograph showing morphometric measurements of Cell diameter (H&E x40)

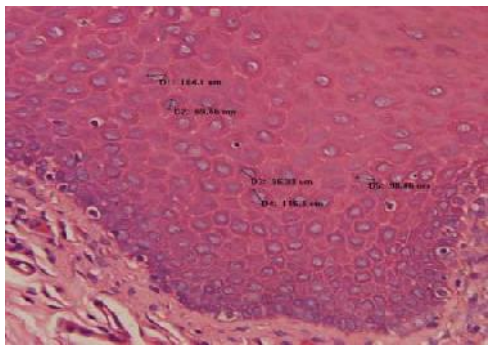

Figure 4 Photomicrograph showing morphometric measurements of nuclear diameter (H&Ex40)

Statistical Analysis

One Way Anova with Post-Hoc was used for comparing the parameters of different study groups. Anova was done to determine whether there are any significant differences between the three independent (unrelated) study groups followed by post-Hoc test which shows the definite significance between two study groups.

RESULTS

Considering the three study groups, all five parameters were analyzed using One-way Anova test which revealed more values in spinous compartment than parabasal compartment (Table-2). In case of OSMF, though all four parameters were more in spinous layer, only CA was significantly increased in parabasal compartment (Table – 3). In Dysplasia, CA, NA & ND were significantly increased in spinous compartment (Table-4).

Table 1 Study groups

S.No	Group	Description
1	Group 1	Normal epithelium
2	Group 2a	Early OSMF
3	Group 2b	Moderately Advanced OSMF
4	Group 2c	Advanced OSMF
5	Group 3a	Mild dysplasia
6	Group 3b	Moderate dysplasia
7	Group 3c	Severe dysplasia

On comparing the study groups all five parameters appeared to be increased from normal epithelium, through OSMF and Dysplasia (Table-2). Also, the study groups were compared using One-Way Anova indicated that CA, NA and CD of both the compartments showed significance and NC was significant in Spinous compartment. Further pair wise comparisons were done by Post-Hoc test. The test revealed CA, NA and CD in parabasal compartment were significant in dysplasia followed by OSMF and normal epithelium. However, in spinous compartment, only CA and CD were significant in the similar manner in different study groups (Table-5).

Pair wise comparison of different groups of OSMF in parabasal compartment showed significance in only CA in Group 3(a,b&c) and significance was seen in ND & CD in spinous compartment of group 3(a,b&c). On comparing different grades of dysplasia, CA and CD in parabasal compartment were significant in group all the grades, similarly in spinous compartment CA, NA and CD were significant.

Table 2 Oneway Anova

Study groups	CA		NA		CD		ND		NC	
	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinuous	Parabasal	Spinous
Group 1	424.56± 58.41	561.72± 44.37	200.36± 57.41	345.42 ± 68.60	116.60 ± 23.60	188.30 ± 18.33	63.24 ± 26.84	110.06 ± 47.00	0.42 ± 0.16	0.58 ± 0.04
Group 2a	496.24 ± 8.91	631.26 ± 28.76	262.46 ± 54.06	346.58 ± 32.30	171.58 ± 11.03	192.22 ± 10.26	95.84 ± 52.73	120.90 ± 54.32	0.48 ± 0.13	0.52 ± 0.04
Group 2b	559.76 ± 54.04	627.30 ± 63.86	275.68 ± 36.80	340.40 ± 37.09	175.60 ± 14.68	201.32 ± 26.81	103.34 ± 32.42	121.12 ± 34.84	0.44 ± 0.05	0.58 ± 0.04
Group 2c	572.10 ± 42.72	654.54 ± 44.18	318.96 ± 11.20	364.54 ± 16.42	183.56 ± 15.61	211.24 ± 13.81	95.98 ± 1.88	112.18 ± 3.31	0.52 ± 0.04	0.58 ± 0.04
Group 3a	506.44 ± 23.74	583.76 ± 54.13	253.68 ± 37.07	293.26 ± 17.80	173.22 ± 11.01	204.82 ± 14.27	68.78 ± 13.30	91.48 ± 12.17	0.48 ± 0.08	0.52 ± 0.04
Group 3b	590.84 ± 32.76	684.20 ± 41.11	286.68 ± 33.81	375.84 ± 39.64	199.76 ± 26.56	262.52 ± 53.21	89.94 ± 22.39	112.70 ± 14.67	0.44 ± 0.08	0.58 ± 0.04
Group 3c	672.46 ± 24.00	752.60 ± 10.78	326.42 ± 51.41	377.46 ± 32.73	227.92 ± 39.56	282.72 ± 16.16	90.94 ± 11.09	113.40 ± 30.27	0.46 ± 0.05	0.50 ± 0.07
F value	1.019	2.466	4.938	2.8	3.304	4.255	1.466	0.641	0.587	2.706
P value	0.035	0.026	0.001	0.029	0.043	0.021	0.226	0.696	0.738	0.034

One wayAnova variance:P values < 0.05 and < 0.001 are stastistically significant and

Table 3 Anova between Different groups of OSF

Study groups	CA		NA		CD		ND		NC	
	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinuous	Parabasal	Spinous
Group 2a	496.24 ± 8.91	631.26 ± 28.76	262.46 ± 54.06	346.58 ± 32.30	171.58 ± 11.03	192.22 ± 10.26	95.84 ± 52.73	120.90 ± 54.32	0.48 ± 0.13	0.52 ± 0.04
Group 2b	559.76 ± 54.04	627.30 ± 63.86	275.68 ± 36.80	340.40 ± 37.09	175.60 ± 14.68	201.32 ± 26.81	103.34 ± 32.42	121.12 ± 34.84	0.44 ± 0.05	0.58 ± 0.04
Group 2c	572.10 ± 42.72	654.54 ± 44.18	318.96 ± 11.20	364.54 ± 16.42	183.56 ± 15.61	211.24 ± 13.81	95.98 ± 1.88	112.18 ± 3.31	0.52 ± 0.04	0.58 ± 0.04
F value	5.15	0.474	2.975	0.877	1.016	1.337	0.072	0.093	1.071	3
P value	0.024	0.634	0.089	0.441	0.391	0.299	0.931	0.912	0.367	0.088

P value <0.05 is significant and <0.001 is highly significant.

Table 4 Anova between different grades of dysplasia

Study groups	CA		NA		CD		ND		NC	
	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinuous	Parabasal	Spinous
Group 3a	506.44 ± 23.74	583.76 ± 54.13	253.68 ± 37.07	293.26 ± 17.80	173.22 ± 11.01	204.82 ± 14.27	68.78 ± 13.30	91.48 ± 12.17	0.48 ± 0.08	0.52 ± 0.04
Group 3b	590.84 ± 32.76	684.20 ± 41.11	286.68 ± 33.81	375.84 ± 39.64	199.76 ± 26.56	262.52 ± 53.21	89.94 ± 22.39	112.70 ± 14.67	0.44 ± 0.08	0.58 ± 0.04
Group 3c	672.46 ± 24.00	752.60 ± 10.78	326.42 ± 51.41	377.46 ± 32.73	227.92 ± 39.56	282.72 ± 16.16	90.94 ± 11.09	113.40 ± 30.27	0.46 ± 0.05	0.50 ± 0.07
F value	5.013	2.834	2.757	11.749	3.36	7.434	3.152	9.495	0.333	2.889
P value	0.026	0.035	0.103	0.001	0.069	0.008	0.079	0.003	0.723	0.095

P value <0.05 is significant and <0.001 is highly significant.

Table 5 Post Hoc of both parabasal and spinous compartments between all the study groups

S.No	Comparison of study groups	P Value									
		Cell Area		Nuclear area		Cell Diameter		Nuclear Diameter		N:C	
		Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinous	Parabasal	Spinous
1	Group 1 vs Group 2a	NS	NS	NS	NS	< 0.05	NS	NS	NS	NS	NS
2	Group 1 vs Group 2b	<0.001	NS	NS	NS	< 0.05	NS	NS	NS	NS	NS
3	Group 1 vs Group 2c	< 0.001	< 0.05	< 0.05	NS	< 0.001	NS	NS	NS	NS	NS
4	Group 1 vs Group 3a	<0.05	NS	< 0.05	NS	< 0.05	NS	NS	NS	NS	NS
5	Group 1 vs Group 3b	< 0.001	< 0.05	< 0.001	NS	< 0.001	< 0.05	NS	NS	NS	NS
6	Group 1 vs Group 3c	< 0.001	< 0.001	NS	NS	< 0.001	< 0.001	NS	NS	NS	NS
7	Group 2a vs Group 2b	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8	Group 2a vs Group 2c	< 0.05	NS	NS	NS	NS	NS	NS	NS	NS	NS
9	Group 2a vs Group 3a	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
10	Group 2a vs Group 3b	< 0.05	NS	NS	NS	NS	< 0.05	NS	NS	NS	NS
11	Group 2a vs Group 3c	< 0.001	< 0.05	NS	NS	< 0.05	< 0.001	NS	NS	NS	NS
12	Group 2b vs Group 2c	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
13	Group 2b vs Group 3a	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
14	Group 2b vs Group 3b	NS	NS	NS	NS	NS	< 0.05	NS	NS	NS	NS
15	Group 2b vs Group 3c	< 0.001	NS	NS	NS	< 0.05	NS	NS	NS	NS	NS
16	Group 2c vs Group 3a	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
17	Group 2c vs Group 3b	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
18	Group 2c vs Group 3c	< 0.05	< 0.05	NS	NS	< 0.05	< 0.05	NS	NS	NS	NS
19	Group 3a vs Group 3b	< 0.05	< 0.05	NS	< 0.05	NS	< 0.05	NS	NS	NS	NS
20	Group 3a vs Group 3c	< 0.001	< 0.001	NS	< 0.05	< 0.05	< 0.001	NS	NS	NS	NS
21	Group 3b vs Group 3c	< 0.05	NS	NS	NS	NS	NS	NS	NS	NS	NS

DISCUSSION

It is well established that PMDs such as hyperkeratosis, epithelial dysplasia, erythroplakia and OSMF can transform into OSCC.[12] in the present study, precancerous lesion (leukoplakia with different grades of dysplasia) and condition (OSMF) were compared histomorphometrically using cellular and nuclear parameters. Epithelium of buccal mucosa was used as a control group. Similar histomorphometric studies, conducted on epithelium of various white lesions[4, 13] and squamous cell carcinoma[14] utilized samples only from suprabasal or spinous compartment and from different anatomical sites of the oral cavity. However, study conducted by Raghavendra *et al*[2] emphasized the site wise differentiation while selecting the sample for histomorphometry and noted cellular and nuclear dimensions were more in normal gingival tissue when compared to normal buccal mucosa. In the present study, the pooling of samples in all the three study groups were taken from the corresponding sites i.e., buccal mucosa in order to avoid the site wise differentiation. Also, emphasis was given to the compartment wise comparison of different study groups.

Results of the study showed diverse morphometric parameters in different compartments and study groups, with all the parameters being greater in spinous cell layer than parabasal layer. The standard deviation values of all the parameters in both parabasal and spinous compartments were sequentially increased from normal epithelium through different grades of OSMF and to leukoplakia with dysplasia. Observations were in accordance to a study conducted by Raghavendra *et al*.[2] compartment wise comparison of all the study groups' revealed greater cellular and nuclear measurements in leukoplakia with dysplasia Followed by OSMF and normal epithelium. While the statistical significance was obtained only with CA, NA and CD. These results were in contrast with the previous studies which documented only increase in nuclear parameters of leukoplakia with dysplasia compared to normal epithelium.[14,15] In the present study comparison of different grades of leukoplakia with dysplasia showed increase in all the parameters from mild dysplasia through moderate to severe dysplasia indicating aggressiveness and possible malignant transformation of severe dysplasia. This was in accordance to Shabana *et al*,[4,14] which revealed an increase in cellular parameters in Leukoplakia when compared to Normal mucosa, Keratosis and Lichen planus.

Compartment wise comparison of different grades of dysplasia showed increase in all the parameters in spinous compartment. These values were exponentially increased from mild to moderate and severe dysplasia. However, only ND in spinous compartment was significant. These findings were against the results achieved by Lee *et al*[4] who demonstrated increase in parabasal parameters. Gao *et al*[13] reported only N/C as a significant parameter in spinous cell compartment which was similar to the study conducted by White *et al*.[15] However, present study showed significant increase in CA, NA and CD in spinous compartment. Studies have documented nuclear DNA content and nuclear area were the better indications of aggressiveness and malignant transformation.[16] Study conducted on uterine cervix by Valeri *et al*[17] reported exponential increase in nuclear volume and DNA content from normal mucosa, carcinoma in situ to invasive carcinoma. Also, increase in the epithelial cell size can attribute to mechanical trauma, repeated friction, in healing wounds, after removal of the stratum corneum and after hair plucking.[9]

Considering OSMF, morphometric parameters were in between normal epithelium and dysplasia suggesting that OSMF is an intermediate lesion with the biological behaviour and aggressiveness are in between normal epithelium and dysplasia. Results of the study were in consistent with the

study conducted by GAO *et al.*[13] Further comparison between the three groups of OSMF, revealed increased parameters from early, moderately advanced to advanced stages. Comparing the results of the present study, to the previous studies[5,10] and to draw conclusion on the aggressive behaviour of OSMF was very unlikely, as little morphometric information is available in the literature to compare and many such studies were conducted utilizing different morphometric parameters. Results of OSMF achieved in the present study requires further evaluation with large sample size and long follow up to comprehend the mechanism of malignant changes.

Most of the morphometric studies documented in the previous literature focused on nuclear changes in the development of malignancy.[13] Only few studies have reported cellular and nuclear changes in the dysplasia[4,14] and OSMF.[6] Similar studies focusing on premalignant lesions were conducted from the uterine cervix, bronchi and hamster cheek pouch epithelium.[2] Histopathological assessment and grading of dysplasia and OSMF is rather subjective leading to observers variability. In such instances observer's variability can be eliminated by using histomorphometry. Computer assisted image analysis can be utilized for large number of measurements and calculations by reducing the time with greater accuracy.[18] However, factors such as fixation time, and tissue processing can affect the values of morphometry mandating cautious handling of the tissue.

CONCLUSION

Present study revealed increased parameters in different grades of dysplasia when compared to OSMF and normal mucosa indicating the aggressiveness of the lesion and possible malignant transformation. OSMF was considered as the intermediate lesion between normal mucosa and dysplasia. In all the three study groups, various parameters were greater in spinous compartment when compared to parabasal compartment. Results of the present study have to be further evaluated by utilizing different parameters in large study group with appropriate follow up.

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