

**ORIGINAL**

## Cytomorphometric Analysis for Evaluation of Cell Diameter, Nuclear Diameter and Micronuclei for Detection of Oral Premalignant and Malignant Lesions

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**Abstract :** Purpose : Detection of a precancerous or cancerous lesion when small is one of the most important factors to improve 5-year survival rates of oral cancer. Although surgical biopsy is the Gold Standard for diagnosing oral lesions, it is impractical to routinely subject large numbers of patients to biopsy. This study had been undertaken to evaluate quantitatively the cellular changes in exfoliated cells from different premalignant and malignant lesions in terms of cell diameter (CD), nuclear diameter (ND) and nucleocytoplasmic ratio and micronucleus frequency. Patients and Methods : Oral mucosal smears were obtained from patients presenting with precancer and cancer using a cytobrush immediately before biopsy. The study group consisted of Group I : lesions with no dysplasia, Group II : lesions with mild and moderate dysplasia, Group III : lesions with severe dysplasia and carcinoma *in situ*, Group IV : lesions with invasive carcinoma. Results : Comparison of study group and controls showed a highly significant decrease in mean cellular diameter, increase in the nuclear diameter and a larger nucleo cytoplasmic ratio of exfoliated cells. ( $p < 0.0001$ ) Also micronuclei (MN) frequencies were found higher in SCC patients and in precancer than in control subjects. Conclusions : Cytomorphometric analysis via oral brush biopsy is a valuable adjunct to biopsy & CD, ND and micronuclei evaluation serves as important diagnostic markers.

### Introduction

Oral cancer affects as many as 274,000 people worldwide annually<sup>1)</sup>. Although representing 2—4% of the malignancies in the west, this cancer accounts for almost 40% of all cancers in the Indian subcontinent. Oral Cancer is sometimes preceded by potentially malignant ( premalignant) lesions and conditions such

as leukoplakia, erythroplakia, oral lichen planus and oral submucous fibrosis. Surgical biopsy remains the most definitive and reliable method for diagnosing oral lesions. Sampling of individual epithelial cells has been proposed with the ample data revealing the initial changes that give rise to a clinically visible lesion that are thought to occur within the epithelium. Given that 90% to 95% of oral cancers are squamous cell carcinomas arising from surface epithelium, evaluation of individual cells may give the opportunity for detection of early dysplastic changes<sup>2)</sup>. It is the nucleus that express the genotypic alterations caused

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in the process of malignancy, and exfoliative cytology is a method that gives better insight of the nuclear changes in individual cells<sup>3)</sup>. Exfoliative cytology is a straight forward and non-invasive technique and smears obtained can be analyzed quantitatively and qualitatively. Quantitative cytomorphometric assessment of exfoliated buccal cells have shown measurable changes in cells obtained from malignant and premalignant lesions<sup>4-6)</sup>. Quantitative parameters such as nuclear size, cell size, nuclear to cytoplasmic ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to establish the diagnosis of malignancy. Of these parameters, the nuclear and cytoplasmic areas and the nuclear to cytoplasmic ratio have been shown to be significant in the diagnosis of oral lesions<sup>7,8)</sup>. Also micronuclei are extracellular cytoplasm bodies and are good prognostic indicators. This study had been undertaken 1. To evaluate quantitatively the cellular changes in exfoliated cells from different premalignant and malignant lesions in terms of cellular diameter, nuclear diameter and nucleo-cytoplasmic ratio and to evaluate frequency of micronuclei. 2. To compare CD, ND and CD/ND and micronucleus frequencies between the control group, and precancer and cancer groups.

## Material and Methods

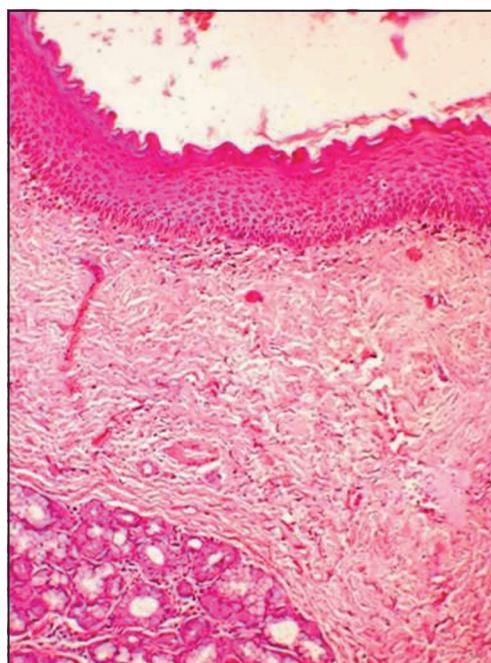
### 1. Patient Selection

Cases for study group Leukoplakia-45, Oral lichen planus-10, Oral sub mucous fibrosis-45, and Oral cancer-100.

Cases for control group : Apparently normal contra lateral healthy mucosal sites served as control (100 cases).

### 2. Collection of exfoliated cells

Following informed consent, brush-cytology scrapings were obtained from the lesions and contra lateral opposite mucosa immediately before biopsy. The surface of the lesions was scraped uniformly, and collected epithelial cells were smeared on an appropriately labeled glass slide and fixed with commercially available spray fixative (available with the rapid



**Fig. 1** Normal epithelium (10×)

Normal parakeratinised stratified squamous epithelium showing presence of salivary gland tissue in the normal appearing connective tissue stroma (10×)

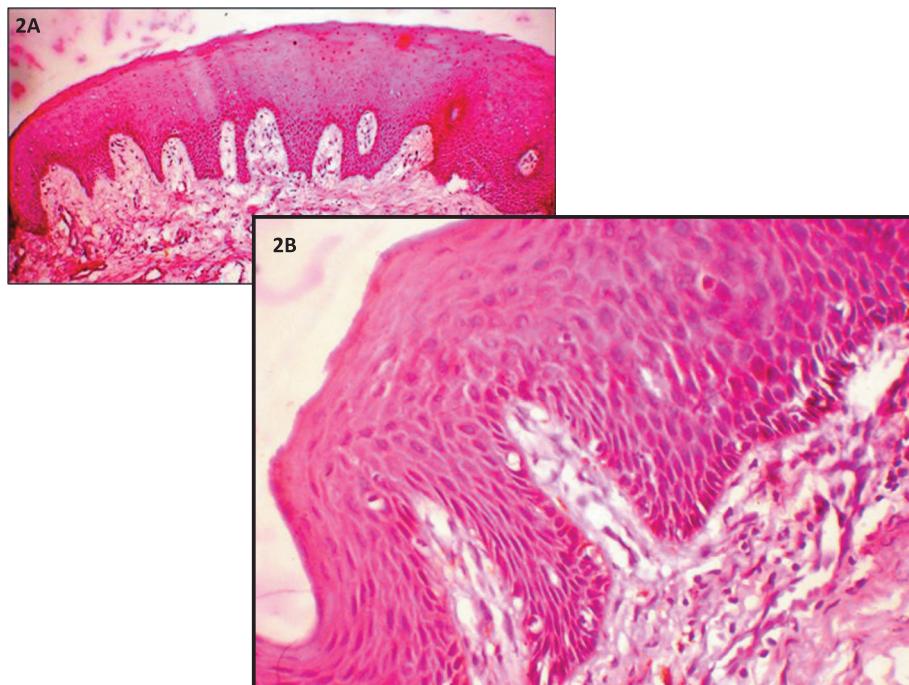
PAP<sup>TM</sup> Kit for 15 min). (Sigma-Aldrich Diagnostic, Mumbai, Maharashtra). All the cytology smears were stained with Papanicolaou's stain

### 3. Biopsy procedure

A 7 mm punch biopsy was performed after the cytological smears. The specimens were placed in 10% formalin for subsequent routine haematoxylin and eosin staining. Depending on histological diagnosis, they were divided into four groups. Group I : Normal or no dysplasia (Fig. 1), Group II : Lesions with mild to moderate dysplasia (Fig. 2A, B and 3A, B), Group III : Lesions with severe dysplasia and carcinoma in situ (Fig. 4A, 4B) and Group IV : Squamous cell carcinoma. (Fig. 5A, 5B, Table 1). Clinical details of Control and study group regarding age and sex of patient, site involvement and possible incriminating factors were noted as given in Table 2A, B and C.

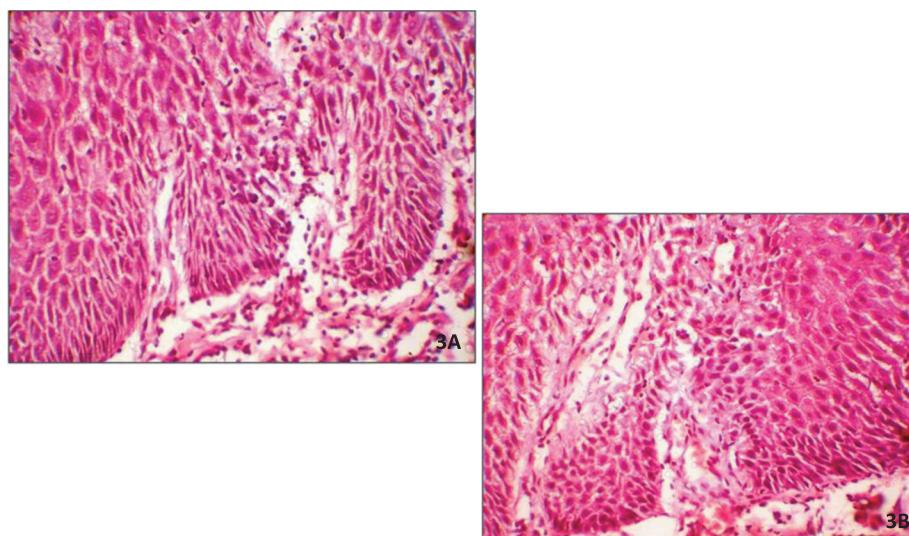
### 4. Cytological evaluation

CD and ND of the cells were measured using a cali-



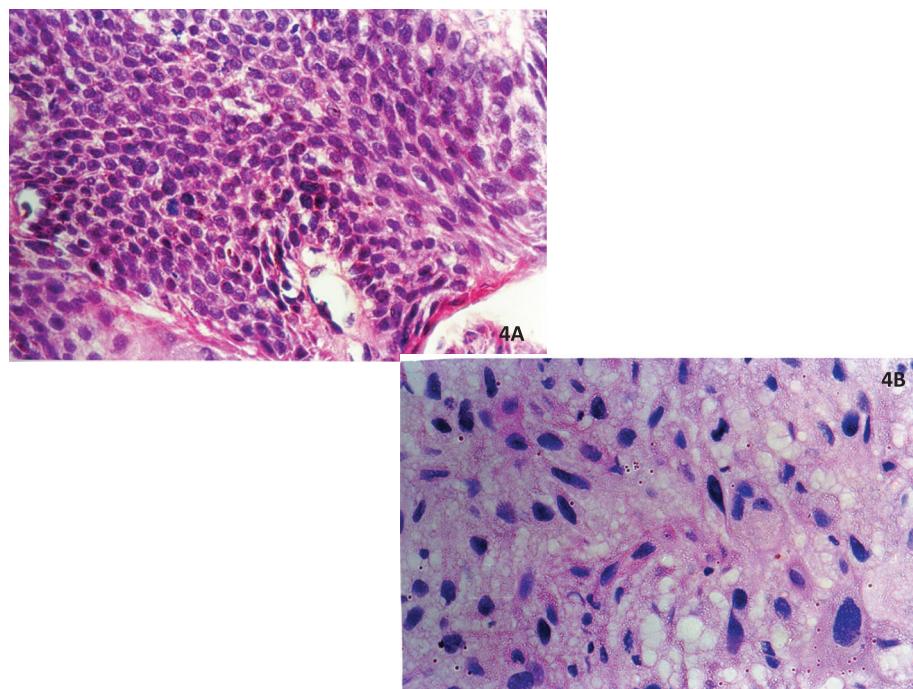
**Fig. 2 A (10 $\times$ ) & B (20 $\times$ ) Mild Dysplasia**

Mild epithelial dysplasia. Cells in the basal layer show cytological atypia including hyperchromatism, but there are no architectural changes and stratification is normal (10 $\times$ )

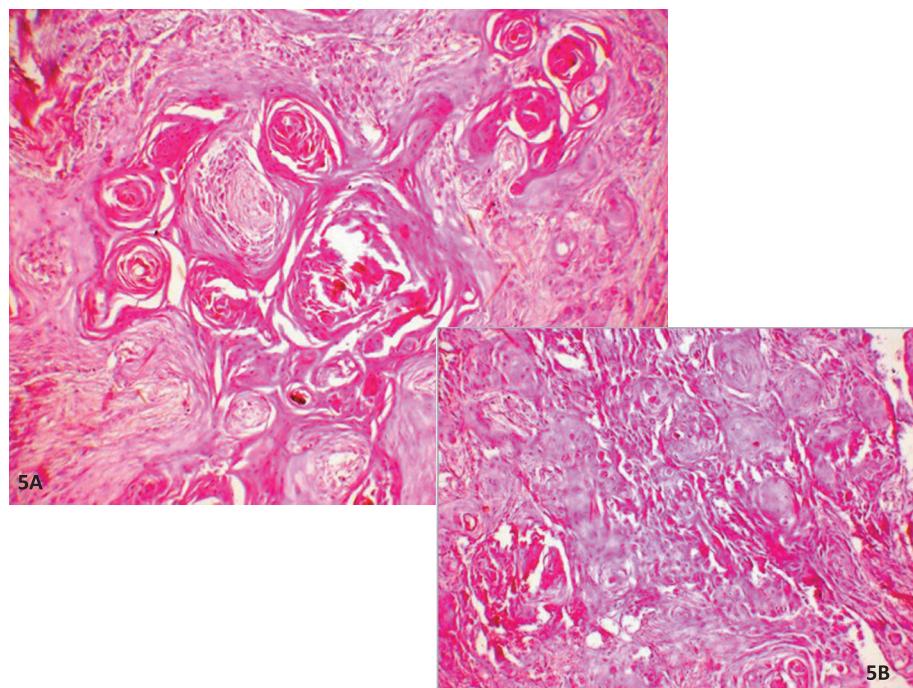


**Fig. 3 A & B Moderate Dysplasia (10 $\times$ )**

Moderate epithelial dysplasia. There is considerable cytological atypia which extends into the middle third of the epithelium. There is disruption of the normal architecture of the epithelium.



**Fig. 4 A & B Severe Dysplasia- (Carcinoma *in situ*) (20 $\times$ )**  
Architecture of the epithelium is almost completely disrupted with atypical cells and hyper chromatic nuclei (20 $\times$ )



**Fig. 5 A & B Well Differentiated Carcinoma (10 $\times$ )**  
Many keratinizing pearls are seen within tumor cells along with scattered hyper chromatic and pleiomorphic nuclei.

**Table 1** Distribution of patients according to histopathological diagnosis

Type of lesion	Total patients	Histopathological Division			
		Group I No Dysplasia	Group II Mild & Mod. Dysplasia	Group III Severe dyspla- sia & Ca <i>in situ</i>	Group IV Invasive SCC
<b>Oral Precancer</b>					
Oral Leukoplakia	45	1 (2.22%)	29 (64.44%)	10 (22.22%)	5 (11.11%)
Oral Lichen Planus	10	5 (50%)	3 (30%)	2 (20%)	0 (0%)
Oral Submucous Fibrosis	45	0 (0%)	25 (55.55%)	18 (40%)	2 (4.44%)
Total	100	6	57	30	7
<b>Oral Cancer</b>					
Control	100	—	—	—	100 (100%)
Total	300	106 (35.33%)	57 (19%)	30 (10%)	107 (35.66%)

brated micrometer. By superimposition of the calibrated eyepiece graticule on the cytological smears, direct measurements of individual epithelial cells were made, with  $100\times$  objective. Values were obtained in both axes of the cells and nuclei (Fig. 6). Cases where cytoplasm was irregular values were obtained in all different axes and mean was taken for calculation. Only clearly defined cells were measured, avoiding clumped or folded cells and unusually distorted nuclei and cells. One hundred cells were measured for CD and ND from each slide. The mean values of CD and ND were obtained for each case. Micro nucleated cells were counted out of 1,000 intact epithelial cells, and they were expressed as percentages. For histopathology, epithelial dysplasia was diagnosed according to the twelve features outlined by the WHO<sup>8</sup>

### 5. Statistical analysis

A correlation analysis was performed between the CD and ND for each group. The study and control groups were compared in terms of mean cellular diameter, mean nuclear diameter, and mean cytoplasm nuclear ratio using one way ANOVA. Micro nucleated cells were counted out of 1,000 intact epithelial cells, and they were expressed as percentages. For statistical evaluation of micronuclei, unpaired *t*-test was applied.

### Results

Clinical details of Control and study group regarding age and sex of patient, site involvement and possible incriminating factors are given in Table 2A, B and C. All the cases had history of tobacco usage in some or other form, the duration and frequency varied in these cases. The pattern of site distribution reflects the tobacco habits, *i.e.* Quid placement followed by Bidi smoking as well as Gutkha chewing are the habits prevalent. The site distribution didn't have any relation to grades of dysplasia ( $p>0.05$ , Chi. Sq. test not significant).

There was no significant difference between the distribution of mild, moderate and severe dysplasia in either different age groups ( $p>0.05$ , Chi. Sq. test not significant) or in males and females ( $p>0.05$ , Chi. Sq. test not significant).

In normal (patient with no dysplasia) smears the mean CD and ND of cells varied from  $39.63\text{ }\mu\text{m}$  to  $115.66\text{ }\mu\text{m}$  and  $9.117\text{ }\mu\text{m}$  to  $22.42\text{ }\mu\text{m}$  respectively (Fig. 7A). The diameter of largest cell seen in patients with no dysplasia was  $138.24\text{ }\mu\text{m}$ . In smears obtained from lesions with mild and moderate dysplasia, the mean CD and ND of cells varied from  $38.82\text{ }\mu\text{m}$  to  $113.07\text{ }\mu\text{m}$  and  $8.65\text{ }\mu\text{m}$  to  $21.36\text{ }\mu\text{m}$  respectively (Fig. 7B). In smears obtained from lesions with

**Table 2.**

A : Age and gender distribution for study groups and control groups of patients

Patients	Age (Years)			Mean $\pm$ SD
	0—25	26—50	51 & above	
Controls				
M	3	22	30	55.36 $\pm$ 21.27
F	5	18	22	52.3 $\pm$ 11.84
Total	8	40	52	58.53 $\pm$ 20.55
Oral Precancer				
M	27	40	16	36.27 $\pm$ 15.13
F	5	6	6	42.80 $\pm$ 17.8
Total	32	46	22	39.53 $\pm$ 16.50
Oral Cancer				
M	1	14	42	56.80 $\pm$ 11.12
F	1	6	36	60.26 $\pm$ 11.55
Total	2	20	78	58.53 $\pm$ 11.33

B : Site involvement in precancer and cancer patients

Site of Involvement	Precancer			Cancer		
	Male	Female	Total	Male	Female	Total
Buccal Mucosa	47	7	54	31	19	50
Gingivae & alveolar sulcus	18	3	21	14	9	23
Tongue	9	3	12	5	6	11
Retromolar Ridge	5	2	7	2	3	5
Palate	3	2	5	3	4	7
Lips	1	0	1	2	1	3
Total	83	17	100	57	43	100

C : Incriminating factors in precancer and cancer patients

Causal Agents	Precancer			Cancer		
	Male	Female	Total	Male	Female	Total
Gutkha	39	3	42	26	7	33
Betel (Paan)	18	9	27	14	27	41
Cigarette/Bidi	17	2	19	12	4	16
Khaini/Snuff	5	1	6	3	1	4
Unknown	4	2	6	2	4	6
Total	83	17	100	57	43	100

severe dysplasia, the mean CD and ND of cells varied from  $31.82 \mu\text{m}$  to  $96.79 \mu\text{m}$  and  $9.96 \mu\text{m}$  to  $23.93 \mu\text{m}$  respectively (Fig. 7C). The cell diameter values were

smaller and nuclear diameter values were larger in cells obtained from SCC (Fig. 8A, B, C). The smallest cell had a mean CD of  $12.9 \mu\text{m}$  and the largest

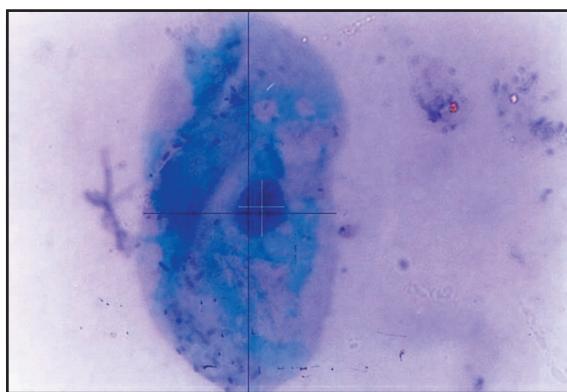
nucleus showed a mean ND of  $38.7 \mu\text{m}$ . The mean CD and ND of cells varied from  $25.61 \mu\text{m}$  to  $84.72 \mu\text{m}$  and  $10.63 \mu\text{m}$  to  $31.55 \mu\text{m}$ , respectively.

Comparison amongst various groups showed increase in mean nuclear diameter as disease stage progresses to oral cancer, and it is proved statistically highly significant ( $p < 0.0001$ ) (Table 3, Fig. 9). Comparison amongst various groups showed significant decrease in mean cellular diameter from Group 1 to Group 4 using one-way ANOVA. This decrease in mean cellular diameter is proven statistically highly significant ( $p < 0.0001$ ) (Table 4, Fig. 10). The

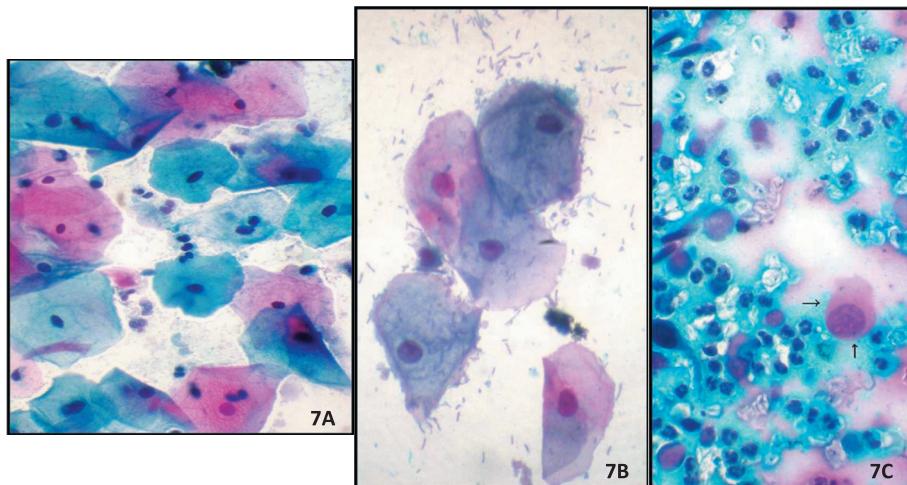
decrease in mean nuclear cytoplasm ratio amongst Groups of 2, 3 and 4 is observed statistically significant when compared to Group I ( $p < 0.0001$ ) (Table 5, Fig. 11). Correlation between CD and ND was positive for no dysplasia (Group I) ( $r = 0.08, p < 0.001$ ) and for lesions with mild and moderate dysplasia (Group II) ( $r = 0.12 ; p < 0.001$ ). However, there were no significant correlations in the case of lesions with severe dysplasia (Group III) and in SCC (Group IV). Frequency of micronuclei ranged from 0% to 0.5% in control group, whereas it varied from 0.7 to 1% in Group 2 (Fig. 12A, B) and slowly increased to 1.5—4.5% in SCC (Fig. 13A, B, C) and it is proved statistically highly significant ( $p < 0.0001$ ) (Table 6).

## Discussion

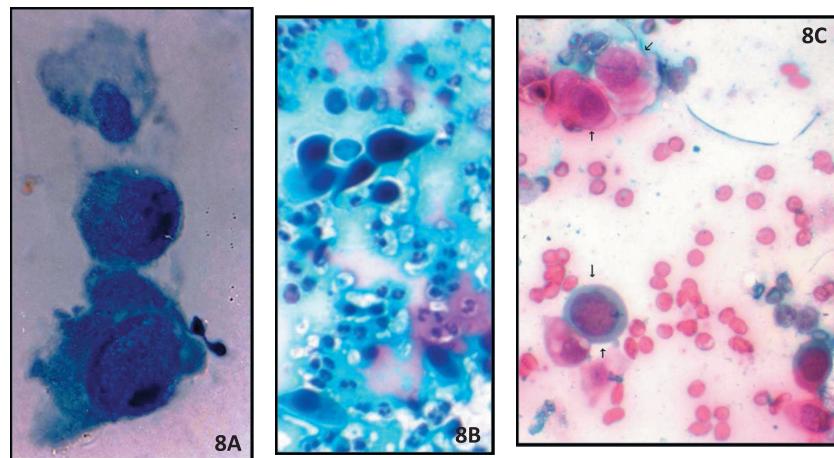
Cancer is one of the major threats to public health in the developed world and is the second most common cause of death<sup>9)</sup>. Oral cancer although prevalent worldwide, it is very common in some developing countries such as India, Pakistan and some parts of France<sup>10)</sup>. In our study 4.44% of OSMF cases have been transformed into carcinoma. In present study 50% of OLP and 2.2% of leukoplakia showed just hyperkeratosis in initial stages but if they become chronic, they may represent premalignant changes as



**Fig. 6** Photomicrograph showing measuring of nuclear and cellular diameter along both the axis (100 $\times$ )



**Fig. 7** Photomicrograph showing distribution of cells in A : Group I smear (20 $\times$ ), B : Group II smear. (20 $\times$ ) & cells in C : Group III smear. (20 $\times$ ). Arrow indicates increased Nuclear diameter and reduced cell diameter.



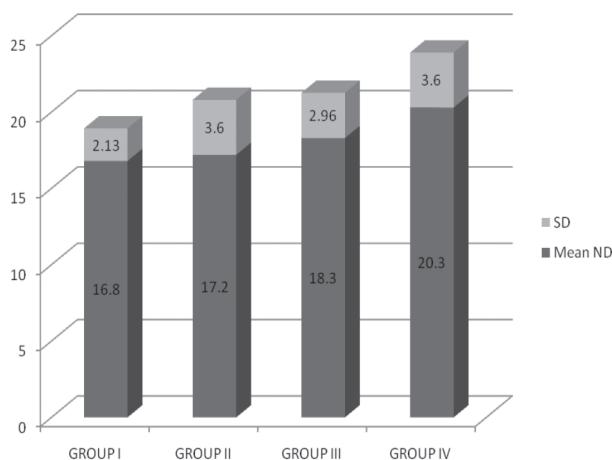
**Fig. 8** A ( $40\times$ ) & B ( $20\times$ ). Photomicrograph showing distribution of cells in Group IV smear. C : Arrow indicates increased Nuclear diameter and reduced cell diameter, pleomorphism ( $20\times$ ) increased nuclear size with rimming of cytoplasm ( $40\times$ )

**Table 3** Comparison of mean ND amongst various groups

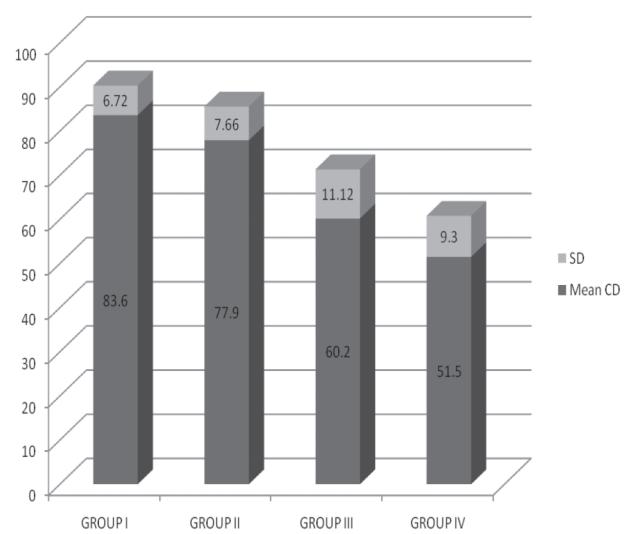
Groups	Mean ND	SD	F	P
I	16.8	2.13		
II	17.2	3.60		
III	18.3	2.96	24.5 ( $1.94 \times 10^{-5}$ )	$p < 0.0001$
IV	20.3	3.60		

**Table 4** Comparison of mean CD amongst various groups

Groups	Mean CD	SD	F	P
I	83.6	6.72		
II	77.9	7.66		
III	60.2	11.12	85.0 ( $2.54 E-018$ )	$p < 0.0001$
IV	51.5	9.30		



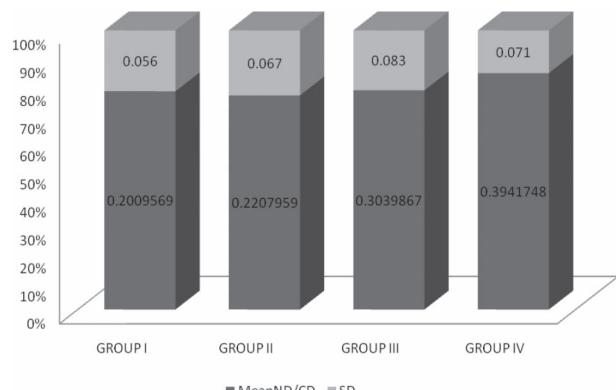
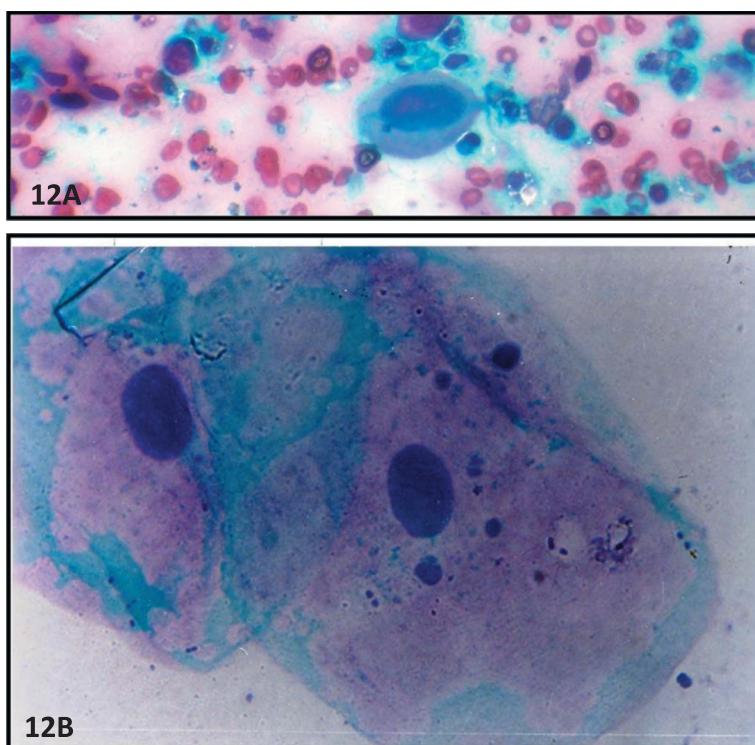
**Fig. 9** Comparison of mean nuclear diameter amongst various groups



**Fig. 10** Comparison of mean cell diameter amongst various groups

**Table 5** Comparison of Mean ND/CD amongst various groups

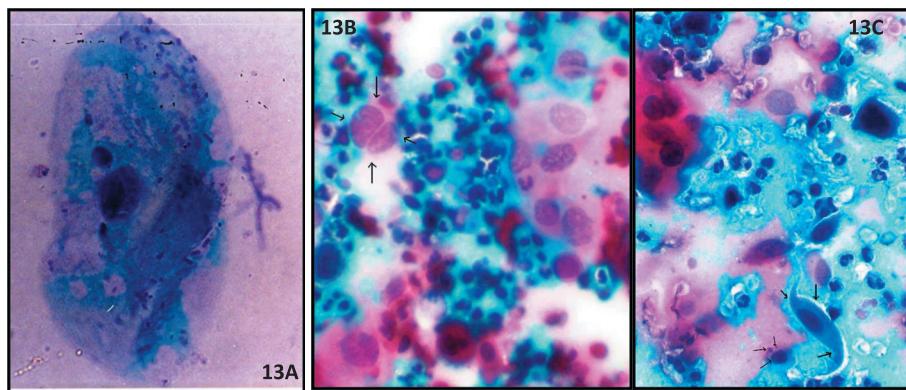
Groups	Mean ND/CD	SD	F	P
I	0.200957	0.02608		
II	0.220796	0.04783	68.1	$p < 0.0001$
III	0.303987	0.10116		(1.79E-025)
IV	0.394175	0.10758		

**Fig. 11** Comparison of mean nuclear cytoplasm ratio amongst various groups**Fig. 12** Photomicrograph showing distribution of cells in Group IV smear along with A : single micronucleus ( $20\times$ ) also B : Presence of micronuclei surrounding central nucleus ( $100\times$ )

seen in Ca *in situ* and SCC.

Cytomorphometric analysis of exfoliated cells could aid in non-invasive follow up of suspicious lesions. The contribution of cytological screening has led its application as a method for the detection of early-

stage oral cancerous and precancerous lesions<sup>4)</sup>. Since then, many authors used Cytomorphometric analysis for this purpose (Mollaoglu *et al.*<sup>11)</sup>, Cowpe *et al.*<sup>12)</sup>, Ogden and Cowpe<sup>13)</sup> and Ramesh *et al.*<sup>8)</sup>). Ramesh *et al.*<sup>8)</sup> reported a significant increase in



**Fig. 13** Photomicrograph showing, A : presence of three micronuclei surrounding central nucleus along with Microbial colonies ( $100\times$ ), B : along with nucleolar fragmentation, arrow indicates binucleation ( $20\times$ ), C : Arrow indicates tadpole like cytoplasm extension and enlarged nucleus also adjacent cell is round with increased size of nucleus and presence of a single micronucleus

**Table 6** Comparison of Mean CD, Mean ND, Mean CD/ND and Micronuclei frequencies between study and control groups

Groups	MN Range%	MN% $\pm$ SD	CD	ND	Mean ND/CD
I	0.0—0.5%	$0.202 \pm 0.151$	83.6	16.8	0.200957
II	0.7—1.0%	$1.378 \pm 0.253$	77.9	17.9	0.220796
III	1.1—1.6%	$2.157 \pm 0.237$	60.2	18.3	0.303987
IV	1.5—4.5%	$4.214 \pm 0.258$	51.5	20.3	0.394175

mean nuclear diameter values during transition from normal mucosal cells to dysplasia and SCC. The mean nuclear diameter was  $7.00$  to  $9.2\text{ }\mu\text{m}$  and  $7.17$  to  $13.75\text{ }\mu\text{m}$  for normal buccal mucosa and SCC lesions respectively. Our study also revealed a statistically significant difference between mean nuclear diameter values for oral cancer, ca *in situ*, mild and moderate dysplasia and healthy mucosal sites of  $20.3\text{ }\mu\text{m}$ ,  $18.3\text{ }\mu\text{m}$ ,  $17.2\text{ }\mu\text{m}$  and  $16.8\text{ }\mu\text{m}$ , respectively (Fig. 9). Similar findings of an increase in nuclear size with dysplasia and in SCC have been reported in experimental lesions in hamster cheek<sup>14)</sup>. Thus, the increase in ND could serve as an early indication of malignant transformation. The CD values showed a reduction from normal samples through lesions with mild to moderate dysplasia and further through severe dysplasia to SCC. This progressive reduction in CD was clearly demonstrated in the graph ; and was in accor-

dance to study of Ramesh *et al.*<sup>8)</sup> This significant reduction in cell size could be an early indication of malignant change, as suggested by Cowpe *et al.*<sup>11,12)</sup>. The analysis of N : C ratio showed an increase from normal smears through lesions with no dysplasia, and further through lesions with dysplasia to SCC. Smaller CD with larger ND and hence an increased nuclear to cytoplasmic ratio in squamous epithelium indicates a lesser differentiation (*i.e.* basal/suprabasal cells.) (Fig. 11). Thus, tumors are distinguished from normal tissues partly by their pronounced variability of nuclear size and nuclear to cytoplasmic ratio. Similarly, there are various other cellular alterations in precancer and cancer. It includes karyorhexis, karyolysis, micronucleus formation (Fig. 13A, C), pyknosis, binucleation (Fig. 13B), broken-egg nucleus, anucleation, etc<sup>8)</sup>. Micronuclei in oral exfoliated cells are marker of chromosomal damage caused by genotoxic

agents from tobacco and tobacco related substances, alcohol, etc. The micronucleus assay has been used to assess the genotoxic damage in OSCC and oral premalignancy. Incidence of micronuclei has been analyzed by various studies in normal patients, oral premalignancy, and OSCC<sup>3,15)</sup>. In the present study, micronuclei in oral exfoliated cells in the control group were observed to be in the range of 0% to 0.5% with a mean micronucleus frequency of  $0.202 \pm 0.151\%$  and these levels are quite similar to those reported by earlier studies, such as  $0.210 \pm 0.168\%$ <sup>3)</sup>,  $0.186 \pm 0.026\%$ <sup>16)</sup>. The overall level of micronuclei was increased significantly from Group I, Group II, and Group III to SCC (Table 6). A significant ( $p < 0.05$ ) stepwise increase was found in the percentage of micro nucleated cells and micronuclei from control to precancer patients, and from precancer to cancer patients in a study by Saran *et al.*<sup>17)</sup>. Though oral biopsy represents the gold standard for determining the nature of a mucosal lesion and for diagnosing SCC, the exfoliative techniques have the advantage of being minimally invasive and use of cytobrush reportedly allows sampling of the full thickness of stratified squamous epithelium (basal, intermediate, superficial) of the oral mucosa, thus giving an opportunity to detect early precancerous changes.

### Conclusions

The reduction in CD, increase in ND, increase in N : C ratio as well as increased percentage of micronuclei could serve as early indicators of malignancy. Increased knowledge of biomarkers and the effect of chemo preventive agents in the oral carcinogenesis pathway can help us in designing better mechanism based prevention studies as well.

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