Comparative cytomorphometric analysis of oral mucosal cells in normal, tobacco users, oral leukoplakia and oral squamous cell carcinoma

ABSTRACT
Background: Squamous cell carcinoma (SCC) is the third most common cause of oral morbidity in India despite the numerous advances made in the treatment protocol.

Aim: To compare the cytomorphometric changes of oral mucosal cells in normal subjects (Group I) with that of tobacco users without any lesion (Group II), tobacco users with oral leukoplakia (Group III), and tobacco users with oral SCC (Group IV) through a semi-automated image analysis system.

Materials and Methods: Oral mucosal cells collected from study subjects (n = 100) stained using rapid Papanicolaou stain. Photomicrograph of 50 nonoverlapping cells captured at 50x magnification with a digital image capture system. Cytomorphometric analysis of cells in the captured images was performed with Image-Pro image analysis software. Image analysis was performed to obtain cell diameter (CD), cytoplasmic area (CyA), nuclear diameter (ND), nuclear area (NA), and nuclear-to-cytoplasmic ratio. These values were statistically compared among the groups using one-way analysis of variance (ANOVA) and Mann-Whitney U test.

Results: The ND, NA, and nuclear-to-cytoplasmic ratio values were found to be increased in the samples collected from leukoplakia and oral SCC. The CD and CyA decreased compared to the normal mucosa in oral SCC samples.

Conclusion: The cytomorphometric changes observed in samples from oral SCC and oral leukoplakia were consistent with the current diagnostic features. Hence, the semi-automated cytomorphometric analysis of oral mucosal cells can be used as an objective adjunct diagnostic tool in the diagnosis of these lesions.

Key words: Cytomorphometry; leukoplakia; oral squamous cell carcinoma (SCC); Papanicolaou stain

Introduction
In the early stages, oral cancer may disguise itself as innocent lesions. Patients are usually unaware of the lesion and report only at a later stage at which more invasive treatment is required. So early detection of potentially malignant and malignant disorders decreases the patient morbidity and improves the patient survival.
Oral cancer is a global health problem with increasing mortality rates. Oral cancer and pharyngeal cancer grouped together is the sixth most common cancer in the world. The annual incidence rate is around 275,000 for oral and 130,000 for oropharyngeal cancers, two-third of the cases occurring in developing countries. The incidence rate in India is around 19 per 100,000 of the population. In India, oral cancer represents up to 40% of all cancers. Oral cancer represents 14.7% of all cancer cases at the Regional Cancer Centre, Kerala, India and the tongue was the most common site. It is the commonest cancer in males and the third most common in females in Kerala, India. Among oral cancers, squamous cell carcinoma (SCC) comprises 90% of all oral malignancies.

Epidemiological and clinical studies suggest a causative role of tobacco use in the evolution of malignant and potentially malignant disorders. Oral cytology is being increasingly important in the early diagnosis of oral cancer. Exfoliative cytology is the microscopic examination of shed or desquamated cells from the epithelial surface, usually the mucous membrane. It also includes the study of those cells that have been collected by scraping the tissue surface or collected from body fluids such as sputum and saliva.

The Papanicolau stain is regarded as the universal stain for cytological preparations. This special stain imparts a different color to the cytoplasm of epithelial cells based on their degree of cellular differentiation.

With the advancements in the quantitative oral exfoliative cytology, oral cytology once again emerged as a diagnostic aid in oral lesions. The accuracy of cytologic technique in diagnosing oral lesions can be enhanced by morphometry. Computer-assisted morphometric analysis of cells improved the ability to accurately measure the various cell parameters such as nuclear diameter (ND), cell diameter (CD), nuclear area (NA), cytoplasmic area (CyA), and nuclear-to-cytoplasmic ratio.

Materials and Methods

The present study was conducted on patients availing treatment from our institution. The study was conducted on subjects in the age group of 25-85 years. The study group consisted of 42 males and 58 females.

Hundred individuals were divided into four groups of 25 individuals each with Group I comprising 25 nontobacco users, Group II comprising 25 tobacco users without any disorder, Group III comprising 25 tobacco users with oral leukoplakia, and Group IV comprising 25 tobacco users with oral SCC. Samples were selected by simple random sampling of tobacco smokers, tobacco chewers, and normal individuals who fitted in the inclusion criteria.

Patients who were histopathologically confirmed as having SCC and leukoplakia, tobacco and paan chewers for at least 10 years without any visible lesions, and normal individuals of a similar age group who were willing to participate were included in the study. The study group with any systemic illness or anemia or under treatment for cancer and normal individuals with any habit of smoking, alcohol, and paan-chewing were excluded from the study to avoid cellular changes associated with these conditions.

Ethical clearance (IEC/IRB No: ACDS/1624/14/12) was obtained from the institutional ethics committee. Patient information sheet was given and the entire procedure was explained to the patient. Informed consent was obtained from each subject. All the needed information of the study subjects were collected using a questionnaire. Blood samples were collected from the study subjects for estimation of hemoglobin. The cytologic study samples were obtained from the oral mucosa. The patient was asked to rinse his/her mouth with water to remove debris within the oral cavity. Gross debris on the lesion was removed with moist swabs. Squamous epithelial cells were collected using a moistened wooden spatula and transferred to a clean dry glass slide and spread thinly and uniformly with a circular motion. The smears were immediately fixed with spray fixative and stained using rapid Papanicolau stain (Biolab, Mumbai, Maharashtra, India), which consumed less time and gave comparable staining characteristics as that of conventional technique.

Rapid PAP kit of Biolab Diagnostics was used. The kit consists of Rapid PAP nuclear stain, hematoxylin stain, cytoplasmic stain, combination of OG-6 and Light Green—eosin, Scott’s tap water buffer, and dehydrant solution, and propanol and xylene. Smears were fixed in tap water followed by Rapid PAP nuclear stain for 45 s. The smears were washed in Scott’s tap water for 30 s followed by Rapid PAP dehydrant for 60 s and cytoplasmic stain for 45 s. Then it was washed in Scott’s tap water followed by dehydrant for 30 s. The slide was air-dried and then dipped in xylene and mount wet.

The stained smears were examined under a microscope under 50x plan apochromat objective of trinocular research microscope (Olympus BX 51, Shinjuku, Tokyo, Japan) loaded with a motorized stage and DP71 image capture system. Photomicrographic images of nonoverlapping cells were captured with the assistance of an image capture and
processing software (Image Pro 3DS6.1, Media Cybernetics, Washington Street, Rockville, USA). Cells were selected by moving the slide from the left to right in a zigzag fashion. The captured pictures were stored and all the measurements were taken after proper calibration of image analysis software. For calculating nuclear and cellular diameters, the maximum and minimum values were measured using image analysis software and the average value was taken. All measurements were imported from the image analysis software to Microsoft Excel for statistical analysis using Statistical Package for the Social Sciences (SPSS) software version 20 (IBM Corp. Released 2010. IBM SPSS Statistics for windows, Version 20.0. Armonk, NY: IBM Corp).

Fifty clearly defined cells were to be measured in each case and the mean value was taken. Image analysis was performed to obtain ND, CD, NA, CyA, cell area (CA), and nuclear-to-cytoplasmic ratio. These calculated values compared in Groups I, II, III, and IV after statistical analysis.

Morphometric parameters studied
Nuclear diameter (ND), cell diameter (CD) — The ND and CD were measured using a digitalized cursor with an interactive measurement tool by tracing two perpendicular lines (maximum and minimum diameter), which was measured by the software and the mean values were taken.

Nuclear area (NA), cell area (CA) — The nuclear and cellular outlines were traced using a digitalized cursor with an interactive measurement tool and the software calculated the area.

Cytoplasmic area (CyA) — The CyA was measured in square micrometers (µm²). The cytoplasmic outline was measured from the difference between CA and NA.

\[ \text{CyA} = \text{CA} - \text{NA} \]

Nuclear-to-cytoplasmic diameter (NCD) ratio — It was calculated using the formula:

\[ \text{NCD} = \frac{\text{Nuclear diameter}}{\text{Cytoplasmic diameter}} \]

Nuclear-to-cytoplasmic area (NCA) ratio — It was calculated using the formula:

\[ \text{NCA} = \frac{\text{NA}}{\text{CyA}} \]

The statistical analysis applied included:
1. One-way analysis of variance (ANOVA) was used to compare the ND, CD, NA, CyA, NCD ratio, and NCA ratio values among the groups.
2. Mann-Whitney U test was used for pair-wise group comparison of the ND, CD, NA, CyA, NCD ratio, and nuclear CyA ratio values within the groups.

Results

Nuclear diameter
The mean ND (in µm) in Group I was 9.81 ± 0.315, in Group II was 9.593 ± 0.233, in Group III was 11.065 ± 0.210, and in Group IV was 11.199 ± 0.276 [Figure 1]. ND was significant among the groups when compared by one-way ANOVA (\(P = 0.000\)). Comparison within the groups using Mann-Whitney U test showed a significant increase in the ND among all groups except between Group I and Group II, and Group III and Group IV.

Cell diameter
The CD (in µm) in Group I was 79.58 ± 2.96, in Group II was 75.24 ± 0.809, in Group III was 67.45 ± 3.32, and in Group IV was 57.90 ± 8.494 [Figure 2]. The CD was significant among the groups when compared by one-way ANOVA (\(P = 0.000\)). Comparison within the groups using Mann-Whitney U test showed a significant decrease in CD among all the groups.

Nuclear area
The NA (in µ²) in Group I was 68.45 ± 3.16, in Group II was 69.63 ± 2.9, in Group III was 80.51 ± 6.21, and in Group IV was 85.87 ± 6.88. The mean NA was highly significant among the groups when compared by one-way ANOVA (\(P = 0.000\)). Comparison among the groups using Mann-Whitney U test showed a significant increase in the NA among all the groups except between Group I and Group II, and Group III and Group IV.

Figure 1: Photomicrographs of nuclear diameter of N1—normal, N2—tobacco users without any lesion, N3—leukoplakia, N4—oral squamous cell carcinoma (Pap, ×500)
Cytoplasmic area
The CyA (in \( \mu m^2 \)) in Group I was 3783.79 ± 382.53, in Group II was 3311.96 ± 99.17, in Group III was 2716.18 ± 269.23, and Group IV was 2080.06 ± 452.022. The mean CyA was highly significant among the groups when compared by one-way ANOVA (\( P – 0.000 \)). Comparison among the groups using Mann-Whitney U test showed a significant decrease in CyA among all the groups.

Nuclear-to-cytoplasmic diameter ratio
The NCD diameter ratio (in \( \mu m \)) in Group I was 0.1265 ± 0.00431, in Group II was 0.1343 ± 0.0066, in Group III was 0.168 ± 0.0089, and in Group IV was 0.213 ± 0.0482. NCD ratio was highly significant among the groups when compared by one-way ANOVA (\( P – 0.000 \)).

Nuclear-to-cytoplasmic area ratio
The NCA ratio (in \( \mu m \)) in Group I was 0.019 ± 0.0019, in Group II was 0.0216 ± 0.0014, in Group III was 0.031 ± 0.0031, and in Group IV was 0.055 ± 0.04. NCA ratio was found to be highly significant among the groups when compared by one-way ANOVA (\( P - 0.000 \)).

Discussion
Tobacco products are one of the prime suspects in the etiology of oral cancer.\(^{[13]}\) Epidemiological and clinical studies suggest a causative role of tobacco in the evolution...
of potentially malignant and malignant lesions of oral cavity. In South Asia, oral cancer ranks first among all cancers in males and is the third most common cancer in females. This study was conducted to find the cytomorphometric changes that occur in tobacco users, leukoplakia, and oral SCC when compared to that of nontobacco users.

Cytopathology is a simple, noninvasive, and low cost technique that can be used to assess the changes in oral mucosa. The basics of cytological diagnosis in malignancies is based on the work done by Papanicolaou and Traut. The differential staining characteristics of PAP gives a pink, blue, and green hue to the cytoplasm according to the maturation pattern and keratin content of cells. Apart from wooden spatula, cotton swabs and cytobrush can be used for the collection of smear. We preferred a wooden spatula for the study as it collects sufficient cells with less trauma to the patient and is cost-effective.

Cytomorphometry is a computer-assisted method for the analysis of cells. A computer analysis a digital microscopic image of the collected cells with the help of a neural network-based image processing system. Morphometric parameters such as NA, CyA, and NCA ratio (NA/CyA) may increase the sensitivity of exfoliative cytology for early diagnosis of malignancy and 50 cells are sufficient to provide indication of malignancy. Tissues undergoing malignant transformation typically shows a reduction in CyA before the reduction in NA. A previous study shows that cytoplasmic diameter was the highest in normal mucosa, lower in dysplastic lesions, and lowest in SCC. The ND was the lowest in normal mucosa, higher in dysplastic lesions, and highest in SCC.

The study group consisted of males and females. The age group of patients varied between 25 years and 85 years. We also compared all morphometric parameters between males and females. But we could not find any significant variation in these parameters between males and females.

Tobacco-chewing and smoking are two common risk factors for oral leukoplakia. Smoking has a higher risk for cancer of the oropharynx and larynx, whereas tobacco-chewing has a higher risk for cancer of oral cavity and hypopharynx. Smokers are more likely to develop oral cancer seven to ten times than nonsmokers. Risk of oral cancer increases with depth of number of cigar smoked per day and the depth of inhalation. Cigarette smoking produces alterations in the mechanism of cell growth control and is considered to be an initiating factor in the process of oral carcinogenesis.

In a previous study ND, CD, NCD ratio in normal mucosa, leukoplakia, and oral SCC were compared. Smears were stained with Papanicolaou stain and cytomorphometric analysis was performed with eye piece graticule. The ND and cytoplasmic diameter values were found to have decreased from normal mucosa to oral SCC with the lowest value. The NCD ratio was found to have increased from normal mucosa to oral SCC with the highest value.
Another study compared ND and NA in smokers and nonsmokers. Smears were stained with Papanicolaou stain and cytomorphometric analysis was performed with a special program Samba IDB.01, Grenoble, France. The ND and NA values were found to be increased from nonsmokers to smokers.\(^{24}\)

The ND and CD in the mucosa of normal subjects, tobacco users without lesion, tobacco users with leukoplakia, and tobacco users with oral SCC were studied in a previous study. Smears were stained with Papanicolaou stain and cytomorphometric analysis was performed with a stage micrometer. The ND values were found to have increased from normal mucosa to oral SCC with the highest value. The CD was found to have decreased from normal mucosa to oral SCC with the lowest value.\(^{25}\)

The NA and CyA were compared among smokers and nonsmokers in another study. Buccal smears were collected with wooden tongue spatula, stained with Papanicolaou stain and cytomorphometric analysis was performed with Vids V image analysis system. The ND values were found to have significantly increased from nonsmokers to smokers. The CD was found to have increased from nonsmokers to smokers but it was not significant.\(^{17}\)

The present study compared ND, CD, NA, CyA, NCD ratio and NCA ratio in nontobacco users, tobacco users without lesion, tobacco users with leukoplakia, and tobacco users with oral SCC. Smears were collected with a wooden spatula, stained with Papanicolaou stain and cytomorphometric analysis was performed with Image-Pro Image analysis software.

The normal oral epithelium is a stratified squamous type. The inner layer is the basal cell layer, then lies the prickle cell layer and then the surface cornified layer. The granular layer will be more if there is overlying keratin. ND decreases and CD increases as we go from the basal layer to the superficial layers.\(^{26}\) The basal cell nucleus is relatively larger. The prickle cells are larger than the basal cells but the nucleus is smaller than the basal cells. As the epithelium matures, the physiologic activity of the nucleus decreases and the nucleus condenses toward the surface. As the cells cornify, the nucleus entirely disappears.\(^{27}\)

The ND value decreased from the normal subject to the tobacco user without any lesion and then was found to have increased with leukoplakia and oral SCC with the highest value. In leukoplakia, atypical cells are characterized by nuclear enlargement with variation in the shape and size of the cells. In leukoplakia, atypical cells are characterized by nuclear enlargement with variation in the shape and size of the cells. In oral smears, malignant cells are characterized by a large nucleus with hyperchromatism and an increase in the nuclear-to-cytoplasmic ratio. In oral smears, malignant cells are characterized by large nucleus with hyperchromatism and an increase in the nuclear-to-cytoplasmic ratio.\(^{28}\)

The NA, NCD ratio, and nuclear CyA ratio values were found to have increased from normal subjects to tobacco users without any lesion to leukoplakia with the highest value in oral SCC.

The CD and CyA was found to have decreased from normal mucosa to tobacco users without any lesion to leukoplakia and oral SCC with the lowest value. Actively dividing cells show a decrease in the cellular diameter and increase in nuclear size.\(^{29}\)

The comparison of mean values using ANOVA and Mann-Whitney \(U\) test is given in Tables 1 and 2. The comparison of mean values obtained from the present study is compared with previous studies in Table 3.

The rationale of exfoliative cytology lies in normal physiology of the epithelium. Deeper layers of cells are strongly adherent in normal conditions. In case of pathological alterations, the deeper layers become loose and exfoliate, along with normal epithelial cells.\(^{18}\) In SCC, more of the basal and parabasal cells can be seen.

### Table 1: One Way ANOVA - Comparison of nuclear diameter, cell diameter, nuclear area, cytoplasmic area, nuclear cytoplasmic diameter ratio, nuclear cytoplasmic area ratio

| Group   | Nuclear diameter [µ] | Cell diameter [µ] | Nuclear area [µm²] | Cytoplasmic area [µm²] | Nuclear cytoplasmic diameter ratio | Nucleur cytoplasmic area ratio |
|---------|----------------------|-------------------|--------------------|------------------------|----------------------------------|-------------------------------|
| Group I | 9.81 ± 0.315         | 79.58 ± 2.96      | 68.45 ± 3.16       | 3783.79 ± 382.53       | 0.1265 ± 0.0043                  | 0.019 ± 0.0019                |
| Group II| 9.593 ± 0.233        | 75.24 ± 0.809     | 69.63 ± 2.9        | 3311.96 ± 99.17        | 0.1343 ± 0.0066                  | 0.0216 ± 0.0014               |
| Group III| 11.065 ± 0.210      | 67.45 ± 3.32      | 80.51 ± 6.21       | 2716.18 ± 269.23       | 0.168 ± 0.0089                   | 0.031 ± 0.0031                |
| Group IV| 11.199 ± 0.276       | 57.90 ± 6.494     | 85.87 ± 6.88       | 2080.06 ± 452.02       | 0.213 ± 0.0482                   | 0.055 ± 0.041                 |
| \(F\)-value | 190.356             | 97.917            | 68.803             | 125.919                | 62.437                           | 16.488                        |
| \(P\)-value | 0.000               | 0.000             | 0.000              | 0.000                  | 0.000                            | 0.000                         |
Table 3: Comparison of the cytomorphometric analysis of different studies

| Present study | Comparison with other studies |
|---------------|-------------------------------|
| Cytoplasmic area in non tobacco users 3783.79±382.53 μm² | Ogden et al. — 3098.96±766 μm²[31] |
| Nuclear cytoplasmic diameter ratio in non tobacco users 0.1265±0.0043 | Sridhar Reddy et al. — 0.17 to 0.20[32] |
| | Hedge V et al. — 0.1629 to 0.18[33] |
| | Anuradha A et al. — 0.18 to 0.2[30] |
| | Nayar AK et al. — 0.16 to 0.18[29] |
| Nuclear cytoplasmic area ratio in non tobacco users 0.019±0.0019 | Patel PV et al. — 0.02±0.003 |
| | to 0.03±0.008[34] |
| Nuclear diameter in tobacco users 9.593±0.233 μm | Batista AB et al. — 0.2[29] |
| Cell diameter in tobacco users 75.24±0.809 μm | Khandelwal et al. — 7.47±0.34 μm[34] |
| | Hande AK et al. — 8.98±1.08 μm[32] |
| Nuclear area in tobacco users 69.63±2.9 μm² | Hande AK et al. — 68.30±3.02 μm²[33] |
| Cytoplasmic area in tobacco users 3311.96±99.17 μm² | Hande AK et al. — 29.71±5.73 μm²[33] |
| Nuclear diameter in leukoplakia 11.065±0.210 μm | Hande AK et al. — 9.12±1.06 μm²[33] |
| Cell diameter in leukoplakia 67.45±3.32 μm | Hande AK et al. — 57.75±4.66 μm²[33] |
| Nuclear area in oral cancer 85.87±6.88 μm² | Hande AK et al. — 41.32±0.13 μm²[33] |
| Nuclear cytoplasmic diameter ratio in leukoplakia 0.169±0.0089 | Hedge V et al. — 0.217±0.0319[28] |
| Nuclear diameter in oral cancer 11.199±0.276 μm | Joshi et al. — 10.11 μm[33] |
| | Khandelwal et al. — 10.41±1.12 μm²[34] |
| | Hande AK et al. — 11.04±1.46 μm²[33] |
| | Ramesh T et al. — 10.10±0.56 μm²[33] |
| Cell diameter in oral cancer 57.90±4.894 μm | Joshi et al. — 47.94 μm²[30] |
| | Khandelwal et al. — 35.81±2.48 μm²[34] |
| | Hande AK et al. — 54.51±4.66 μm²[33] |
| Nuclear area in oral cancer 85.87±6.88 μm² | Joshi et al. — 60.04 μm²[30] |
| | Khandelwal et al. — 81.54±4.31 μm²[34] |
| Nuclear cytoplasmic diameter ratio in oral cancer 0.213±0.0482 | Ogden et al. — 0.25±0.036[29] |

**Conclusion**

In the early stages, oral cancer may disguise itself as innocent lesions. Patients are usually unaware of the lesion and report only at a later stage at which more invasive treatment is required. So early detection of potentially malignant and malignant disorders decreases patient morbidity and improves patient survival.

Biopsy is an invasive technique with psychological implication for most of the patients. Exfoliative cytology is a simple, noninvasive technique that could be used in the diagnosis of oral leukoplakia and oral SCC. Quantitative parameters such as morphometry are reproducible and eliminate the observer bias as it is obtained by software analysis and hence, improves the accuracy in the diagnosis of these lesions.

Results of the present study suggest that oral SCC and oral leukoplakia produce definite cytomorphometric changes. Hence, the cytomorphometric analysis of oral mucosal cells in leukoplakia and oral SCC could be used as an adjunct diagnostic tool in the detection of these lesions.

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**Conflicts of interest**

There are no conflicts of interest.

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