Original Article

Cytomorphometric analysis of squames obtained from normal mucosa, leukoplakia and oral squamous cell carcinoma

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Abstract

Introduction: Exfoliative cytology is becoming increasingly important in the early diagnosis of oral cancers, as a procedure for obtaining cell samples, which can be analyzed by sophisticated diagnostic techniques. Quantitative techniques, based on the evaluation of parameters such as nucleus area, cytoplasm area and nucleus-to-cytoplasm area ratio, may increase the sensitivity of exfoliative cytology for early diagnosis of oral cancers, since these techniques are precise, objective and reproducible.

Objectives: This study was undertaken to employ the cytomorphometric quantitative techniques to determine the value of exfoliative cytology and to assess this method to analyze the effectiveness in providing additional diagnostic test for the detection of early oral malignancy.

Materials and Methods: The study consisted of three groups. The experimental group comprised of 20 subjects with oral leukoplakia and 20 subjects with oral squamous cell carcinoma of the buccal mucosa. The control group comprised of 10 apparently healthy subjects. They were subjected to clinical examination and cytosmear of the buccal mucosa. A standard wooden tongue spatula/metal spatula moistened with normal saline was used to obtain scrapings of buccal mucosa. The scrapings were spread on plain glass slides and immediately fixed in Biofix spray, followed by staining with the Papanicolaou technique. Then, the microscopic pictures were captured onto a computer and are cytomorphometrically analyzed using an image analyzer. Since this study involved multiple groups, one-way analysis of variance (ANOVA) was used for comparing the parameters for multiple groups. Where significant difference was seen, the ANOVA test was followed by Mann–Whitney test, for pairwise comparison.

Results: The mean count of nuclear area and nuclear diameter showed an increase from Group I to Group II, Group I to Group III and Group II to Group III, which was statistically significant. The mean count of cell area and cell diameter showed a decrease from Group I to Group II, Group I to Group III and Group II to Group III, which was statistically significant. The mean count of nuclear/cytoplasm area ratio showed an increase from Group I to Group II, Group I to Group III and Group II to Group III, which was statistically significant.

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Conclusion: With the advancements in the field of quantitative exfoliative cytology, interest in oral cytology has once again emerged in the diagnosis of oral premalignancy and malignancy. Computer-aided analysis with the sophisticated software increases the speed and accuracy of cytological measurements, which are repeatable. These versatile systems facilitate quantitative oral cytological assessments, which may become a viable procedure for the early detection of oral premalignancy and malignancy.

Keywords: Cytomorphometrical analysis, exfoliative cytology, Papanicolaou technique

INTRODUCTION

Head and neck cancers accounts for up to 30%–40% of malignancies in India.[1] Oral squamous cell carcinoma is the sixth most common cancer in the world,[2] and encompass at least 90% of all oral malignancies.[3] The morbidity and mortality rate associated with oral squamous cell carcinoma are still unacceptably high and prognosis is generally poor. This low survival rate can be reduced by the direct examination of oral cavity as it can be accessed and subjected for diagnostic procedures. Further oral squamous cell carcinoma is very often preceded by mucosal changes known as precancer, which aids in early recognition and elimination. Despite improvements in surgery, radiotherapy and chemotherapy, the 5-year survival rate for oral squamous cell carcinoma has remained approximately 50% for the past several decades.[4]

These potential harmful oral lesions may not be possible to determine by clinical observation alone, as they remain

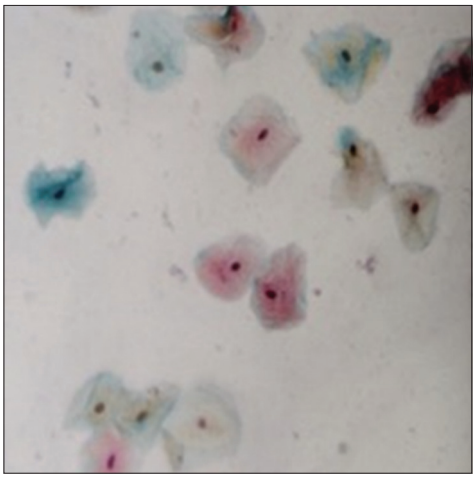

Figure 1: Photomicrograph showing exfoliated cells from a patient belonging to Group I (×10)

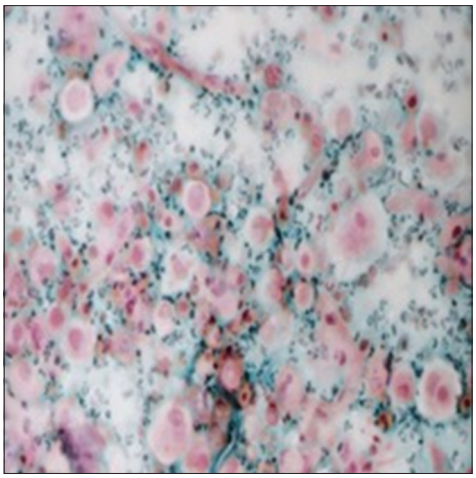

Figure 2: Photomicrograph showing exfoliated cells from a patient belonging to Group II (×10)

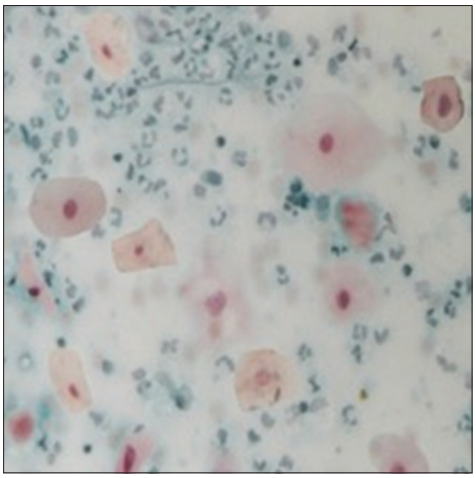

Figure 3: Photomicrograph showing exfoliated cells from a patient belonging to Group III (×10)

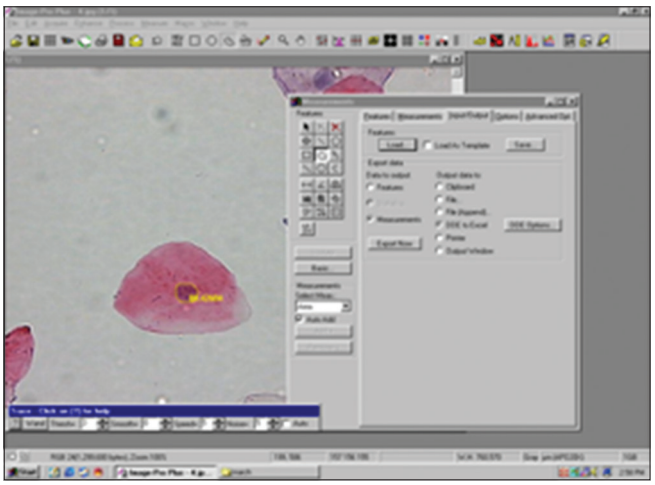

Figure 4: Photomicrograph showing image analysis measurements of cell area

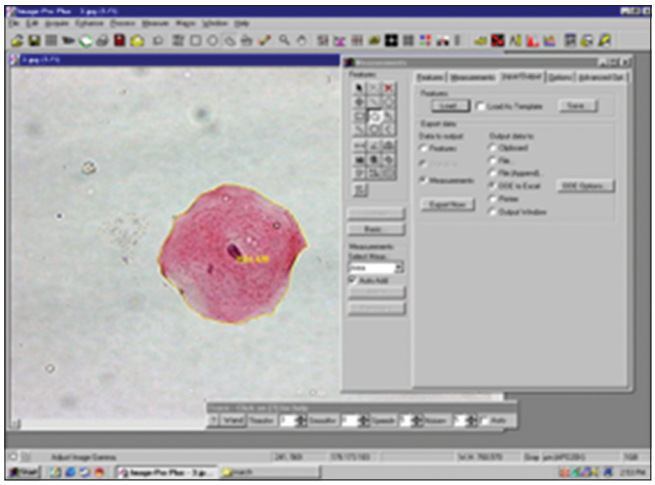

Figure 5: Photomicrograph showing image analysis measurements of nuclear area

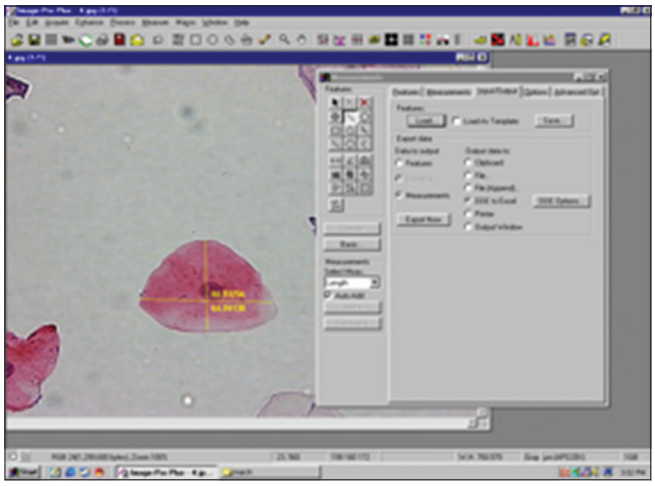

Figure 6: Photomicrograph showing image analysis measurements of cell diameter

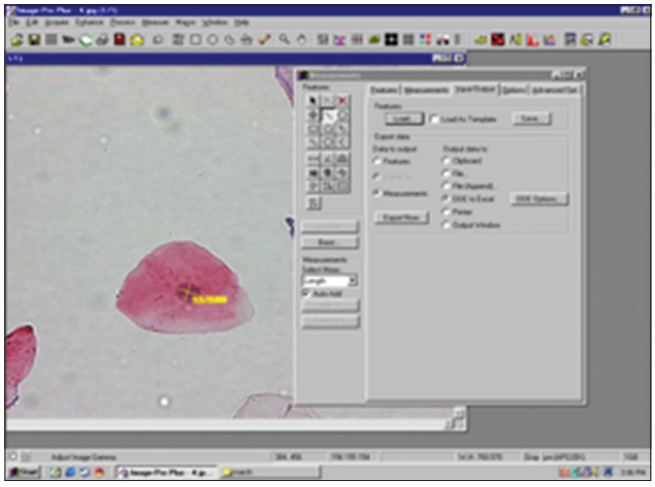

Figure 7: Photomicrograph showing image analysis measurements of nuclear diameter

innocuous, undiagnosed and progress to a more advanced stage. They may also cause some changes in the oral mucosa within the epithelium, which can be biopsied and confirmed.

Ever since Papanicolaou described exfoliative cytology technique, which is a nonpainful, noninvasive procedure, it has become a valuable tool for cancer screening.[6,7] Ziskin was the first person to have reported the use of exfoliative cytology in oral cavity. Montgomery and Von Hamm in 1951 used exfoliative cytology for the diagnosis of oral cancer.[8] This being a noninvasive procedure, patients are more likely to agree for the investigations. The sampling of individual cells might improve the chance of detecting dysplastic change. The loss of normal cellular architecture and the decrease of mutual cellular adhesion combined with the increased production of new cells makes cytology successful in the diagnosis of very early and minute tumors.

Cytomorphometry can be used selectively on structures or samples, which are difficult to assess accurately, such as variation in cell and nuclear size and staining intensity. As nuclear changes are the most important criteria for diagnosing precancerous and cancerous lesions and since no single structural change is diagnostic by itself, a combination of several abnormalities is always necessary. Oral epithelium renews itself rapidly and it sheds off its superficial cells into oral cavity through the process known as desquamation. These cells reflect the physiological or pathological changes of underlying tissue, which can be studied both qualitatively and quantitatively.[9]

This study was carried out to compare the cytological features of buccal squames of normal, oral premalignant and malignant lesions by computer-aided cytomorphometry, which may improve the definitive diagnostic value of oral exfoliative cytology.

Aims and objectives

1. To study the following cellular and nuclear changes of the squames obtained from the premalignant and malignant lesions using exfoliative cytology and to compare the same with normal oral buccal mucosa
   i. Nuclear area
   ii. Cell area
   iii. Nuclear diameter
   iv. Cell diameter
   v. Nuclear/Cytoplasmic area ratio.
2. To explore the effectiveness of the use of quantitative cytomorphometry in early diagnosis of oral premalignant and malignant lesions
3. To compare the cytomorphometric values of squames obtained from both study group and control group and to infer the significance of the same.

MATERIALS AND METHODS

This study was conducted in the Department of Oral Pathology and Microbiology Bapuji Dental College and Hospital, Davangere. The patients above the age of 40 years were included in the study irrespective of sex, caste and socioeconomic status. A prestructured pro forma was used for each individual case to collect relevant information and cytomorphometric measurements of cells.

The experimental groups comprised of 20 subjects of oral leukoplakia and 20 subjects of oral squamous cell carcinoma of the buccal mucosa. In each case, smears were obtained from the lesion in cases of leukoplakia and around the lesion in cases of squamous cell carcinoma. This was followed by incisional/excisional biopsy of the lesion. Only those cases with adequate smears and that were confirmed histopathologically were included in the study.

The control group comprised of 10 apparently healthy controls, age and sex matched with the experimental group, who had never consumed tobacco, arecanut or alcohol in any form and had no history of viral disease or any medication during the preceding 6 months. These patients were subjected to clinical examination and cytosmear of the buccal mucosa.

In the control group, subjects were asked to rinse their mouth with water and 2 smears were taken using a wooden/metal cement spatula moistened with normal saline were used to obtain scrapings from the normal buccal mucosa.

In the experimental group, cytosmears two smears were taken using a moist wooden/metal cement spatula from the site of lesion in leukoplakia and around the lesion in cases of squamous cell carcinoma. Subjects were made to rinse their mouth with water to remove debris and necrotic material prior to performing the cytosmears. The scrapings were smeared on plain glass slides and immediately fixed in spray fixative followed by staining with the Papanicolaou technique.

The scrapings were spread on plain glass slides and immediately fixed in Biofix spray, followed by staining with the Papanicolaou technique. Then the microscopic pictures were captured onto a computer and are cytomorphometrically analyzed using an image analyzer.

A high-resolution CCD camera attached to a research microscope was used to capture the images of the fields at × 1000 magnification and stored in computer. From each slide, 50 cells were selected for cytomorphometric analysis using Sophisticated Image Analysis Software (Image AQ27 Proplus V-4.1.0.0 Media, Cybernetics, USA) [Figures 1-7].

The cytomorphometric parameters studied and compared between the various groups were; nuclear area, cell area, nuclear diameter, cell diameter and nuclear/cytoplasm area ratio.

RESULTS

Since this study involved multiple groups, one-way analysis of variance (ANOVA) was used for comparing the parameters for multiple groups. Where significant difference was seen [Table 1], the ANOVA test was followed by Mann–Whitney test, for pairwise comparison.

The mean count of nuclear area and nuclear diameter showed an increase from Group I to Group II, Group I to Group III and Group II to Group III, which was statistically significant.

The mean count of cell area and cell diameter showed a decrease from Group I to Group II, Group I to Group III and Group II to Group III, which was statistically significant.

The mean count of nuclear/cytoplasm area ratio showed an increase from Group I to Group II, Group I to Group III and Group II to Group III, which was statistically significant.

DISCUSSION

Oral cancer development is a multi-step and multi-focal process involving field carcinogenesis and intraepithelial clonal spread. Large areas of normal mucosa are replaced by a population of genetically altered cells of monoclonal origin (referred to as an expanding field), within such a field additional genetic hits may lead to the emergence of multiple genetically related subclones, and some cells eventually develops into cancer. Multiple oral tumors and second primary tumors are a major problem in head and neck oncology.[10] The World Health Organization predicts a continuing worldwide increase in the incidence of oral cancer, extending this trend into the next several decades.[5]

The concept of a two-step process of cancer development in the oral mucosa, from the initial presence of a precursor (pre-malignant, pre-cancerous) lesion subsequently developing into cancer, is well established. The main purpose of identifying oral premalignant lesions is

Table 1: Comparison of cytomorphometric parameters in various groups with normal buccal mucosa

Groups	NA (μ^2)	CA (μ^2)	ND	CD	N/C area ratio
Group I	76.20±6.00	3177.3±288.1	8.60±0.67	50.1±2.8	0.02±0.01
Group II	93.24±3.47	2179.7±211.3	9.89±0.99	41.57±1.77	0.04±0.01
Group III	112.02±8.51	1001.1±76.6	11.03±1.01	39.38±1.78	0.11±0.01
ANOVA, *F*	111.1, *P*<0.001	468.8, *P*<0.001	22.9, *P*<0.001	96.7, *P*<0.001	521.2, *P*<0.001

One-way ANOVA. ANOVA: Analysis of variance

to prevent malignant transformation by initiating adequate intervention. A precancerous lesion has been defined as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart.[11,12] Leukoplakia is the most common premalignant, potentially malignant or precancerous lesion of the oral mucosa. It is not known how many oral squamous cell carcinomas arise from precursor lesions and how many develop from apparently normal oral mucosa. Indian house-to-house survey showed, about 80% of oral cancers were preceded by oral pre-cancerous lesions or conditions.[13] Others consider the vast majority of oral cancers to arise from otherwise clinically normal mucosa.[14]

The automated instruments, capable of objective and quantitative cell analysis, have been used in descriptive morphology, for the assessment of tumor cell heterogeneity. Diagnostic and prognostic techniques are continually being developed and refined to detect cancer in its early stages. It is believed that the detection of oral cancer tumors when they are small provides an opportunity for less invasive treatment, thus improving the patient's quality of life and contributing to a better prognosis. Cytology is a simple and relatively pain-free procedure, which can be carried out repeatedly with minimum discomfort to patients. If its accuracy could be enhanced then it could provide a valuable adjunct to clinical evaluation of lesions. The most common stain used for the cytomorphological assessment of cells present in cytological smears is the Papanicolaou stain.[15,16]

The advantage of this stain is dehydration and clearing solutions helps in causing cellular transparency. This detects the overlapped cells better, which otherwise would be confused for a giant cell or bi/multinucleated cell. The second advantage being differential staining for different degrees of differentiation, green-blue cytoplasm for basal cells and yellow-orange for spinous or granular cell with the stability of the stains over long periods, stability of color and better reproducibility.[17]

Of late interest has turned toward applying sophisticated technique of computer-assisted cytomorphometry to investigate the nuclear and cellular changes. The results have been more reliable, objective and reproducible. Many investigators have evaluated the use of nuclear morphometry for grading and for predicting prognosis in esophageal, laryngeal, renal, bladder, breast, prostrate and colonic carcinomas.[18]

The length and size estimates made by microscopists (human eye) are neither accurate nor highly repeatable. Quantitative cytomorphometric analysis offers an accurate and precession comparison that is not achievable by human eye. It is the purpose of all types of image quantitation to eliminate observer-to-observer variation and produce evaluations that are accurate and repeatable.[18]

Image analysis software also permits the creation of "scripts" or "macros." A significant advantage of defined scripts is that they ensure that a particular type of measurement is performed in an identical manner each time it is performed. Scripts also ensure that data are collected in precisely the same manner regardless of who performs the analysis.

CONCLUSION

Digital image quantification has numerous potential advantages, including improved objectivity and consistency, enhanced sensitivity, and shorter turnaround times, especially for projects in which measurements can be automated. Additionally, it provides a permanent record of the data and of data collection, produces results that are highly amenable to statistical analysis, and allows the user to collect data that would otherwise be difficult or impossible to obtain (for example, area measurements of irregular things).[18]

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Conflicts of interest

There are no conflicts of interest.

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