

DIAGNOSIS OF ORAL PREMALIGNANT AND MALIGNANT LESIONS USING CYTOMORPHOMETRY

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INTRODUCTION

Oral cancer is a major health problem in South East Asia including Sri Lanka (1). A large majority of oral cancers are squamous cell carcinomas (2). Many oral carcinomas arise within regions that previously had premalignant lesions (1, 4). The most common premalignant lesion seen in the oral cavity is leukoplakia with associated dysplasia.

Early diagnosis and prompt treatment of early malignant lesions offer the best hope of improving the prognosis in patients with oral squamous cell carcinoma. Currently, biopsy is the diagnostic test routinely carried out for diagnosing oral premalignant and malignant lesions.

Oral exfoliative cytology is a simple and non-invasive diagnostic technique that could be used for early detection of oral premalignant and malignant lesions. It has been used for this purpose in other centres but has not gained wide acceptance due to the occurrence of false negative results (5-8). Analysis of the histopathological findings on an objective basis, using stereology and computer aided analysis gave encouraging results in the detailed diagnosis of oral premalignant and malignant lesions (9 -11). Cowpe et al advocated the idea of using morphometry for enhanced diagnosis of oral lesions using cytological techniques (12, 13). The measurements of nuclear and cytoplasmic area combined with nuclear DNA assessment have shown to improve the diagnostic sensitivity of oral Cytology (14). Our own study using nuclear and cell diameter values has shown that significant changes occur in these parameters in lesions with dysplasia and malignancy.

This study was carried out to determine whether oral exfoliative cytology with morphometric analysis of cells was, useful in detecting dysplasia and malignancy. Our study was confined to leukoplakia and oral squamous cell carcinoma.

METHODS

The study group consisted of patients with oral leukoplakia and squamous cell carcinoma of the buccal mucous membrane who required a biopsy for diagnosis of their lesions. Prior approval for this study was obtained from the Research and ethical review committee of the University of Peradeniya. Informed consent was obtained from all patients to obtain a cytological smear and a blood sample. Forty volunteers (20 males and 20 females) with normal buccal mucosae served as the control group.

All patients used in this study were over the age of 40 years. Each patient underwent routine venepuncture to determine the haemoglobin levels. The patients found to be anaemic (ie : female patients with the haemoglobin concentration of less than 12 g/dl and male patients with less than 13 g/dl) were excluded from this study (16).

Scrapings of the lesions were obtained by using a standard wooden tongue spatula moistened with normal saline. The entire lesion was scraped if possible. If not, a representative area was scraped. In cases where a heavy keratinized surface was present, fissured or reddish areas were scraped to obtain the sample. The scrapings were smeared on two plain glass slides and fixed immediately in 95% methyl alcohol for one hour and stained with the Papanicolaou stain. This was followed by a biopsy of the lesion.

Measurements were made using an eyepiece graticule (Graticule Ltd, England). The calibrated eyepiece graticule (x 10 magnification) was superimposed on the cytological smear slide and direct measurements of the individual epithelial cells were made blinded to the underlying histopathology of the lesion. All measurements were done using the oil immersion objective

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of x 100 magnification. In all cases the diameter of the nucleus (ND) and the cell (CD) were measured in both the long and short axes in micrometers. Only clearly defined cells were measured, avoiding clumped or folded cells and unusually distorted nuclei and cells. 100 cells were measured in each smear and the mean values of the ND and CD were obtained for each case.

Biopsies of the oral lesions were subjected to histopathological examination by the first and third authors, blinded to the cytomorphometrical readings of the smears. Each lesion was classified as non-dysplastic, dysplastic or squamous cell carcinoma. Epithelial dysplasia was diagnosed according to the criteria outlined by the WHO (17). When there was disagreement, concordance was reached following consultation. The normal mucosa was not biopsied for ethical reasons.

Smear samples were grouped for cytomorphometrical analysis.

- * Normal controls constituted the Group 1.
- The test smear samples were grouped according to their histopathological diagnosis as :
- * lesions with no dysplasia - Group 2,
- * lesions with dysplasia - Group 3 and,
- * squamous cell carcinoma - Group 4.

The ND and CD values obtained in each of the above groups were displayed as scattergrams for the combination of the following groups, Groups 1 and 2, 1 and 3, 1 and 4 and 2 and 3. In the scattergram, the ND values were plotted against the CD values for each sample in the groups. The ND and CD values were subjected to linear discriminant analysis as quoted by Morrison in order to discriminate the different groups (18). From the discriminant analysis, equations were obtained for the discriminant lines. The discriminant lines were superimposed on the scattergrams. In order to assess the diagnostic value of oral cytology in separating dysplastic lesions from non-dysplastic lesions (Group 3 and 2), the samples classified according to discriminant analysis were subjected to evaluation in terms of sensitivity, specificity, positive predictive value and negative predictive value.

RESULTS

In this study smears were collected from 150 lesions clinically diagnosed as oral leukoplakia or squamous cell carcinoma. These samples consisted of 124 males

and 26 females. Comparison of the males and females by a t test showed no significant difference in the nuclear diameter and cell diameter for the normal control group.

Therefore, the samples obtained from the lesions of both males and females were pooled for the analysis. Of the 150 samples, smears obtained from 5 lesions were inadequate and 9 lesions had only anucleated squames. Therefore only 136 smears were suitable for the cytomorphometric study. The study groups and the number of samples are given in Table 1. The means of ND and CD obtained from different groups are displayed as scattergrains (Figures 1, 2, 3 and 4).

Figure 1 : Scattergram displaying the ND vs CD values for cells collected from normal (Group 1) and lesions with no dysplasia (Group 2)

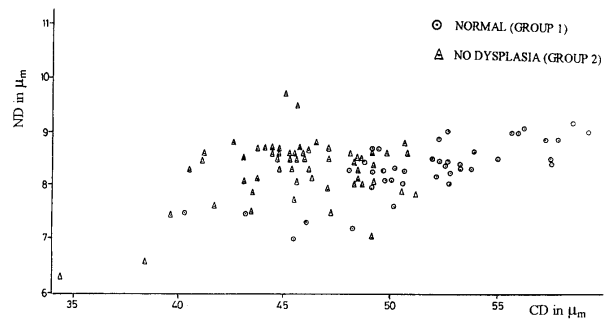


Figure 2 : Scattergram displaying the ND vs CD values for cells collected from normal (Group 1) and lesions with dysplasia (Group 3)

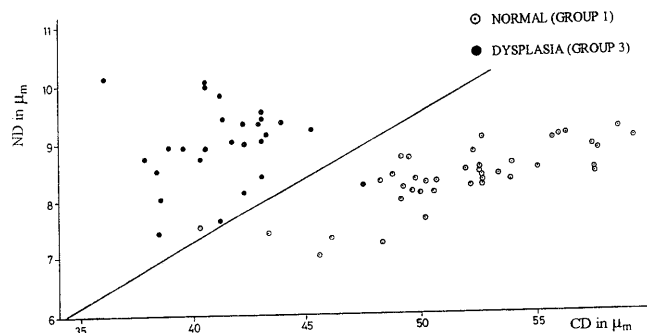


Figure 3 : Scattergram displaying the ND vs CD values for cells collected from normal (Group 1) and squamous cell carcinoma (Group 4)

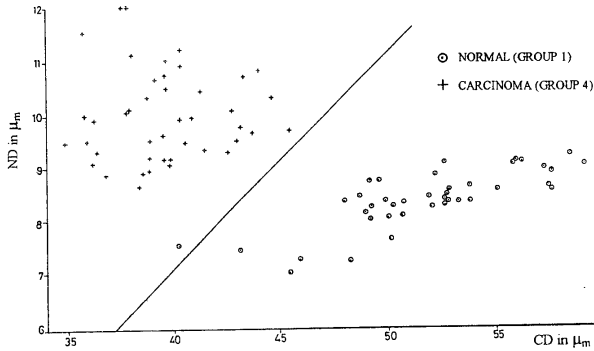
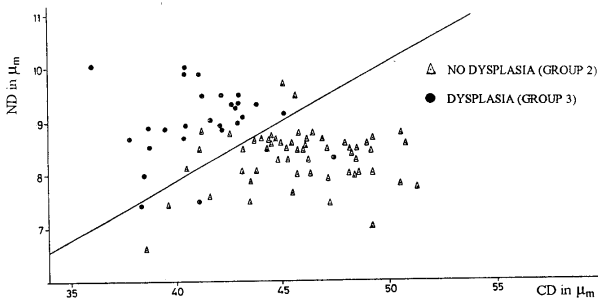


Figure 4 : Scattergram displaying the ND vs CD values for cells collected from lesions with no dysplasia (Group 2) and lesions with dysplasia (Group 3)



Scattergrams and discriminant analysis

Normal controls (group 1) and lesions with no dysplasia (group 2).

The scattergram of ND vs CD values for normal samples and lesions with no dysplasia is shown in Figure 1. Although, there was a shift of the values obtained for the lesions with no dysplasia (Group 2) to the left when compared with the normal samples (Group 1), the values obtained from the lesions did not show a clear separation from the normal in the scattergram. When the discriminant analysis was carried out, 6 normal samples displayed values similar to those obtained from the non-dysplastic lesions. Twelve smears from non-dysplastic lesions displayed values similar to that of normal control smears. It was observed that the discrimination between these two groups based on ND and CD values was not possible.

Normal controls (group 1) and lesions with dysplasia (group 3)

The scattergram of ND vs CD values displayed for the lesions with dysplasia showed a marked shift of the values to the left when compared with the normal samples (Fig. 2).

When the discriminant line obtained from the discriminant analysis was superimposed on the scattergram, it showed that the values of normal samples were lying below the line and the values obtained from dysplasia were lying above the line. Two samples (ie : one smear obtained from normal buccal mucosa and one from lesion with dysplasia) were misclassified. The separation between the normal samples and lesions with dysplasia was significant ($p < 0.001$). The discrimination between the two groups is given by the equation: $W = 1.35 CD - 6.09 ND - 9.62$. When the ND and CD values are inserted in this equation for new lesions, if W is found to be greater than zero ($W > 0$), then the oral mucosa of that individual is considered to be normal. In other words, if W is less than zero ($W < 0$), then the lesion is considered as having dysplasia.

Normal controls (group 1) and squamous cell carcinoma (group 4)

The scattergram of ND vs CD values displayed for the lesions with squamous cell carcinoma showed a well marked shift of the values to the left when compared with the normal samples (Fig. 3). Only one sample obtained from the normal buccal mucosa was misclassified with lesions of squamous cell carcinoma. The separation between the normal samples and squamous cell carcinoma was significant ($p < 0.001$). The discrimination between the two groups is given by the equation :

$$W = 0.92 CD - 2.29 ND - 20.57$$

When the ND and CD values are inserted in this equation for new lesions, if the weight (W) is less than zero ($W < 0$), then the lesion is considered as squamous cell carcinoma.

Lesions with no dysplasia (group 2) and lesions with dysplasia (group 3).

The scattergram of ND vs CD values displayed for the lesions with no dysplasia and dysplasia is given in Figure 4. When the discriminant line obtained from the discriminant analysis was superimposed on the scattergram, nine samples (ie : 3 smears obtained from lesions with dysplasia and 6 from lesions with no dysplasia) were misclassified.

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The separation between the lesions, with no dysplasia and lesions with dysplasia was significant ($p < 0.001$). The discrimination between the two groups is given by the equation: $W = 0.63 \text{ CD} - 2.74 \text{ ND} - 3.68$.

When the ND and CD values are inserted in this equation for new lesions, if W is found to be less than zero ($W < 0$), then the lesion is considered as having dysplasia. In other words, if W is greater than zero ($W > 0$), then the lesion is considered as having no dysplasia.

Of the 27 lesions with dysplasia analysed in this study, three were misclassified with the non-dysplastic lesions giving a false negative, results of 11.1 % of the 58 non-dysplastic lesions, 6 were misclassified with the dysplastic lesions giving a false positive results of 10.3%. The cytological diagnosis based on cytomorphometry and discriminant analysis achieved a sensitivity of 89% and specificity of 89.7%. A positive predictive, value of 80% and negative, predictive value of 94.5% were obtained for dysplastic lesions.

DISCUSSION

When the scattergram was plotted for normal control samples and leukoplakia with no dysplasia there was a shift of values to the left in the latter group when compared with the normal samples. Similar finding was observed by Cowpe et al in their scattergram of nuclear area values against the cytoplasmic areas values (14). The discrimination between these two groups based on ND and CD values was not possible. Therefore it was concluded that these two groups are similar cytomorphometrically and cannot be discriminated easily. Although the histology of non-dysplastic lesions show superficial keratinization and hyperplasia, their cellular features are similar to that of normal epithelial cells and the cytomorphometric measurements also agree with this.

Promising results were obtained in the scattergrams plotted for normal and dysplastic lesions and also for normal and squamous cell carcinoma. There was a marked shift of values to the left in the cases of epithelial dysplasia and squamous cell carcinoma and they displayed the ND and CD values above the discriminant line. This shows that the cells obtained from the lesions with dysplasia and squamous cell carcinoma could be easily discriminated from the

normal cells. The lesions which displayed ND and CD values above the discriminant line would always be subjected to biopsy for the confirmation of the diagnosis.

In this study, when the ND and CD values of non-dysplastic lesions and dysplastic lesions were subjected to discriminant analysis, it gave a false negative results of 11.1 % and false positive results of 10.3%. The 3 lesions that gave false negative results histopathologically showed mild epithelial dysplasia. This shows that the lesions with mild epithelial dysplasia may fall into the non-dysplastic group. The lesions that lie above the discriminant line need histopathological investigation and follow up. A few of the non-dysplastic lesions (6 lesions) displayed ND and CD values above the line, giving false positive results. However, much more serious are the false negative values, where 3 of the dysplastic lesions were misclassified with the non-dysplastic lesions (negative predictive value 94.5%). This suggest that lesions which displayed ND and CD values close to the discriminant line in the scattergram also referred to as grey area need to be followed up and if necessary biopsied.

The present study on quantitative exfoliative cytology gave a sensitivity of 89%, specificity of 89.7% and the positive predictive value of 80%. Very low sensitivity of 37% was reported by Dabelsteen et al in a qualitative assessment of oral smears obtained from dysplastic lesions (5). Shklar et al. could not detect the dysplastic changes in cytological smears obtained from 21 lesions of dysplasia in a qualitative assessment (19). As opposed to these finding based on qualitative assessment, the present study of quantitative assessment of oral smears shows high sensitivity.

Cowpe et al. reported 9% of false negative results in a quantitative study with the measurements of nuclear and cytoplasmic area (14). More recently, Brickley et al carried out a study using a computer simulated neural net work to discriminate between normal, non-dysplastic, dysplastic and malignant oral smears based on nuclear and cytoplasmic area values (20). A sensitivity of 76% and specificity of 82% was achieved when comparing normal and non-dysplastic lesions with dysplastic and malignant lesions. The network trained to differentiate non-dysplastic lesions from, dysplastic and malignant lesions showed a sensitivity of 88%. This is similar to our findings.

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In the present study the cells were separated using discriminant analysis based on ND and CD. There was no significant variation in ND and CD values between males and females. In a study using nuclear and cytoplasmic areas Cowpe et al. reported no significant difference between males and females (21). There are conflicting reports about the factors such as age, anaemia, tobacco habits and radiotherapy which might influence the nuclear and cell sizes in normal oral mucosa. These factors could also affect the cells obtained from the oral lesions (21-28). Therefore, it is important to consider these factors when assessing abnormal oral smears.

It has been shown that the nuclear area varies with age in those under 40 years of age, there being no significant difference in nuclear and cell sizes above this age. All the patients included in this study were over 40 years of age. The effect of anaemia on nuclear and cell sizes is not conclusive (22-24). All the subjects included in this study were non-anaemic. Previous studies suggest that, in normal buccal mucosa smoking tobacco does appear to influence the nuclear size (25, 26). It was found that in oral cancer patient's tobacco was not responsible for changes in cell size (27). Most of the patients with leukoplakia, and squamous cell carcinoma had history of tobacco habits either in the form of smoking or chewing or both and it was difficult to control this factor in this study. Increase in nuclear and cell areas were reported in normal buccal cells up to 6 weeks following radiotherapy (28). Smears used in the present study were collected from the oral cancer patients before any treatment started. Therefore, this factor would not have affected the parameters analysed in this study.

These results show that the application of cytomorphometry to oral smears would increase the diagnostic value of oral cytology. There were difficulties in obtaining a representative sample from some of the lesions, as the smears obtained from 5 lesions were

inadequate and 7 lesions, presented with only anucleated squames. Repeat smears were not obtained in this study and further more even if repeated the smears of the hyperkeratotic lesions would give anucleated squames. For these lesions biopsy is the only diagnostic test of choice. There is no doubt that histological assessment remains the accepted method of diagnosis. The cytological procedure is a simple and non-invasive technique that would be a very useful screening procedure. It could also be used in follow up procedures in treated oral cancer patients to detect recurrences (29, 30) and in extensive lesions to choose the site for biopsy.

CONCLUSIONS

Cytomorphometric analysis of smears from buccal lesions was useful in differentiating dysplastic and malignant squamous cells from normal squames using discriminant analysis based on ND and CD values. A sensitivity of 89%, specificity of 89.7%, positive predictive value of 80% and negative, predictive value of 94.4% were achieved, when comparing non-dysplastic lesions with dysplastic lesions.

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Table 1 : Study groups according to their histopathological diagnosis

Groups	Histology	No of cases
1	Normal	40
2	Lesions with no dysplasia	58
3	Lesions with dysplasia	27
4	Squamous cell carcinoma	51
Total		176

SUMMARY

In this study cytomorphometry has been applied to smears collected from the buccal mucosa. Normal cells and the cells collected from lesions with no dysplasia, dysplasia and squamous. cell carcinoma were differentiated using discriminant analysis based on nuclear and cell diameter values. Cytomorphometrically the dysplastic and malignant cells were well discriminated from the normal cells. A sensitivity of 89%, specificity of 89.7%, positive predictive value of 80% and negative predictive value of 94.4% were obtained when comparing non-dysplastic lesions with dysplastic lesions.

Key words : Cytomorphometry, oral cancer, oral exfoliative cytology, oral leukoplakia, oral premalignant lesion.

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