

Cytomorphological analysis in oral squamous cell carcinoma lesions and normal controls using rub and rinse technique

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ABSTRACT

Context: Early diagnosis of oral cancer requires simple noninvasive screening tools. **Aim:** To analyze the cytomorphological features of keratinocytes in smears obtained from the oral mucosa of oral squamous cell carcinoma (OSCC) lesions and normal controls using oral rub and rinse technique. **Settings and Design:** Oral smears were prepared using oral rub and rinse method in subjects with OSCC cases ($n = 35$) and apparently healthy normal controls ($n = 35$). They were subjected to cytomorphometric analysis. **Materials and Methods:** The smears prepared with the rinse method were stained with Papanicolaou stain. Quantitative assessment of nuclear diameter (ND), cytoplasmic diameter (CD), cellular area (CA), nuclear area (NA), and nuclear cytoplasmic ratio (N:C) was carried out. **Statistical Analysis Used:** Unpaired Student's *t*-test was used to compare the mean value between the groups. **Results:** There was a significant difference between ND, CD, CA, NA, and N: C of oral cancer cells and that of the normal controls. There was increase in the mean ND, NA, and N: C; and decrease in CA and CD of cancer subjects when compared to that of normal controls. **Conclusion:** Cytomorphometric analysis of keratinocytes obtained with oral rinse method can serve as a useful adjunct in the early diagnosis of OSCCs.

Key words: Cytomorphometry, exfoliative cytology, oral cancer, oral rinse

INTRODUCTION

Oral cancer is currently the sixth most common malignancy in the world.^[1] In India, it is the most common malignancy among men and one of the five most common malignancies among women.^[2] Despite advances in surgery, radiotherapy, and chemotherapy; the five year survival rate of oral squamous cell carcinoma (OSCC) patients have remained unchanged at approximately 50%.^[3] This poor survival rate can be attributed mainly to the lack of early detection and treatment. Thus, early

detection of the lesion is the key aspect in controlling oral cancer. Oral biopsy is invasive and involves both psychological implications for the patient and technical difficulties for the health practitioner.

Oral exfoliative cytology is examining desquamated cells from the surface of the oral mucosa. Miller *et al.*, were the first to study the cytology of the normal oral epithelium.^[4] Alterations in the epithelial cells serve as reliable indicators of dysplastic or neoplastic changes. Montgomery and his associates applied the principle of exfoliative cytology for the diagnosis of oral cancer.^[5]

Ideally, a diagnostic procedure should be neither time-consuming nor complicated and, in addition to high sensitivity, should have the potential for automation. The oral cytology technique is one such simple, noninvasive, relatively painless technique tolerated well by patients. It can be used for diagnosis and identification of recurrent potentially malignant and malignant lesions.^[6] Cytology

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optimally meets all the requirements, particularly when it is supplemented by an adequate image analysis method.^[7]

However, conventional exfoliative cytology has failed to gain popularity among the private dental practitioners revealed by the fact only 10% of all dentists had ever done an oral cytology smear, only 42% were taught how to do a smear and that 96.9% of dental offices lacked necessary materials.^[8] Hence, a simpler alternative to the conventional exfoliative cytology may prove to be a more practical solution. As diagnostic fluid, oral rinse offers distinct advantage as it can be collected noninvasively by individuals with modest training and with minimum armamentarium in contrast to the conventional exfoliative cytology.

In the present study, keratinocytes were obtained using oral rub and rinse method, and were subjected to cytomorphometric analysis. The Papanicolaou stain imparts a different color to the cytoplasm of the epithelial cells based on their degree of cellular differentiation;^[9] hence, used in this study for cytological evaluation of oral keratinocytes. The cytomorphometric features of keratinocytes obtained through oral rinse from OSCC lesion and normal controls were analyzed and compared.

MATERIALS AND METHODS

Thirty-five histologically confirmed cases of OSCC patients and 35 age- and sex-matched controls were selected for the study from the Department of Oral Medicine, A. B. Shetty Memorial Institute of Dental Sciences, Deralkatte.

The study protocol was approved by the Committee on Ethics of the Nitte University, Mangalore, Karnataka, India. Patients were informed with regard to the research objectives, methods, possible benefits and potential risks, and a written consent was obtained from all participants.

The case group included 35 patients with squamous cell carcinoma in the oral cavity. They were diagnosed according to the World Health Organization (WHO) classification of tumors, which describes it as a malignant epithelial tumor with squamous cell differentiation, presenting microscopically with cells that resemble keratinocytes, intercellular bridges, and/or keratinization.^[10] None of the selected patients had started treatment and none presented with other neoplasia elsewhere. History and physical examination were performed. The latter included careful examination of the oral cavity and was followed by smear preparation using oral rinse technique in previously diagnosed cases of squamous cell carcinoma, in the case group, and of the clinically normal areas, in age- and sex-matched control group.

Operational definitions/Techniques employed

Oral rub and rinse technique was used to collect oral cells. Patient was asked to swish his/her mouth with water and expectorate. Then, the suspected oral lesion was rubbed on firmly by the clinician or by the patient himself using their tongue in for at least 30 s. While swishing phosphate buffered saline (PBS), pH 7.2 was used and patient was asked to expectorate into a sterile container. Once the sample was obtained, it was labeled and centrifuged at 1,000 rpm for 5 min. Supernatant fluid was discarded and cells were collected with a micropipette and smears prepared.

All the slides were immediately fixed in absolute alcohol and consequently stained with Papanicolaou stain. Two smears were prepared for each of the cases and controls.

Cytomorphometric analysis

All the stained smears were observed under a research light microscope. Each smear was assessed using the Motic Microscope (Motic image analyzing system, 2003, 1.3 version) with a $\times 40$ objective lens. Fifty randomly selected cells were measured in a stepwise fashion. Cytoplasmic diameter (CD), nuclear diameter (ND), cellular area (CA), and nuclear area (NA) were measured. CD and ND were measured drawing a line with a digital cursor from one axis to the other along both X- and Y-axis. The average of X- and Y-value was taken as final diameter. For measurement of CA and NA, the nucleus and cell outline was traced on the screen and the software automatically calculated the area. Nuclear cytoplasmic ratio (N: C) was calculated by subtracting NA from the cytoplasmic area and further dividing the NA by the subtracted area. For repeatability, 10% of the slides were reevaluated by the researcher. For reproducibility, 10% of the smears were evaluated by a qualified oral pathologist.

Statistical analysis

Since this study involved two groups, unpaired *t*-test was used to compare the means between the two groups. The $P < 0.05$ was considered to be significant.

RESULTS

The study included 70 subjects of which 82.85% were males and 17.14% were females. History regarding use of tobacco/areca nut products and alcohol are listed in Table 1. Quantitative analysis of the cellular parameters showed a significant difference between ND, CD, CA, NA, and N: C of oral cancer and that of the normal controls [Table 2]. There was increase in ND, NA, and N: C and decrease in CA and CD of cancer subjects when compared to that of normal controls [Table 2]. All the measurements were in microns. The mean value of the ND (10.098 ± 0.651), nuclear area (352.779 ± 50.568), and N: C (0.0417 ± 0.007)

of keratinocytes in cancer group smears were higher when compared with those of the control group ND (9.426 ± 0.58), NA (274.6 ± 19), and N: C (0.02170 ± 0.004). Conversely, CD and CA in oral cancer group was 45.812 ± 4.820 and $8,765 \pm 1,345$ and in normal controls the mean values of CD and CA were 53.27 ± 4.48 and $12,893 \pm 1,829$, respectively.

DISCUSSION

Use of cytology as a screening tool for prevention and early diagnosis of oral cancer is well documented. Also, the computer-assisted exfoliative cytology (oral CDX) is considered as an accurate method for detecting premalignant and malignant buccal lesions. This is a computer-assisted method for the analysis of exfoliative cytology material, which adds to clinical examination in the differentiation of premalignant and malignant alterations found in “benign” lesions, the purpose being to find carcinomas of innocuous appearance, at the most curable and early stages.^[11] CDX brush is designed to make abrasions in the oral mucous membrane so that the dysplastic or malignant cells which are mostly present in the basal layer of epithelium will be caught up in the sample for cytologic analysis. But, few authors are of the opinion that oral cancer is a poor man’s

disease and the methods of diagnosis should be cheap and accurate and they have no doubt in their minds to say that the oral CDX brush biopsy is irrelevant for oral cancer detection in developing countries.^[12]

Routinely shed oral epithelial cells can be detected in saliva and oral rinses, making cytologic and molecular analysis of this fluid attractive for oral cancer screening. Oral rinse technique has been in use for long mainly for microbiological purposes, especially to analyze oral candidal colonization.^[13] The same technique was utilized to detect OSCC. The aberrant methylation of a combination of marker genes present in oral rinse samples was used to detect OSCC with > 90% sensitivity and specificity.^[14]

Epidemiological studies conducted earlier concluded that both a 10 ml oral rinse sample and 2 ml whole saliva sample provide sufficient DNA quantity and better quality DNA for genetic epidemiological studies than do the commonly used buccal swab and brush techniques.^[15] Since the present technique utilized phosphate buffer saline (PBS) for collection of cells, remaining cells may be stored and used later for advanced studies. In a randomized crossover study, which compared the deoxyribonucleic acid (DNA) yield, quality, and associated costs of buccal cell DNA collected using cytobrushes (three brushes per collection) and swish (i.e., mouthwash) in self-administered procedures, there was a nonstatistically significant higher yield from the mouthwash.^[16]

In the present study, we found that the mean NA and ND N:C value of keratinocytes was higher in OSCC lesions when compared with those from the mucosa of normal controls. Malignant squamous epithelial cells display a significant increase in mean NA.^[17] Some authors state that

Table 1: Characteristics of the subjects included in the study

Characteristic	Cancer cases	Normal controls
Male (%)	29 (82.85)	29 (82.85)
Female (%)	6 (17.14)	6 (17.14)
Smoker	18	-
Other tobacco/ areca nut products	31	-
Nonsmoker	12	35
Drinker	21	-
Nondrinker	8	35

Table 2: Comparison of the mean values of the nuclear area, nuclear diameter, cytoplasmic area, cytoplasmic diameter, and nuclear cytoplasmic ratio among the two groups

	Normal controls			Cancer cases			P value
	Mean	Standard deviation	Standard error	Mean	Standard deviation	Standard error	
ND	9.426 95% CI for mean difference: (-0.958, -0.39) t=-4.71, DF=65	0.538	0.091	10.098	0.651	0.110	<0.0001
CD	53.27 95% CI for mean difference: (5.24, 9.68) t=6.71, DF=67	4.48	0.76	45.812	4.820	0.815	<0.0001
NA	274.6 95% CI for mean difference: (-93.6, -55.9) t=-8.00 DF=42	19.0	3.2	352.779	50.568	8.548	<0.0001
CA	12,893 95% CI for mean difference: (3,362; 4,895) t=10.76 DF=62	1,829	309	8,765	1,345	227	<0.0001
N:C	0.02170 95% CI for mean difference: (-0.02282, -0.0171) t=-14.07 DF=58	0.00463	0.00078	0.0417	0.00701	0.0012	<0.0001

CI=Confidence interval, DF=Degrees of freedom, N:C: Nuclear cytoplasmic ratio, ND: Nuclear diameter, CD: Cytoplasmic diameter, NA: Nuclear area, CA: Cytoplasmic area

increase in the nuclear size is related to an increase in the nuclear contents required for replication and conversely decrease in the cytoplasmic size is related to diminished cytoplasmic maturity in active cells.^[18]

The mean value for the CA and cellular diameter of keratinocytes obtained from the mucosa of normal controls was significantly greater than those of OSCC lesions. Decrease in the cellular diameter and increase in the nuclear size are two significant changes that occur in actively proliferating cell.^[19] Accordingly, the study evidenced that with the progression from normal to malignancy, the nuclear dimensions increased and cellular dimensions decreased with a gradual increase in N:C.

Ogden *et al.*, suggested that quantitative techniques, based on the evaluation of parameters such as NA, cytoplasmic area (CA), and nucleus-to-cytoplasmic area ratio (NA/CA), may increase the sensitivity of exfoliative cytology for early diagnosis of oral cancers, since these techniques are precise, objective, and reproducible.^[20] Exfoliative cytology is capable of detecting malignant changes, through estimation of NA/CA using the planimeter method in Papanicolaou-stained smears. Their study, published in 1985, concluded that 50 cells were sufficient to provide indication of malignant changes.^[21]

Cytomorphometric techniques were used in another study, to assess ND and CD in normal oral mucosa, in dysplastic lesions and in squamous cell carcinomas.^[22] They found that CD was highest in normal mucosa, lower in dysplastic lesions, and lowest in SCCs. By contrast, ND was lowest in normal mucosa, higher in dysplastic lesions, and highest in SCCs. These studies suggested that reduced nuclear size and increased cytoplasmic size are useful early indicators of malignant transformation, and thus exfoliative cytology is of value for monitoring clinically suspect lesions and for early detection of malignancy.^[23]

In yet another study, oral smears were obtained from clinically normal appearing mucosa of OSCC patients and from the mucosa of smokers, and apparently healthy individuals were used as controls. They found statistically significant reduction in cytoplasmic area and increase in NA in cancer subjects when compared to normal controls, while there was a significant reduction in the CA of keratinocytes from OSCC lesion when compared with those from oral smears of tobacco users.^[24]

In summary, present study supports and extends the view that cytomorphometric evaluation of keratinocytes obtained from oral rinse can serve as a useful diagnostic screening aid for early detection of oral cancer. Evaluation of a greater

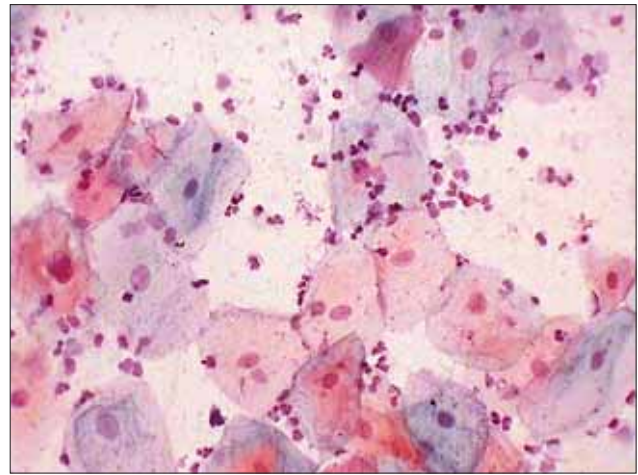


Figure 1: Photomicrograph of a smear prepared with oral rinse method (Papanicolaou stain, x400)

number of cases using oral rinse technique is essential to establish the cut-off values of these parameters so that they can be used as definitive indicators. The technique can be used as an easier alternative to exfoliative cytology as a first level test and is not a substitute for scalpel biopsy. It is also noticed that the smears thus prepared are superior to the smears of conventional exfoliative cytology [Figure 1]. They exhibit minimum overlapping of cells in a clear background aiding easy visualization of dysplastic cells. The collection of cells via oral rinse method being a simple, noninvasive technique can be used very efficiently in resource challenged areas.

CONCLUSION

Cytomorphometric analysis of smears obtained with oral rinse can serve as a convenient screening tool in detection of OSCC cases.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: 2000 Globocan. *Int J Cancer* 2001;94:153-6.
2. Boyle P, Macfarlans GJ, Maisonneuve P, Zheng T, Scully C, Tedsceo B. Epidemiology of mouth cancer in 1989: A review. *J R Soc Med* 1990;83:724-30.
3. National Cancer Institute. SEER Cancer Statistics Review: 1973-1998. Available from: http://www.seer.cancer.gov/csr/1973_1998/index.html. [Last accessed on 20-6-2013].
4. Miller SC, Soberman A, Stahl SS. Study of cornification of oral mucosa in young male adults. *J Dent Res* 1951;30:4-11.
5. Montgomery PW, von Hamm E. Study of exfoliative cytology in patients with carcinoma of the oral mucosa. *J Dent Res* 1951;30:308-13.
6. Moralis A, Kunkel M, Reichert TE, Kosmehl H, Driemel O. Identification of a recurrent oral squamous cell carcinoma by brush cytology. *Mund Kiefer Gesichtschir* 2007;11:355-8.
7. Remmerbach TW, Meyer-Ebrecht D, Aach T, Würflinger T, Bell AA, Schneider TE, *et al.* Toward a multimodal cell analysis

- of brush biopsies for the early detection of oral cancer. *Cancer* 2009;117:228-35.
8. Horowitz AM, Drury TF, Goodman HS, Yellowitz JA. Oral pharyngeal cancer prevention and early detection. Dentists' opinions and practices. *J Am Dent Assoc* 2000;131:453-62.
 9. Sivapathasundharam B, Kalasagar MY. Yet another article on exfoliative cytology. *J Oral Maxillofac Pathol* 2004;8:54-7.
 10. MacDonald DG. Tumors of the Oral Cavity. In: Fletcher CD, editor. *Diagnostic Histopathology of Tumors*. London: Churchill Living Stone; 2000. p. 209-30.
 11. Sciubba JJ. Improving detection of precancerous and cancerous oral lesions. *JADA* 1991;130:1445-56.
 12. Babu M, Sayed MN. The relevance of CDX brush biopsy in diagnosing early oral cancer in developing countries. *Oral Maxillofac Pathol J* 2011;2:115-6.
 13. Coulter WA, Murray SD, Kinirons MJ. The use of a concentrated oral rinse culture technique to sample oral candida and lactobacilli in children, and the relationship between candida and lactobacilli levels and dental caries experience: A pilot study. *Int J Paediatr Dent* 1993;3:17-21.
 14. Nagata S, Hamada T, Yamada N, Yokoyama S, Kitamoto S, Kanmura Y, *et al.* Aberrant DNA methylation of tumor-related genes in oral rinse: A noninvasive method for detection of oral squamous cell carcinoma. *Cancer* 2012;118:4298-308.
 15. King IB, Satia-Abouta J, Thornquist MD, Bigler J, Patterson RE, Kristal AR, *et al.* Buccal cell DNA yield, quality, and collection costs: Comparison of methods for large-scale studies. *Cancer Epidemiol Biomarkers Prev* 2002;11:1130-3.
 16. Rogers NL, Cole SA, Lan HC, Crossa A, Demerath EW. New saliva DNA collection method compared to buccal cell collection techniques for epidemiological studies. *Am J Hum Biol* 2007;19:319-26.
 17. Cowpe JG. Quantitative exfoliative cytology of normal and abnormal oral mucosal squames: Preliminary communication. *J R Soc Med* 1984;77:928-31.
 18. Frost JK. Pathologic processes affecting cells from inflammation to cancer. In: Bibbo M, editor. *Comprehensive Cytopathology*. 2nd ed. Philadelphia: WB Saunders Company; 1997. p. 845.
 19. Cañado RP, Yurgel LS, Filho MS. Comparative analysis between the smoking habit frequency and the nucleolar organizer region associated protein in exfoliative cytology of smokers' normal buccal mucosa. *Tob Induc Dis* 2004;2:43-9.
 20. Ogden GR, Cowpe JG, Wight AJ. Oral exfoliative cytology: Review of methods of assessment. *J Oral Pathol Med* 1997;26:201-5.
 21. Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of normal oral squames: An age, site and sex related survey. *J R Soc Med* 1985;78:995-1004.
 22. Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of abnormal oral mucosal smears. *J R Soc Med* 1988;81:509-13.
 23. Ramaesh T, Mendis BR, Ratnatunga N, Thattil RO. Cytomorphometric analysis of squames obtained from normal oral mucosa and lesions of oral leukoplakia and squamous cell carcinoma. *J Oral Pathol Med* 1998;27:83-6.
 24. Khandelwal S, Solomon MC. Cytomorphological analysis of keratinocytes in oral smears from tobacco users and oral squamous cell carcinoma lesions - A histochemical approach. *Int J Oral Sci* 2010;2:45-52.

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