ABSTRACT

Aims: To determine the cellular and nuclear area of keratinocytes in smears obtained from the oral mucosa of tobacco users, those with oral squamous cell carcinoma (OSCC), and from normal healthy persons and resolve if any significant difference exists in these three groups.

Materials and Methods: The study group comprised 100 subjects 20 controls, (40 OSCC patients-20 from lesional sites and 20 from nonlesional sites, 20 tobacco smokers and 20 tobacco chewers) in the age group of 25-75 years. Oral mucosal smears obtained by using a cytobrush were stained with Papanicolaou (PAP) stain and using 20X objective in trinocular Olympus model BX53 with Jenoptik scientific grade-dedicated microphotographic camera images were taken. With ProgRes version 8.0 image analysis software, 20 cells with defined borders were evaluated from each slide. Finally, one-way analysis of variance (ANOVA) was used to compare the above parameters in the studied groups.

Statistical Analysis Used: Minitab and Excel software were used to analyze the data. One-way ANOVA was used to compare the above parameters in the studied groups.

Results: The mean value of the cell area for groups I, II, III, IV, and V were 2838 ± 275.2, 2762.1 ± 511.4, 2861.9 ± 512.9, 2643.8 ± 333.3, and 3064.3 ± 362.7, respectively, the nuclear area (NA) was 83.88 ± 9.86, 106.19 ± 13.45, 95.11 ± 14.24, 85.55 ± 21.11, and 80.83 ± 13.45, respectively, and nuclear-to-cellular (N:C) ratio was 0.0297, 0.03924, 0.0337, 0.03257, and 0.02678, respectively.

Conclusions: Thus, our study elucidates that cytomorphology gauges the effect of tobacco on the oral mucosa and possibly establishes a link between premalignant and malignant transformations even before a lesion is visibly noted.

Key words: Cellular area (CA); exfoliative cytology; nuclear-to-cellular (N:C) ratio, nuclear area (NA); oral squamous cell carcinoma (OSCC)

Introduction

The oral cavity is known to be a miniature screen of the whole body reflecting the total health and adverse habits of an individual. Most commonly encountered habits are smoking and quid chewing with an insight into the genesis of oral cancer suggesting a causative role of tobacco. The etiology of oral squamous cell carcinoma (OSCC) is multifactorial but the use of tobacco still continues to be a prevalent risk factor.[1] The attributable risk of tobacco for oral cancer development in men is 90% and in women, 59%.[2] Oral exfoliative cytology.

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Udayashankar U, Guduru VS, Ananthaneni A, Ramisetty SD, Kuberappa PH, Namala S. Evaluation of cytomorphometric changes in tobacco users and diagnosed oral squamous cell carcinoma individuals. J Cytol 2016;33:125-9.
which is a quick, simple, less technically demanding, painless, noninvasive yet quite a simple laboratory procedure that can be repeated frequently with little discomfort to patients, with a vital role in diagnosis in the study of Nayar et al. Previous studies show a reduction in cell diameter and increase in nuclear diameter which could be used as an early indicator of malignant transformation. Using this contemplation, the present study has been conducted to evaluate the cytomorphometric changes of buccal mucosal cells in tobacco users and OSCC patients and to study the morphometric differences between the lesional and non-lesional exfoliated cells. Hence, nuclear area (NA), cellular area (CA), nuclear to cellular ratio of keratinocytes from the oral smears were evaluated and the values of each group with that of the normal were compared and the differences if any in these parameters, were analysed.

Almost 95% of the cancers in the head and neck region are squamous cell carcinomas (SCCs) and 30-40% of malignant neoplasms in the oral cavity are OSCCs, which is known for early metastasis and high rates of locoregional recurrence. Previous studies using exfoliative cytology based on nuclear and cellular alterations were able to differentiate dysplastic and malignant cells from normal ones, and also detect tissue alterations associated with tobacco habits in the oral cavity.

**Materials and Methods**

The study group consisted of 100 subjects in total who were divided into 5 groups:

1. Group I: 20 healthy subjects without any tobacco consumption habit and any other systemic disease.
2. Group II: 20 histopathologically diagnosed OSCC patients (Smears taken from lesional site).
3. Group III: 20 non-lesional sites of diagnosed OSCC patients (Smears taken from non-lesional site).
4. Group IV: 20 tobacco chewers.
5. Group V: 20 tobacco smokers.

**Methodology**

The present study was conducted in the Department of Oral Maxillofacial Pathology and Microbiology. A total of 100 cases were evaluated as per the routine protocol. The significance and method of the study were explained to each individual and an informed consent was obtained from all of them. Exhaustive clinical history with specific insight into tobacco habits was drawn from the study groups.

**Inclusion criteria**

1. Individuals with the habit of consuming tobacco in any form for more than 2 years in the age group of 25-60 years were taken.
2. Histopathologically diagnosed cases of OSCC are included in groups II and III.

**Exclusion criteria**

1. Individuals with known local/systemic disorders.
2. Medically compromised and immunocompromised patients.
3. Patients with anemia and blood dyscrasia.
4. Patients aged <20 years and >60 years.

General oral mucosal examination was done using a mouth mirror (CDC company). All the patients were asked to rinse their mouth with water prior to preparation of the smear. Photographs were taken for each case. Oral smears were taken using a cytobrush (AXIOM tm company, New Delhi). The exfoliated cells were transferred from the cytobrush onto the glass slide with firm pressure and the smears were made. All the slides were fixed in 95% alcohol for 24 h and stained using Papanicoloau (PAP) staining method. Using 20X objective in trinocular Olympus model BX53 with Jenoptik (Olympus UCMAD3, T7, Tokyo, Japan) scientific grade-dedicated microphotographic camera with ProgRes version 8.0 image analysis software (Progres Capture Pro 2.8.8, 2011, Germany), 20 cells with defined borders were evaluated from each slide. The cell and nuclear outlines were traced on the screen and the CA and NA were obtained automatically by the software in all the study groups [Figures 1 and 2a-d]. The nuclear-to-cellular ratio was given by dividing the NA by the CA. Minitab and Excel software version 16 were used to analyze the data. One-way analysis of variance (ANOVA) was used to compare the above parameters in the studied groups.

**Results**

The mean CA was found to be decreased in group II ($P = 0.562$) though insignificant and it was significant in group V ($P = 0.032$) but no significant increases in group III ($P = 0.856$) and group IV ($P = 0.052$) were present in comparison to the control group and when the groups were compared with each other, the mean CA significantly increased in group V when compared to groups II ($P = 0.038$) and IV ($P = 0.001$). There was no significant difference between group III and group V ($P = 0.158$), group IV and group II ($P = 0.391$), and group IV and group III ($P = 0.119$) [Table 1]. The mean NA increased in group II ($P = 0.0003$) and group III ($P = 0.006$) but no significant increase was seen in group IV ($P = 0.751$) in group V ($P = 0.417$) in comparison to control group and when comparison was done among the groups, there was a significant increase in group II when compared to groups IV ($P = 0.004$) and V ($P = 0.0002$) and it was insignificant compared to group III ($P = 0.066$). Also, a significant increase was seen in group III to group V ($P = 0.002$).
0.002) and no significant difference was seen in group III to group IV ($P = 0.102$) and group IV and group V ($P = 0.404$) [Table 2]. The NA-to-CA area ratio significantly increased in group II ($P = 0.001$) and group III ($P = 0.008$) with no significant difference in group IV ($P = 0.162$) but a significant decrease in group V ($P = 0.059$) in comparison to the control group. In comparison between the groups, a significant increase in group II to group III ($P = 0.021$), IV ($P = 0.017$), V ($P = 0.002$), and group V to groups III ($P = 0.003$) and IV ($P = 0.011$) was seen. There was no significant difference between group III and group IV ($P = 0.592$) [Table 3].

Discussion

OSCC is the sixth most common cancer worldwide, accounting for 90% of cancers in the oral cavity. Tobacco abuse has been proved to be the major risk factor in the development of OSCC. In its early stages, oral cancer may disguise itself and appear as an innocent lesion. Early detection of such premalignant or cancerous oral lesions promises to improve the survival and morbidity of patients suffering from these conditions. In the last few years, the interest for oral exfoliative cytology as a diagnostic and prognostic methodology for monitoring patients in oral precancer and cancer has increased. Miller et al. were the first to study the cytology of the normal oral epithelium.\(^9\) The superficial epithelial cells do contain nuclei, thus alterations in these cells can serve as reliable indicators of dysplastic or neoplastic changes. It is the nucleus that expresses the genotypic alterations caused in the process of malignancy. Quantitative cytomorphometric parameters such as the NAs, cytoplasmic areas, and the nuclear-to-cytoplasmic (N:C) ratio have shown significant alteration in the diagnosis of oral lesions. Ramesh et al. in a study have reported a positive correlation between the ND and CD for normal buccal mucosa and lesions with no epithelial dysplasia and an insignificant correlation in lesions with epithelial dysplasia and SCC lesions and hence, suggested that ND and CD could

---

Table 1: Summary statistics of mean cellular area and $P$ value between groups (area ± standard deviation in $\mu m^2$)

| Groups    | Normal          | Cancer          | Contra          | Quid            | Smokers         |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mean Cellular Area ± SD | 2838 ± 275.2 | 2762.1 ± 511.4 | 2861.9 ± 512.9 | 2643.8 ± 333.3 | 3064.3 ± 362.7 |
| Normal    | —               | —               | —               | —               | —               |
| Cancer    | $P = 0.562$     | —               | —               | —               | —               |
| Contra    | $P = 0.856$     | 0.542           | —               | —               | —               |
| Quid      | $P = 0.052$     | 0.391           | 0.119           | —               | —               |
| Smokers   | $P = 0.032$     | 0.038           | 0.158           | 0.001           | —               |

Table 2: Summary statistics of mean nuclear area and $P$ value between the groups (area ± standard deviation in $\mu m^2$)

| Groups    | Normal          | Cancer          | Contra          | Quid            | Smokers         |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mean nuclear area ± SD | 83.88 ± 9.86 | 106.19 ± 13.45 | 95.11 ± 14.24 | 85.55 ± 21.11 | 80.83 ± 13.45 |
| Normal    | —               | —               | —               | —               | —               |
| Cancer    | $P = 0.0003$    | —               | —               | —               | —               |
| Contra    | $P = 0.006$     | 0.066           | —               | —               | —               |
| Quid      | $P = 0.751$     | 0.004           | 0.102           | —               | —               |
| Smokers   | $P = 0.417$     | 0.0002          | 0.002           | 0.404           | —               |
be sensitive parameters in the diagnosis of oral premalignant and malignant lesions.\textsuperscript{10} Suneet Khandelwal \textit{et al} and Monica Charlotte Solomon \textit{et al} conducted a similar study and concluded that cytomorphometric analysis of keratinocytes can serve as a useful adjunct in the early diagnosis of OSCC.\textsuperscript{11} In another study, Hedge \textit{et al} have suggested that a decrease in the CD of premalignant lesions could serve as an early indicator of dysplastic changes, especially in lesions that appear to be histologically benign.\textsuperscript{11} Therefore, an attempt was made to identify the cytomorphometric features of keratinocytes that could signify an impending malignant transformation.

In the present study, when group II was compared to the control group, there was a decrease in the cell area though it was not significant, which was consistent with other studies;\textsuperscript{12} this could have been due to the diminished ability of the cytoplasm to mature in cells with increased activity and also, in actively proliferating cells the amount of cytoplasm present in relation to the nucleoplasm is less.\textsuperscript{13} When group III was compared to the control group, there was a mild increase in the cell area but it was not significant, which was in contrast to the study of Suneet \textit{et al}. This may have been due to early increased functional activity of the cytoplasm to cope with the demand of proteins required as a result of increased nuclear activity. When group IV was compared to the control group, there was a significant decrease in the cell area, which could be the first evidence for field change in oral cancer at the light microscope level and was consistent with Ogden \textit{et al}.\textsuperscript{14} When Group V was compared to the control group, there was an increase in the cell area, which was in consistency with the study of Hillman \textit{et al}.,\textsuperscript{15} which might have been due to temperature effect, production of carcinogenic hydrocarbons from destructive distillation of cigarette components, and carcinogens from unburnt cigarettes produced by pyrolysis. All these together stimulate the cells of the oral mucosa to undergo increased cellular activity.\textsuperscript{14} When comparing the mean cell area among the groups, a significant increase was seen in group V compared to group II and in group IV compared to group V. This reverse scenario could have been due to the additive effects of combustion products by tobacco, heat produced, and change in intraoral pH in smokers.\textsuperscript{17} There was no significant difference between group III and group V as the field changes in group III might have been still occurring at the molecular level and hence, cytomorphological cellular changes are not evident. There was no significant change in the cell area when group IV was compared to groups II and III; there was also a similar comparison between groups II and III.

When the control group was compared to group II and group III, the NA significantly increased, which was related to an increase in the nuclear contents required for replication.\textsuperscript{13} When group IV was compared to the control group, the NA slightly increased but was insignificant, consistent with the study of Ramesh \textit{et al}.\textsuperscript{18} that might have been due to exposure of the mucosa to a high concentration of carcinogens such as reduced pyridine alkaloids, area-derived nitrosamine, and N-nitrosamines that act over the nucleus directly.\textsuperscript{19,20} When group V was compared to the control group, the NA was not much increased though it was not significant, which was in contrast to Suneet \textit{et al} and Ramesh \textit{et al}.\textsuperscript{1,18} that might indicate that the altered cell mechanism at the molecular level in response to the stimulation effect by combustion products.\textsuperscript{17} The mean NA significantly increased in group II when compared to group IV and group V although when compared to group III, there was a slight increase in group II, which was insignificant. When group III was compared to group V, a significant increase was seen in group III though it was not significant when compared to group IV, which may have been due to an impending slow transformation of premalignant change in tobacco users.\textsuperscript{21} Also, there was no significant difference between group IV and group V \textsuperscript{20} (\(P = 0.404\)), which was in contrast to the increase in cell area in group V as described earlier, indicating that the effect of combustion products and heat is lagging behind in inducing any morphological change in the nucleus.

The nuclear-to-cell area ratio was the highest in group II when compared to all other groups and it was least in group V. A significant difference in nucleus-to-cell area was between group II to group III, group IV, group V, and group III to group V and group IV to group V. Likewise, Freitas \textit{et al} used image analysis techniques to compare the nucleus area, cytoplasm area, and NA-to-CA ratio between oral mucosa cells from oral carcinoma and healthy subjects and concluded that the NA-to-CA ratio of keratinocytes of the oral carcinoma lesions was significantly greater than those of the mucosa of normal, healthy individuals.\textsuperscript{22} When group III and group IV \textsuperscript{21} \((P = 0.592)\) were compared, there was no significant difference with regard to cell area and NA and hence, N: C ratio was insignificant as well, whereas other studies by Franklin and Smith reported that the N:C ratio

### Table 3: Summary statistics of mean nuclear-to-cellular area ratio and \(P\) value between the groups

| Groups   | Normal | Cancer | Contra | Quid | Smokers |
|----------|--------|--------|--------|------|---------|
| Mean N:C area | 0.0227 | 0.03924 | 0.0337 | 0.03257 | 0.02678 |
| Normal   | —      | —      | —      | —    | —       |
| Cancer   | \(P=0.001\) | —      | —      | —    | —       |
| Contra   | \(P=0.008\) | 0.021  | —      | —    | —       |
| Quid     | \(P=0.162\) | 0.017  | 0.592  | —    | —       |
| Smokers  | \(P=0.059\) | 0.002  | 0.003  | 0.011 | —       |
has the advantage of relating nuclear volume to cytoplasmic volume and possibly represents the significant changes that occur in the cell, more accurately at a morphological level.[23]

Thus, in cancer patients there was an increase in NA and decrease in cell area. On the contralateral nonlesional site of cancer patients and quid chewers, though there was an increase in NA and decrease in cell area it was insignificant, which could have been due to field changes taking place at the molecular level. And lastly, in the smokers group the cell area increased and the NA remained almost the same, indicating a slow transformation into a potentially malignant lesion.

**Conclusion**

Thus, to conclude exfoliative cytology, along with cytomorphicmetric analysis can aid in motivating individuals to withdraw the use of tobacco, as the acceptance in reliability of measurable values increases. Thus, our study elucidates that cytomorphology is a valuable parameter to assess the influence of tobacco on the oral mucosa and in establishing a link to premalignant and malignant transformations before a lesion is noted. Further studies with larger sample sizes should be encouraged to confirm these findings and a cytomorphicmetric grading system should be used to further explore the advantages of this technique.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**

1. 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.
2. Schmidt BL, Dierks EJ, Homer L, Potter B. Tobacco smoking history and presentation of oral squamous cell carcinoma. J Oral Maxillofac Surg 2004;62:1055-8.
3. Nayyar AK, Sundharam BS. Cytomorphometric analysis of exfoliated normal buccal mucosal cells. Indian J Dent Res 2003;14:87-94.
4. Khalili J. Oral cancer: Risk factors, prevention and diagnostic. Exp Oncol 2008;30:259-64.
5. Joshi PS, Kajikar MS. Cytomorphometric analysis of premalignant and malignant lesions using feulgen stain and exfoliative brush cytology. J Interdiscip Histopathol 2013;1:204-11.
6. Sirsat MV, Vatsala M. Buccal mucosa with tobacco chewing. Br J Cancer 1967;2:277-84.
7. Bundgaard T, Bentzen SM, Søgaard H. Histological differentiation of oral squamous cell cancer in relation to tobacco smoking. Eur J Cancer B Oral Oncol 1995;31:118-21.
8. Ringdahl BE, Johnson GK, Ali RB, Organ CC. Effect of nicotine on arachidonic acid metabolites and epithelial parameters in rat oral mucosa. J Oral Pathol Med 1997;26:40-5.
9. Miller SC, Soberman A, Stahl SS. A study of the cornification of the oral mucosa of young male adults. J Dent Res 1951;30:4-11.
10. 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.
11. Hegde V. Cytomorphometric analysis of squames from oral premalignant and malignant lesions. J Clin Exp Dent 2011;3:441-4.
12. Cançado RP, Yurgel LS, Filho MS. Evaluation of nuclear organizer region associated proteins in exfoliative cytology of normal buccal mucosa. Effect of smoking. Oral Oncol 2001;37:446-54.
13. Frost JK. Pathologic processes affecting cells from inflammation to cancer. In: Bibbo M, editor. Comprehensive Cytopathology. 2nd ed. Philadelphia: WB Saunders Company; 1997. p. 68-78.
14. Ogden GR, Cowpe JG, Green MW. Quantitative exfoliative cytology of normal buccal mucosa: Effect of smoking. J Oral Pathol Med 1990;19:53-5.
15. Hillman RW, Kissin B. Oral cytologic patterns in relation to smoking habits. Some epithelial, microfloral, and leukocytic characteristics. Oral Surg Oral Med Oral Pathol 1976;42:366-74.
16. Bock FG, Moore GE, Crouch SK. Tumor-promoting activity of extracts of unburned tobacco. Science 1964;145:831-3.
17. Einstein TB, Sivapathasundharam B. Cytomorphometric analysis of the buccal mucosa of tobacco users. Indian J Dent Res 2005;16:42-6.
18. 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.
19. Hecht SS, Chen CB, Hirota N, Oraif RM, Tso TC, Hoffmann D. Tobacco-specific nitrosamines: Formation from nicotine in vitro and during tobacco curing and carcinogenicity in strain A mice. J Natl Cancer Inst 1978;60:819-24.
20. Ashby J, Styles JA, Boyano E. Betel nut arecaandine and oral cancer. Lancet 1979;1:112.
21. Hande AH, Chaudhary MS. Cytomorphometric analysis of buccal mucosa of tobacco chewers. Rom J Morphol Embryol 2010;51:527-32.
22. Diniz-Freitas M, Garcia-García A, Crespo-Abelleira A, Martins-Carneiro JL, Gándara-Rey JM. Exfoliative cytology of the oral mucosa: A cytomorphometric comparison of healthy oral mucosa in oral cancer patients and healthy subjects. Rev Bras Pathol Oral 2003;2:2-6.
23. Franklin CD, Smith CJ. Stereological analysis of histological parameters in experimental premalignant hamster cheek pouch epithelium. J Pathol 1980;130:201-15.