Original Article

Quantitative analysis of agnor counts of buccal mucosal cells of chewers and non chewers of gutkha: A comparative cytologic study

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ABSTRACT

Aims and Objectives: The present study was taken up to evaluate the AgNOR counts in the buccal mucosa cells of gutkha chewers and compare that with the sex-matched controls.

Materials and Methods: In all, 100 gutkha chewers and 50 sex-matched non-chewers (controls) were chosen. None of the patients in both groups had any clinical oral lesions or systemic diseases. After rinsing with 0.9% sodium chloride, cytologic smears were prepared and stained using the AgNOR method and observed in immersion oil at 1000 × magnification. Finally, 50 cells were selected at random; AgNOR dots were counted and their mean was recorded. The student *t*-test was used for analysis of data.

Results: Comparison between mean AgNOR counts of gutkha chewers (2.68 ± 0.23) and non-chewers (2.01 ± 0.14) was found to be statistically significant.

Conclusion: Cytology associated with AgNOR staining can effectively detect the early molecular changes within buccal mucosa cells of oral mucosa.

KEY WORDS: AgNOR, cytology, cytobrush, gutkha, smear

INTRODUCTION

Squamous cell carcinoma is one of the commonly occurring malignancies in oral cavity. The early diagnosis and consequent treatment of oral cancer could prevent a large number of deaths due to this disease.[1] It is an established fact that oral squamous cell carcinomas are preceded by oral precancerous lesions (potentially malignant disorders) in most of the cases. There exists a strong relationship between gutkha chewing and squamous cell carcinoma.[2] Molecular changes appear much prior to any lesion being evident clinically. These ultra-structural changes can be detected by special techniques like flow cytometry, AgNOR or micronuclei count technique.[3,4] AgNOR in cytology has not been used as frequently and efficiently as in paraffin embedded sections. No particular technique or diagnostic modality is designed to predict the accurate malignancy conversion rate of potentially malignant disorders.[5] Also, there is no specific technique that would accurately predict whether the potentially malignant disorder would transform into a malignancy. It is important that techniques are developed to aid in the diagnosis of early oral cancer especially in predicting the behaviour of those lesions which display epithelial dysplasia but no overt malignancy.[6] Proliferation markers like proliferating cell nuclear antigen (PCNA), ki-67 and economical substitutes like AgNOR and micronuclei evaluation techniques can give an arbitrary estimate of the changes the cells undergo in potentially malignant disorders or even the changes that cells undergo before the lesions appear.[7,8] If not precise, but an arbitrary prediction about the molecular changes that take place in the buccal mucosa cells of gutkha chewers in individuals without any lesion, can hint towards occurrence of any lesion in those individuals in future. Hence, this study was conducted with an intention of detecting changes in buccal mucosa cells of gutkha chewers by comparing their AgNOR counts with the counts of buccal mucosalcells of non-gutkha chewers (normal).

MATERIALS AND METHODS

This study comprised of cytological examination of buccal mucosa cells in 100 gutkha chewers as study sample group and 50 sex-matched non-chewers as controls using AgNOR staining.

Criteria for inclusion of subjects

The study group consisted of gutkha chewers with the following criteria:

- Consumption of minimum of five commercially available packs or sachets of gutkha per day
- Duration of consumption of gutkha for more than three years

- Clinical absence of any apparent lesion in the oral cavity
- No history of any systemic disease/malignancy/recurrence.

The control group comprised of age- and sex-matched subjects without any lesion, systemic illness or any other tobacco habits resulting in alteration of buccal mucosa cells.

Materials used

- Cytobrush for brush biopsy
- AgNOR stain
- Binocular light microscope
- Microscopy slides
- Fixative (90% ethyl alcohol).

Procedure employed

The subjects from the study group and control group were well informed about the study and their consent was obtained prior to the commencement of the study. The details of the subjects and controls were noted on the case history proforma and examined for presence of any lesion. After rinsing with 0.9% sodium chloride, scrapings from buccal mucosae of the individuals were taken and fixed on microscopy slides using 90% ethyl alcohol. The slides were stained with AgNOR stain using Ploton's method. The number of AgNOR has been thought to be related to cellular activity. After horizontally screening of the sections from left to right, AgNORs were counted in the nuclei of the first 50 non-overlapped nucleated cells. Cells with pyknotic nuclei were not counted. The AgNOR count was made adopting the method described by Ahmed and Babiker[9] by using a binocular light microscope at 1000 × magnification (oil immersion power) by three different observers. The average AgNOR count per smear was calculated by dividing the sum of nucleolar organizing regions counted in the cells by the total number of cells counted.

RESULTS

AgNOR counts of fifty cells per smear of subjects and control were counted by three observers and the mean of the three was taken. An overall mean of AgNOR count per smear was counted in subjects and controls and a correlation was evaluated using student *t*-test and the difference was found to be statistically significant (*P* value = 0.0000). The average AgNOR count was found to be 2.6811 ± 0.2306 in gutkha chewers whereas the average AgNOR count in control group was found to be 2.0114 ± 0.1478 [Table 1 and Figure 1].

A comparison of AgNOR counts was done between two groups of gutkha chewers; for more than 10 years (2.6943 ± 0.2683) and less than 10 years (2.6735 ± 0.2061) and the difference was not statistically significant.

DISCUSSION

It is not correct to perform an incisional biopsy on a clinically normal appearing mucosa and in such a case, exfoliative cytology can be a better substitute for biopsy procedures.

Table 1: Comparison of chewers and non-chewers with respect to average counts by *t*-test

Group	*N*	Mean	SD	*t* value	*P* value
Chewers	100	2.6811	0.2306	18.6881	0.0000*
Non chewers	50	2.0114	0.1478		

*$P<0.05$. SD=Standard deviation

Exfoliative cytology has proved to be a fair adjunct for routine histopathological techniques. Newer techniques like flow cytometry, or the economical substitutes like AgNOR staining procedures can be combined with exfoliative cytology for effective evaluation of cells for molecular changes.[10] AgNOR stains have been used successfully on paraffin-embedded sections previously but use of AgNOR stain in cytology has not been explored.

Since prevalence of potentially malignant disorders and malignancies is more associated with habits like smoking, gutkha chewing and tobacco chewing,[11] detection of molecular changes in individuals with habits becomes more relevant and rational.[12] A study done by Illana Kaplan *et al.*, suggested that AgNOR method seems to be sensitive and enables earlier identification of nuclear changes.[13] Mean AgNOR counts increased gradually from normal epithelium to non-dysplastic to dysplastic leukoplakia to squamous cell carcinoma.[14] In spite of the rampant and fast increasing prevalence of gutkha chewing habit in India, no study using AgNOR in cytology of gutkha chewers has been documented in the literature.

This cytology based study to evaluate the AgNOR counts in normal appearing mucosa of gutkha chewers shows increased number of AgNOR dots in gutkha chewers [Figure 2] as compare to non-gutkha chewers [Figure 3]. The increased AgNOR count in gutkha chewers is due to the ingredients of gutkha. The study done by Jeng JH *et al.*, concluded that areca nut induces unscheduled DNA synthesis in keratinocytes.[15] The non-significant difference between the AgNOR counts of gutkha chewers that were categorized depending upon gutkha chewing period could be attributed to the variable amount of gutkha being chewed per day. Similar studies were done on smokers that have shown that cellular proliferation is significantly higher in smokers and this causes an increase in the nuclear dimensions of oral mucosal cells.[16]

Various follow-up studies have been done that provide a significant correlation between the AgNOR count and prognosis was in pre-malignant and malignant lesions of the cervix[17], colorectal cancer[18], benign and malignant effusions[19], adenoid cystic carcinoma[20] and breast carcinoma.[21] However, it is proven to be of prognostic value in ovarian cancer,[22] transitional cell carcinoma of the bladder[23] and glottic cancer.[24]

CONCLUSION

Occurrence of keratotic and white lesions has consistently been seen in association with gutkha consumption habit.

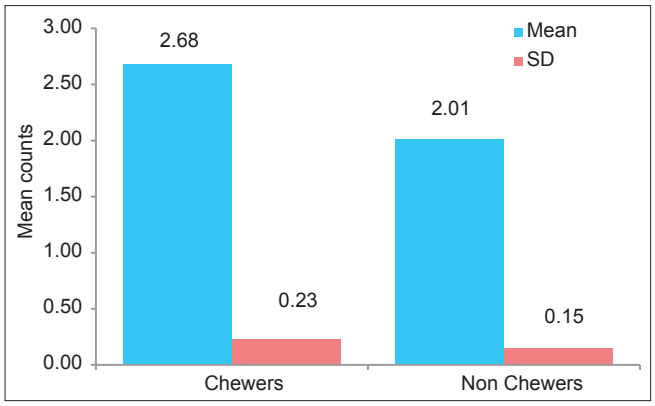

Figure 1: Graphical comparison of chewers and non-chewers with respect to average counts

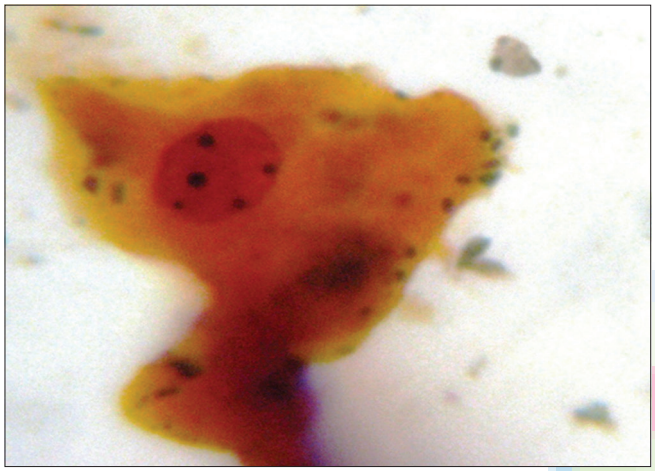

Figure 2: Photomicrograph showing four AgNOR dots in epithelial cells of gutkha chewer (AgNOR stain, ×40 magnification)

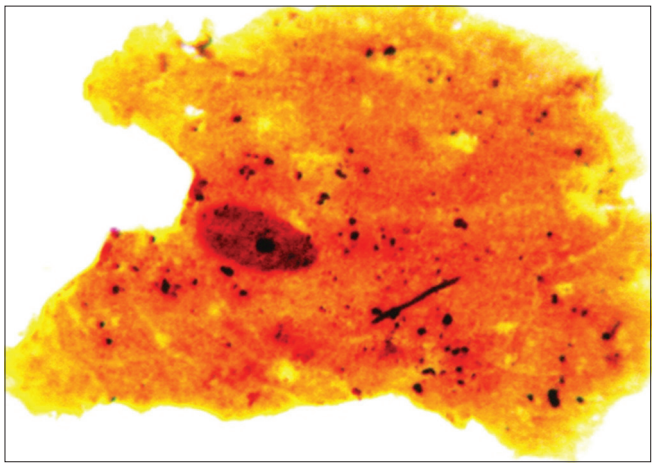

Figure 3: Photomicrograph showing one AgNOR dot in epithelial cells of Non-gutkha chewer (AgNOR stain, ×40 magnification)

Timely detection of molecular changes prior to occurrence of clinically evident lesions can be helpful in creating awareness in the individuals with habits. Thus, cytological screening of the patients, with high risk of oral neoplastic lesions and without any macroscopically apparent oral lesion, can be of extreme importance.[9]

Exfoliative cytology combined with AgNOR is an economical noninvasive procedure that can be helpful in evaluating high-risk group individuals. Follow-up of the individuals associated with the habit, is advised in situations where they present with increased AgNOR counts.

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