Original Article

Assessment of micronuclei frequency in individuals with a habit of tobacco by means of exfoliated oral buccal cells

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Abstract

Aims and Objectives: To study the genotoxic effects of tobacco on the exfoliated buccal epithelial cells in patients with oral precancerous lesions (OPLs) and Patients with tobacco habit but without oral precancerous lesion(habit controls) by using micronucleus assay as well as the quantification and detection of the biomarkers in these premalignant lesions which will be helpful in finding those patients who are at higher risk for malignant transformation. **Materials and Methods:** Forty samples were collected from the right and left side of buccal epithelial cells obtained from 20 individuals, i.e., 10 patients with habit control and 10 patients with OPLs. Statistical analysis was performed by the Statistical Package for the Social Sciences version 21.0 Unpaired t-test was performed to determine the micronucleated cell (MNC) and micronuclei (MN) frequencies in individuals; significance was set at $P > 0.05$. **Results:** There was an increase in both the MNC and MN frequency from habit controls to OPLs, indicating that the number of cells with chromosomal damage and extent of chromosomal damage in each cell was high in OPLs. **Conclusion:** The MN count can be used as a noninvasive tool for early detection, educating patients, screening a large population, and to check the risk for malignancy, which in turn may help in treatment planning.

Key words: *Biomarkers, leukoplakia, micronucleus, oral cancer*

INTRODUCTION

Every year there are approximately 500,000 new cases of oral malignancies, that is, 3% of all malignancies, which creates a remarkable health issue all over the world.[1] In spite of many advanced treatment modalities, early detection of oral cancer and remedies are the best way to ensure patient survival, which in turn improves the quality and span of life.[2,3]

The incidence rate of oral malignancy in India is 12.6 in 100,000 population, however, in recent years, it has increased according to certain reports.[4] In India, the high incidence of oral malignancies are mostly seen in individuals with the habit of chewing and smoking tobacco.[5]

Leukoplakia was considered to be one of the premalignant lesion, however, now it is included under potentially

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malignant disorders. It is mainly associated with chewing and smoking tobacco. It has a risk of malignant transformation if the risk factors are not eliminated. In 1877, Schwimmer proposed the term leukoplakia.

The definition of a biomarker according to the World Health Organization (WHO) and the International Labor Organization is "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease." The Committee on Biological Markers of the National Research Council/National Academy of Sciences has categorized the biomarkers.[6] They come into broad groups that helps in the detection of exposure, whether they are susceptible to carcinogens, their progression, and the response by the target cellular populations.

Micronuclei is one biomarker that defines chromosomal aberrations by taking up the stain in exfoliated buccal mucosal cells.[7] The micronuclei are defined as a microscopically visible, round-to-oval cytoplasmic chromatin mass next to the nucleus. It is a small extranucleus separated from the main one generated during cellular division. They remain in the cytoplasm of interphasic cells as structures with a constitution and appearance similar to those of the nuclei. A micronucleus is formed during the metaphase/anaphase transition of cell division.

Thus, the present study has been carried out with an objective to evaluate the genotoxic effects of tobacco by means of micronucleus assay in exfoliated cells of buccal mucosa in individuals with different tobacco related habits, and comparing the values in patients with oral precancerous lesions (OPLs) and habit controls without any significant changes in oral mucosa.

MATERIALS AND METHODS

In the present study, 40 samples were collected from the right and left side of buccal epithelial cells obtained from 20 individuals. The study group comprised 10 patients with habit controls and 10 patients with OPLs. The number of cells scored from each sample was 1500, that is, 3000 from an individual.

The participants were recruited from the Department of Oral Medicine and Radiology, Dr. D. Y. Patil Dental College and Hospital, Nerul, Navi Mumbai, Maharashtra, India for a period of 6 months from June 2013 to November 2013. An informed consent was

taken from individuals and personal history of each participant was recorded thoroughly. Ethical clearance was obtained from the ethical committee of Dr. D. Y. Patil Dental college and hospital, Nerul, Navi Mumbai, Maharashtra, India.

The participants for the study were grouped as:
- Habit controls: Individuals with a history of significant consumption of tobacco in various forms and without any clinically examinable lesions in the oral mucosa were selected as habit controls
- Oral precancerous lesions (OPLs): Individuals with histopathologically proven lesions of leukoplakia comprised this group.

Only those patients were included in the study who had a history of tobacco consumption with or without presence of oral leukoplakia. Patients having oral leukoplakia without any history of tobacco consumption and those who were treated or were undergoing treatment for leukoplakia were excluded from the study.

Sample collection procedure

The entire procedure was explained to the patients in the language they best understood. The participants were asked to rinse their mouth thoroughly with tap water 2–3 times immediately before the sampling procedure to reduce the oral bacterial load. Pre-moistened separate wooden spatula was used to scrape the oral buccal mucosa cells (right and left buccal mucosal cells) by the observer under the guidance of an expert from the Department of Oral and Maxillofacial Pathology, Dr. D.Y. Patil Dental College and Hospital, Nerul, Navi Mumbai, Maharashtra, India. The cells were then smeared over clean, coded glass slides. The slides were then air dried and fixed in fixative for 20 minutes and stained with Giemsa.

Scoring criteria

The type of microscope used in the study was a Light microscope-Nikon Eclipse 50i. First, slides were scanned in low power magnification (10×) to determine the quality of preparation and then scored under 100× magnification. Fifteen hundred intact epithelial cells from each side (right and left buccal mucosa) were scored to determine the frequency of micronucleated cells. Thus, 3000 cells in each individual were analyzed. Only cells free from clumping or overlapping and those containing intact cytoplasmic boundaries were included in the scoring. Scoring of the micronuclei in buccal epithelial cells was done by the observer who was also under the guidance of an expert from the Department of Oral and Maxillofacial Pathology,

D. Y. Patil Dental College and Hospital, Nerul, Navi Mumbai, Maharashtra, India. It was done according to the criteria defined by Tolbert e*t al.* (1992)[8] and Holland e*t al.* (2008).[9] The criteria were as follows:

- The micronucleus staining intensity and pattern should be similar to that of the main nucleus
- Their borders should be distinctly recognizable indicating the presence of a nuclear membrane
- They should be circular and distinctly separated from the main nucleus
- They should be in the same optical plane with that of the main nucleus and should be similar in texture
- Their size should be equal to or less than one-third the diameter of the main nucleus.

To understand the pattern of distribution of micronucleated cells and micronucleus frequency in the study group, overall distribution of their frequency in each group was analyzed by using the Statistical Package for the Social Sciences software version 21.0. For that purpose, the results were analyzed by Mann–Whitney test. A *P* value less than 0.05 was considered to be significant. Unpaired *t*-test was performed to determine the micronucleated cells and micronucleus frequencies in individuals that showed $P > 0.05$.

RESULTS

Table 1 shows that the age range in OPLs group was 22 to 60 years, and the mean age was 37.8 years. The age range for the habit control group was 23 to 52 years, and the mean age was 31.5 years.

Table 2 shows that incidence of patients with oral leukoplakia habituated to more than one habit such as tobacco chewing and tobacco smoking was 30%, whereas in habit control it was 10%. Tobacco chewing and alcohol drinking accounted for 70% in the OPL group whereas in habit control group it was 10%. In OPL group, patients with the habit of smoking and drinking was 40%, and in the habit control group it was 20%. Patients with all the three habits in OPL group were 30%, whereas in habit control group no one was exposed to all the three habits.

We analyzed percentage of micronucleated cell frequency in habit controls and OPLs. We also determined percentage of micronucleus frequency in these groups as it indicates the extent of chromosomal damage in the cells [Tables 3 and 4]. Figure 1 shows buccal epithelial cell with no micronucleus, single micronucleus, and multiple micronuclei.

Table 1: Age distribution

Study group	No. of study subjects (*n*)	Mean±SD	Median (Range)
Habit controls	10	35.3±10.58	31.5 (23-52)
OPL	10	37.8±12.8	37 (22-60)

SD=Standard deviation

Table 2: Showing mixed tobacco habit patterns in the study groups

Mixed habit	Status	Habit controls *n* (%)	OPLs *n* (%)
Chewing + Smoking	Yes	1 (10%)	3 (30%)
	No	9 (90%)	7 (70%)
Chewing + Drinking	Yes	1 (10%)	7 (70%)
	No	9 (90%)	3 (30%)
Smoking + Drinking	Yes	2 (20%)	4 (40%)
	No	8 (80%)	6 (60%)
Chewing + Smoking + Drinking	Yes	0	3 (30%)
	No	10 (100%)	7 (70%)

OPLs=Oral precancerous lesions

### Table 3: Distribution of average %MNC frequency in the two groups

Study group	*n*	Mean±SD	*P* value
Habit controls	10	5.90±3.28	0.073
OPL	10	9.08±4.14	

(P≤0.05 – Significant), OPL=Oral precancerous lesion, SD=Standard deviation

Table 4: Distribution of average %MN frequency in the two groups

Study group	*n*	Mean±SD	*P* value
Habit controls	10	14.84±9.04	0.1105
OPL	10	23.46±13.49	

(P≤0.05 – Significant), OPL=Oral precancerous lesion, SD=Standard deviation
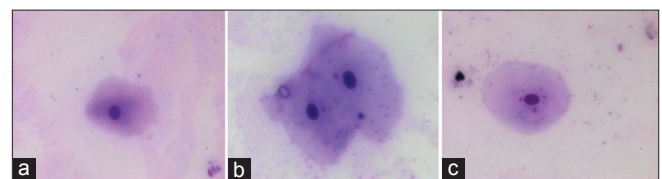
Figure 1: Buccal epithelial cell with (a) no micronucleus, (b) single micronucleus, (c) multiple micronuclei

DISCUSSION

The aim of our study was to evaluate micronucleus as a biomarker for DNA damage in oral leukoplakia and habit control group. The most commonly studied confounders were age, gender, habits, percentage micronuclei (%MN) and % micronucleated cell (%MNC) frequency. In the present study, exposure to tobacco is the common factor in both the groups (habit

control and OPLs), which is uncommon in the studies done in the past. It was done to assess the frequency and percentage of micronuclei in patients who are habituated to not only one habit but the synergistic effect of one or more habit.

Palaskar *et al.*,[10] Patel *et al.*,[11] and Himanta *et al.*[12] have shown in their study that the frequency of micronuclei is more in patients who are habituated to smokeless tobacco rather than the smoking form of tobacco.

The micronucleus in oral buccal cells is considered to be a biomarker of chromosomal damage caused by genotoxic agents from substances related to tobacco, tobacco itself, and alcohol. The induction of micronucleated cells by carcinogens and mutagens is a sign of the genotoxic effect of such substances.

In buccal mucosal cells, effects are seen in various studies because it is an easily approachable tissue which can be obtained with minimal invasion for sampling, also it does not cause stress to the patients.[13-16] According to Livingston *et al.*,[17] saliva soluble compounds present in tobacco could diffuse into the basal cell layer and disturb the reproductive mechanism of the underlying proliferating cell population, thereby causing genotoxicity and formation of nuclear aberrations. Nagler and Dayan[18] showed that the interactions between redox active metals in saliva and the low reactive free radicals during chewing and smoking of tobacco enhance the potency of genotoxicity.

Increased frequency of nuclear aberrations in buccal mucosal cells of tobacco and alcohol users indicates a high risk group of oral cancer.[19,20] The assessment of micronucleus in buccal epithelial cells is an appropriate tool for the research of malignant cells, and it can be used in the detection of chromosome breakage.[21]

In our study, we found that the patients with all the three habits in OPL group were 30% while in the habit control group no one was exposed to all the three habits. This shows that the synergistic effect of the mixed habit is more vulnerable to oral mucosal cells and shows more frequency of micronuclei in exfoliated buccal epithelial cells. These results were in concordance with the studies done previously by various authors. For example, in the study by Kohn *et al.*, approximately 80% of patients with a habit of alcohol consumption also smoked.[22] In an another study by Marks *et al.*, the dependency of nicotine was more severe in smokers along with a history of alcohol.[23] Individuals with both the habits of smoking and drinking are almost 38 times more prone to develop oral cancers than those who do not have any deleterious habit.[24]

In our results, the micronucleated cell and micronuclei frequency in oral exfoliated cells was more in individuals with OPLs in comparison with habit controls indicating DNA damage. The multiple micronucleation in the target tissue indicates extensive genetic damage resulting in chromosomal instability, which is a hallmark of human tumors.[25] Our findings are in accordance with other studies carried out on oral mucosal cells.[26,27]

However, there are limitations of sample size in the study which could have been larger to show that the risk of malignancy is significantly higher in OPLs due the synergistic effect of mixed habit pattern.

CONCLUSION

Micronuclei assay is an effective tool that reflects severity of disease. Even though tobacco-induced cancers are preventable, banning the use of tobacco has not been possible for social and political reasons. The count of micronuclei is observed as a noninvasive tool for detection, education of patients, screening of mass population, and also checking for the treatment efficacy.

Future scope of the study is to consider the fact that oral cancer develops after a long latency period, and hence, it is difficult to determine the clinical outcome. The detection of an oral cancer lesion in an early stage will improvise the rate of survival, which will help in the reduction of the morbidity to a greater extent while treatment.

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Conflicts of interest

There are no conflicts of interest.

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