Original Article

Evaluation of genotoxicity by micronucleus assay in oral leukoplakia and oral squamous cell carcinoma with deleterious habits

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Abstract

Background: Oral cancer is the 12th most common cancer in women and the 6th in men. Of all oral malignancies, more than 92-95% is Oral Squamous Cell Carcinoma (OSCC). The high risk was due to Lifestyle-related habits such as smoking, alcohol consumption, chewing of areca nut related products which are considered as the major risk factors in OSCC. The exogenous carcinogens from tobacco smoke may induce a defective DNA damage response, which may alter the expression of genes that protect us against cancer that may result in genomic instability and this DNA damage can be assessed by studying the chromosomal aberrations, sister chromatid exchanges and the varied forms of the micronucleus.

Aims and Objectives: The aim of this study was to evaluate the risk of development of oral leukoplakia (OLP) and OSCC due to DNA damage by studying micronuclei count in the east coast of Andhra Pradesh population with tobacco consumption habit and habit-free controls using Fluorescent microscopy.

Materials and Methods: A total of 60 subjects, 20 normal controls, 20 oral leukoplakia and 20 OSCC patients were selected from the outpatient patients of GITAM Dental College and Hospital, Rushikonda, Visakhapatnam and peripheral cancer hospitals in and around Visakhapatnam. Exfoliated cells were collected by giving 5-6 gentle strokes with spatula in a continuous unidirectional movement and then were uniformly spread on the previously cleaned microscopic slide. Fluorescent stain 4′, 6′-diamidino-2 phenylindole (DAPI) was used for MN analysis.

Results: Mean of cells with MN in controls, leukoplakia and OSCC cases was observed to be 1, 5.1,10.1 ($F = 112.396$, $P < 0.001$) respectively. Mean of the cells with MN in different grades of leukoplakia. ($F = 35.594$, $P < 0.001$) Mean of the cells with MN in different grades of OSCC. ($F = 39.752$, $P < 0.001$).

Conclusion: The present study revealed an increase in mean frequency of cells with micronucleus from healthy individuals however similar studies in larger sample has to be done. This study concludes that MN index can be used as a screening test among high risk groups.

Keywords: Chromosome, leukoplakia, micronucleus, mitotic apparatus, oral cancer, squamous cell carcinoma

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INTRODUCTION

Oral cancer is the 12th most common cancer in women and the 6th in men.[1] Of all oral malignancies, >92%–95% is oral squamous cell carcinoma (OSCC).[2]

Prevalence of oral leukoplakia in India varies from 0.2% to 5.2% of all potentially malignant disorders. The high risk in the Indian population was due to lifestyle-related habits such as smoking, alcohol consumption, and chewing of areca nut-related products which are considered as the major risk factors in oral cancer. The exogenous carcinogens from tobacco smoke may induce a defective DNA damage response, which may alter the expression of genes that protect us against cancer that may result in genomic instability.[3]

The DNA damage can be assessed by studying the chromosomal aberrations, sister chromatid exchanges and the varied forms of the micronucleus (MN).

A MN is a small additional nucleus readily identifiable by light microscopy because it is morphologically identical to but smaller than the main nucleus. It is usually situated around the main nucleus, within inner half of the cytoplasm except signet-ring cells or mucin-filled cells where it can be seen at the periphery of the cell. Chromatin texture and staining intensity are similar to the main nucleus. Biologically, micronuclei (MNi) are the chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. It results from failure of the mitotic apparatus and is detectable in the cytoplasm of the interphase cell as a small additional nucleus or an acentric chromosome (fragment) due to chromosome breakage.[4]

An evaluation of the literature shows that a variety of different stains are used in MN studies. Among the DNA-specific stains, the ones most widely used are Feulgen and acridine orange; in some experiments, 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide were also used. About 30% of the studies in epithelial cells were conducted with nonspecific stains (Giemsa, May-Grünwald-Giemsa, Papanicolaou and less frequently orcein and hematoxylin and eosin).

The smear prepared from exfoliated buccal mucosal cells is arguably the least invasive and easily accessible method available for measuring DNA damage in humans, especially in comparison to obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies. The buccal mucosa cells with MN assay was first proposed in 1983 and continues to gain popularity as a biomarker of genetic damage in numerous applications. Due to the reasons, MN assay is being predominantly used day by day.

MN is induced in cells by a variety of substances, including genotoxic agents and carcinogenic compound in smoke and smokeless forms of tobacco. Tobacco-specific nitrosamines have been reported to be potent clastogenic and mutagenic agents which are thought to be responsible for the induction of chromatid/chromosomal aberrations resulting in the production of MN.

It is a known fact that oral deleterious habits are associated with increased risk of developing potentially malignant disorders and malignancy. The carcinogenic effect of the above-mentioned habits may be related to inducing genotoxic effect on oral mucosal cells. Investigations of MN frequencies support the widely accepted assumption that MN are a product of early events in human carcinogenic processes, especially in oral regions, especially because they are virtually absent in unexposed mucosa. The MN assay from exfoliated buccal cells was also used to study cancerous and precancerous lesions and to monitor the effects of a number of chemopreventive agents.[5]

MATERIALS AND METHODS

Patient selection

A total of 60 patients, of which 20 normal controls, 20 oral leukoplakia and 20 OSCC patients were selected from the outpatient patients of GITAM Dental College and Hospital, Rushikonda, Visakhapatnam and Peripheral Cancer Hospitals in and around Visakhapatnam. Patients were selected with suspected oral lesions. Relevant history of each patient, including their oral habits was recorded thoroughly. Only those patients who were subsequently diagnosed histopathologically with epithelial squamous cell carcinoma in addition to who had not received any therapy were included in the OSCC group. Twenty patients clinically and histopathologically proven cases of oral leukoplakia were included under the second group. All the patients in the two groups had oral deleterious habits. Age- and sex-matched healthy controls have no obvious oral lesions or habits of consumption of tobacco, other tobacco-related substances were included under control group. Written informed consent was obtained before the study.

Collection of exfoliated cells

Prior to the sampling, all the participants were asked to rinse their mouth with plain water to remove residual particles. The cells were collected by giving 5–6 gentle

strokes with spatula in a continuous unidirectional movement and then were uniformly spread on the previously cleaned microscopic slide. This procedure was repeated and 3–4 slides were collected from each patient. The slides were allowed to air-dry and then fixed in 3:1 methanol/acetic for 15 min. Fluorescent stain DAPI was used for MN analysis. DAPI staining was done in Genome Foundation Lab. Briefly, the slides smeared with buccal cells were washed with ×1 PBS for 5 min for three times. The stock solution of DAPI (Invitrogen) was diluted (1 μg/ml) with distilled water and stored at 4°C. Fixed slides were stained for 20 min, rinsed thrice in ×1 PBS, air-dried and mounted in the same buffer. Observations were carried out in a dark room using a fluorescence microscope (Leica DM 3000, Leica Microsystems) equipped with a band-pass filter of 450–490 nm (excitation range: Blue). Stained slides were read at ×40. A total of 100 cells per individual were evaluated for the presence of MN. In order to avoid bias, two observers were used to score cells with MN. The measures of DNA damage were evaluated as MN frequency and expressed as percentages.

Quantitating micronucleated cells

The micronuclear assessment was performed using pathologist in blinded fashion. All the stained slides were observed under fluorescent microscope under at ×40 for counting the MNi. A total of 100 cells were counted from the smears collected from each individual. Zigzag method was used for screening of the slides. Cells with intact nuclei and cell boundaries were counted in the smears of each patient for the presence and micronucleated cells (MN cells) and MN.

The frequency of MN was evaluated by scoring 100 cells from each smear collected from each individual. MN was scored only in epithelial cells. Criteria used for the identification of MNi were as per Tolbert *et al*.[6]

The same procedure was followed for normal healthy controls, wherein the smears were taken by scraping with wooden spatula on the buccal mucosa and the scraping transferred on a clean glass slide. The staining procedure was carried out similar to that of the study group following which the slides were studied for the presence of micronucleated cells.

Criteria to be satisfied by the cell to be scored (Tolbert *et al*.)

- Intact cytoplasm and relatively flat cell position on the slide
- Little or no overlap with adjacent cells
- Little or no debris
- Nucleus normal and intact; nuclear perimeter smooth and distinct.

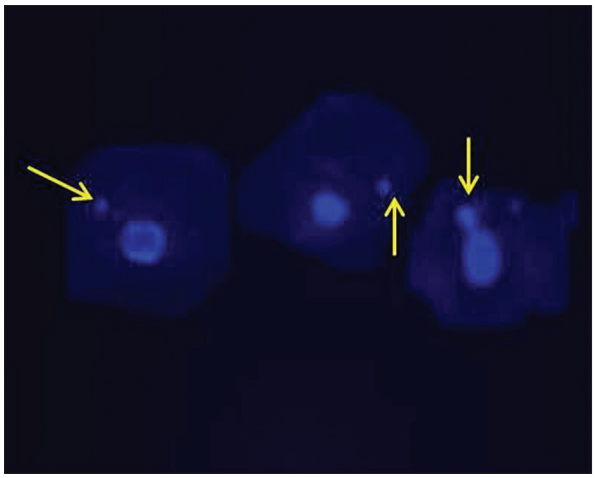

Figure 1: Micronuclei in leukoplakia

Criteria for identifying MNi (Tolbert *et al*.):

- Rounded smooth perimeter, suggestive of a membrane
- Less than a third the diameter of the associated nucleus, but large enough to discern shape and color
- Staining intensity similar to that of the nucleus.

Data entry and statistical analysis

Ethical clearance was taken by the institute before commencing the study.

Once the MNi were scored all the results were entered in a sheet (Excel 2007, Microsoft Office) and analyzed using the statistical analysis software SPSS version 18 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant. Comparison of gender was made using the Chi-square test. Comparison of MNi between the two groups was made using ANOVA test.

RESULTS

The MN was observed in leukoplakia as shown in Figure 1 and in OSCC in Figure 2.

In the present study, the MN test has been applied to patients having leukoplakia and squamous cell carcinoma.

The study included a total of 60 patients and they were divided into three groups.

- Group I was the control group, in which 20 healthy controls with no deleterious habits were selected
- Group II consisted of 20 cases of leukoplakia with deleterious habits and this group showed a male predominance

- Group III consisted of 20 cases of OSCC with deleterious habits and this group showed a female predominance.

The results of the present study are summarized in Tables 1-3.

Mean of cells with MN in controls, leukoplakia and OSCC cases were observed to be 1, 5.1, 10.1 (F = 112.396, P < 0.001), respectively.

Mean of the cells with MN in different grades of leukoplakia are tabulated in Table 2. (F = 35.594, P < 0.001).

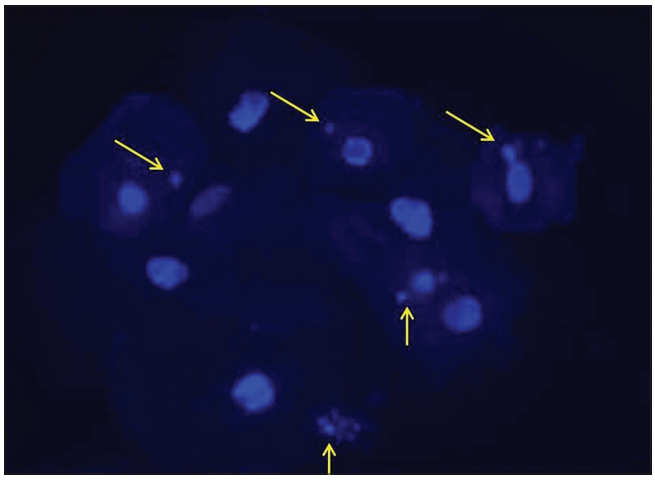

Figure 2: Micronuclei in oral squamous cell carcinoma

Table 1: Comparing mean frequency of cells with micronucleus in different study groups

Different study groups	Number of cases	Mean	SD	Number of MN	
				Minimum	Maximum
Group I	20	1	0.617	1	3
Group II	20	5.1	1.619	3	8
Group III	20	10.1	2.315	6	15

F=112.396, P<0.001. SD: Standard deviation, MN: Micronucleus

Mean of the cells with MN in different grades of OSCC are tabulated in Table 3 (F = 39.752, P < 0.001).

DISCUSSION

Oral premalignant and malignant lesions are one of the most debilitating diseases afflicting humankind. In spite of the best efforts of researchers and clinicians, the global incidence of cancer is high today. It is an establishing fact that tobacco and related products are one of the leading causative agents for oral cancer; their use is still very prevalent. The effect of smoking on the oral cavity has been well studied in Asian countries, mainly India. Oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body through inhalation and ingestion. As the focus shifts to finding methods to detect early genotoxic damage, the MN test provides a simple, noninvasive and a reliable screening technique for assessing early genotoxic damage much before any clinical or histological signs of cancer are evident.[5]

Various groups have found analysis of MN in buccal mucosal cells to be a sensitive method for monitoring genetic damage in human populations (Foiles 1989; Sarto 1990; Kayal 1993, Tolbert 1991, Stich 1990, Urgaz 1995).[6]

The use of cells with MN assay to assess smoking-induced genotoxic damage has been done since as early as 1982 by Stich *et al*. who applied the MN assay in residents of Bihar, India and found an increase in cells with MN frequency. They developed a protocol for MN assay with exfoliated buccal cells, which was widely used in occupational and lifestyle studies.[7]

In the present study, mean of cells with MN in leukoplakia cases was observed to be 5.1 which to a large extent

Table 2: Comparing mean micronucleus frequencies in various histological grades of leukoplakia (Group II)

Group II leukoplakia	Number of cases	Mean	SD	Number of MN	
				Minimum	Maximum
Mild epithelial dysplasia	10	3.8000	0.78881	3	5
Moderate epithelial dysplasia	8	6.0000	0.75593	5	7
Severe epithelial dysplasia	2	8.0000	0.00000	8	8

F=35.594, P<0.001 vhs. SD: Standard deviation, MN: Micronucleus

Table 3: Comparing mean of cells with micronucleus frequencies in various histological grades of squamous cell carcinoma (Group III)

Group III OSCC	Number of cases	Mean	SD	Number of MN	
				Minimum	Maximum
Well differentiated	8	8.0000	1.06904	6	9
Moderately differentiated	9	10.6667	1.00000	9	12
Poorly differentiated	3	14.0000	1.00000	13	15

F=39.752, P<0.001 vhs. SD: Standard deviation, MN: Micronucleus, OSCC: Oral squamous cell carcinoma

confirms the findings given by Buajeeb *et al*.[8] and a study by Halder *et al*.[9] gave the same information with much lower mean MN count 0.63.

MN index was observed to be two folds more in malignant cases when compared with premalignant patients. There are reports showing stepwise increase in the percentage of MN cells from control to oral precancer patients and from precancer to cancer patients suggesting that MN are a biomarker of neoplastic progression in oral as well as other cancer types. According to Palve and Tupkari,[10] level of MN in the OSCC group was observed to be in the range of 1.1%–3.0%, whereas it ranged from 1.4% to 9.15% Kumar *et al*.[11] In the present study, it ranged with a mean of 10.1%.

The MN assay has been reported to correlate well with the histological grading of OSCC and leukoplakia with the highly statistically significant result.

Another observation in the study by Kumar *et al*. that the frequency of MN increased significantly from Grade I to Grade II to Grade III, respectively, in squamous cell carcinoma group.

In the present study, observation was similar to those reported by Kumar *et al*., Palve and Tupkari, where the frequency of cells with MN increased significantly from Grade I to Grade II to Grade III, respectively, in OSCC group.

In the present study, there was a gradual increase of cells with mean MN from well differentiated to poorly differentiated, and they ranged from 8% to 14%.

In accordance with the Sangle *et al*.,[12] the assessment of MN exfoliated oral epithelial cells is a promising tool for the study of epithelial carcinogens and can be used to detect chromosome breakage of mitotic interference, thought to be relevant to carcinogenesis. MN index can be used as a screening test among high-risk groups.

The present study revealed an increase in mean frequency of cells with MN from healthy individuals; however, similar studies in the larger sample have to be done.

CONCLUSION

The present study revealed an increase in mean frequency of cells with micronucleus from healthy individuals however similar studies in larger sample has to be done. This study concludes that MN index can be used as a screening test among high risk groups.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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