Evaluation of micronuclei in betel quid chewers, potentially malignant oral disorders, and oral squamous cell carcinoma patients: A cytological assay

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Abstract

Background: The micronuclei are round to oval extranuclear cytoplasmic bodies associated with chromosomal aberrations. Micronuclei are reported to be prognostic biomarkers in the screening of potentially malignant disorders (PMDs) and oral cancer. The present study was done to determine, compare, and correlate micronuclei count in normal participants, betel quid chewers, PMDs, and oral squamous cell carcinoma patients (OSCC).

Materials and Methods: The buccal smears in each group were stained by Papanicolaou technique using commercially available staining kit RAPIDPAP®. Micronuclei were identified and counted in all the cases using standardized techniques. Comparative statistical evaluation was done within and among the groups.

Results: The median value of micronuclei in each group was assessed using Kruskal–Wallis test. Intergroup comparison was done using Dunn’s Multiple Comparison Test. A significant increase in the median values of micronuclei in betel quid chewers was found compared to normal participants. The median values of micronuclei were significantly higher in OSCC patients followed by oral submucous fibrosis (OSF), leukoplakia, oral lichen planus, and betel quid chewers as compared to normal participants.

Conclusion: The median values of micronuclei are indicative of cytogenetic damage induced by potential carcinogens. Micronuclei counts are reliable cytogenetic tools in the progression of carcinogenesis.

Key words
Biomarker, epithelial cells, micronuclei, oral squamous cell carcinoma

Introduction

Epithelial cells derived from the basal layer are known to have a limited DNA reparative ability compared to peripheral blood lymphocytes.[1] Epithelial cells are primary cells in the body, which are in direct contact with ingested or inhaled genotoxic agents and their metabolites.[2] They form a protective barrier against the carcinogenic agents and also metabolize them to their reactive products. Epithelial cells migrate to the surface layer in a duration of 5-14 days and are representative of nuclear damage in a cell occurring at that period.[3] Micronuclei present in exfoliated epithelial cells are reported to be an accurate representative of the early genotoxic effects of potential carcinoma in the body that occurred 1-3 weeks earlier in the basal cell layer.[4,5] These cells are the source of origin in 90% of all cancers. Micronuclei occur during early cell division of oral epithelial cells due to chromosomal breakage induced by potential carcinogens. It is known as a prognostic biomarker of genotoxic effects of potential carcinogens in epithelial tissue, potentially malignant disorders (PMDs), and oral cancer.[6,8]

The micronuclei are round to oval extranuclear cytoplasmic bodies associated with chromosomal aberrations.[6] In Asia-Pacific region, betel quid habit is one of the main causative agents of PMDs.[7] The N-nitrosamine presents in tobacco content of betel quid is clastogenic and mutagenic in nature. They cause chromosomal aberration and induce formation of micronuclei in oral epithelial cells. Tobacco, betel nut, and calcium hydroxide also promote release of reactive oxygen species from arecanut extracts and cause damage to DNA.[7] In the published reports, micronuclei are considered to be a reliable genomic biomarker in the screening of PMDs as compared to normal oral mucosa with a significant increase in the frequency of micronuclei in oral exfoliated cells of PMDs.[6,10] Oral cancer is associated with complex karyotypes, which involve several
chromosomal deletions, chromosomal translocations, and structural abnormalities. Chromosomal aberrations reported in oral cancer make micronuclei a prognostic indicator of genotoxic exposure in oral cancer patients.\textsuperscript{[11]}

The present study was done to determine, compare, and correlate micronuclei count in RAPIDPAP stained buccal smears of normal participants, betel quid chewers, and PMDs such as leukoplakia, oral lichen planus, oral submucous fibrosis, and oral squamous cell carcinoma (OSCC) patients with a history of betel quid chewing habit to understand the role of potential carcinogens in inducing formation of micronuclei in the carcinogenesis.

**Material and Methods**

The study was carried out after obtaining permission from the institutional review board. The informed consent of all the study participants was obtained before the procedure.

**Patient selection**

The study involved the age- and gender-matched normal participants with no habit association as control group, and the other groups included in the study were betel quid with tobacco chewers and patients diagnosed histopathologically as PMDs and OSCC. Relevant case history was recorded from all participants. The informed consent was obtained before the procedure. The participants of study were grouped into four groups:

- **Group 1**: Normal participants with no association of any habits ($n = 10$).
- **Group 2**: Betel quid chewers with a mean duration of habit for 10 years ($n = 10$).
- **Group 3**: PMD patients such as leukoplakia, oral lichen planus, and oral submucous fibrosis ($n = 5$ in each PMDs).
- **Group 4**: OSCC patients ($n = 10$).

**Sample collection**

Participants were informed to rinse their mouth gently with water before the procedure. Buccal mucosal cells were scraped from the lesional site of betel quid chewers, PMDs, OSCC patients, and control group using a cytobrush. The samples were immediately smeared on precleaned microscopic slides and were labelled. The smears were fixed with a commercial fixative provided in the RAPIDPAP kit for 15 mins. The slides were stored in dust free boxes until further evaluation.

**Cytological staining and evaluation**

The cytological smears were stained by Papanicolaou technique using commercially available staining kit RAPIDPAP. The stained slides were mounted and observed under the light microscope using low magnification ($\times 10$) for screening and high magnification ($\times 40$) for counting of micronuclei.

**Scoring criteria**

The zigzag method was followed for the screening of the slides. The criteria developed by Tolbert et al. was used for identification of micronuclei.\textsuperscript{[12]} It includes:

1. Round, smooth perimeter suggestive of a membrane
2. Less than a third the diameter of associated nucleus
3. Staining intensity similar to nucleus
4. Same focal plane as nucleus
5. Texture similar to nucleus
6. The absence of overlap with or bridge to the nucleus.

**Statistical analysis**

The median values of micronuclei in each group were calculated using Kruskal–Wallis test.

**Results**

The RAPIDPAP stained cytological smears of each study group were screened for the presence of micronuclei positive exfoliated buccal epithelial cells. The cytological smears showed cells with micronuclei ranging from 1 to 5 with size ranging from 1/3 to 2/3 the size of cell nucleus [Figure 1]. The median value of micronuclei was found to be significantly different in each study group. The highest median values were in OSCC group (10) followed by OSF (5), betel quid chewers (4), leukoplakia (2), oral lichen planus (1), and least in the control group [Table 1].

Intergroup comparison was done using Dunn’s multiple comparison test. $P < 0.05$ was considered to be significant. Statistically significant results were found in control versus betel quid chewers group, OSF group, and OSCC with the highest significance between control and OSCC ($−34.20$) groups followed by control versus OSF ($−26.40$) and control versus

![Figure 1: The RAPIDPAP stained buccal smears revealing micronuclei (a) cell with one micronucleus, (b) cell with two micronuclei, and (c) cell with five micronuclei](image-url)
betel quid chewers groups (−19.35). Statistically significant results were also found in leukoplakia versus OSCC (−22.80) and OSF versus OSCC groups (−28.80) [Table 2].

The box plot was used for comparison of median values of micronuclei among study groups. The median values of micronuclei were found to be distributed in the following order - OSCC > OSF > betel quid chewers > leukoplakia > oral lichen planus > control group [Figure 2].

Discussion

Oral exfoliative cytology is used in screening of OSCC and PMD patients. It reveals details of cellular alteration caused by potential carcinogens due to their genotoxic effect. Bloching et al. suggested micronuclei to be cellular alteration formed due to genotoxic effect of potential carcinogens which can be used as a prognostic biomarker of cancer. In a study conducted by the International Collaborative Project on Micronuclei Frequency in different populations and cell types, an increase in the micronuclei frequency in the target tissues and peripheral lymphocytes in cancer patients was found which further supports micronuclei to be a reliable cytogenetic biomarker.

In the present study, the median values of micronuclei were calculated. The median frequency of micronuclei was found to be significantly different in each study group. The highest median values were in OSCC group (10) followed by OSF (5), betel quid chewers (4), leukoplakia (2), oral lichen planus (1), and least in control group. This could be explained as a genotoxic effect of potential carcinogens present in the betel quid such as tobacco and reactive products released by calcium hydroxide from betel nut extracts.

Intergroup comparison was done using Dunn’s multiple comparison test. P < 0.05 was considered to be significant. Statistically significant results were found in control versus betel quid chewers group, OSF group, and OSCC with the highest significance between control and OSCC (−34.20) groups followed by control versus OSF (−26.40) and control versus betel quid chewers groups (−19.35). Statistically significant results were also found in leukoplakia versus OSCC (−22.80) and OSF versus OSCC groups (−28.80). These findings indicate a genotoxic damage of genotoxic agents and their metabolic products to exfoliated buccal epithelial cells. We found similar results in studies done by Parvathi et al. and Halder et al. In the past, there are studies reported with significant micronuclei values in PMDs as compared to normal participants. In our study, we also found similar results. Samantha and Dey have...
suggested that the chromosomal loss or breakage, chromosomal aberrations, mitotic apparatus dysfunction, aneuploidy, and genetic instability occurring in PMDs may induce the formation of micronuclei.\textsuperscript{[10]} In a study by Saran \textit{et al.}, a gradual increase in the frequency of mean micronuclei from control group to precancerous group and from precancer to cancer patients was reported.\textsuperscript{[17]} Our study results showed an increase in the mean micronuclei frequency from normal to precancerous to cancerous lesions which were similar to Casartelli \textit{et al.}, who suggested micronuclei to be a genomic biomarker in the carcinogenesis process.\textsuperscript{[21]}

**Conclusion**

In the present study, a significant increase in the median values of micronuclei in betel quid chewers compared to normal participants was observed. The median value of micronuclei was significantly higher in OSCC patients followed by PMDs participants was observed. The median value of micronuclei in betel quid chewers compared to normal participants was significantly higher in OSCC patients followed by PMDs and betel quid chewers as compared to normal participants. The present study concludes micronuclei to be a cytogenetic tool for assessing genotoxic damage induced by potential carcinogens present in betel quid, and it is a reliable genomic biomarker in the progression of carcinogenesis.

**References**
