Micronuclei as a Predictor for Oral Carcinogenesis

Kamini Kiran, Padmanidhi Agarwal¹, Shailesh Kumar¹, Kanav Jain¹
Department of Pathology and ¹Dentistry, All India Institute of Medical Sciences(AIIMS), Rishikesh, Uttarakhand, India

Abstract

Objectives: The aim of the study was to provide a quantitative evaluation of oral mucosal micronuclei (MN) frequency as a biomarker for oral cancer susceptibility in carcinogenesis progression. Methods: 60 patients were included in the study. 30 patients with biopsy proven epithelial dysplasia (ED, 15) and oral squamous cell carcinoma (OSCC, 15) comprised the study group and 30 patients with normal buccal mucosa, reporting for minor surgical procedures formed the controls. After informed consent, exfoliated cells were collected from the affected site using a premoistened wooden spatula and spread on precleaned slides, fixed, stained using modified rapid Papanicolaou method and subjected to microscopic examination. MN were identified and scored according to Tolbert et al. criteria. Results: Maximum patients with ED and OSCC were males and in age groups of 20–40 and 40–60 years, respectively. The most common site was the buccal mucosa. The maximum of MN count/500 cells in OSCC group was 11.93, 4.0 in ED and 1.46 in controls, with the mean and mean MN index ± SD distribution in the three groups showing high statistical significance ($P = 0.000$). A significant difference between mild and moderate ED and between moderately and well-differentiated OSCC was also observed. Conclusion: MN assays can help in early detection of premalignant and malignant lesions, thereby improving survival and reducing morbidity associated with treatment. MN index is thus a feasible and economical method for screening high-risk populations of oral cancer, to be able to timely identify genomic damage in order to prevent the cancer epidemic.

Keywords: Biomarkers, carcinogenesis, DNA damage, micronuclei, mouth neoplasms, precancerous conditions, squamous cell carcinoma

Introduction

Oral cancer is one of the most common cancer worldwide with about 5,75,000 new cases and 3,20,000 deaths occurring annually.[¹] Oral squamous cell carcinoma (OSCC) is an epithelial neoplasm with altered expression, focal clonal overgrowth of altered stem cells and disruption of normal function.[²] Visible physical changes at cellular level (atypia) and tissue level (dysplasia) can occur during progression to cancerous state like genetic/epigenetic changes, surface alterations, and intercellular interactions. These “precancerous” changes are of diagnostic and prognostic relevance, driving cells to neoplastic transformation.[³]

About half of the oral cancers in India have associated precancerous changes, characterized by complex karyotypes with chromosomal deletions, translocations, and structural abnormalities. This instability is contributed to environmental exposure to genotoxins, medical procedures, lifestyle, and genetic changes, reflected either as leukoplakia, erythroplakia, lichen planus, or submucous fibrosis.[⁴]

Diagnosis by cytological study of oral cells is nonaggressive and well-accepted, therefore an attractive option. Oral exfoliative cytology is particularly diagnostic for mass screening purposes with a sensitivity, specificity, and accuracy of 94%, 100%, and 95%, respectively.[⁵] Genotoxic risks can thus be assessed by indicators like deoxyribonucleic acid (DNA) damage and cytogenic markers, chromosomal aberrations, and sister chromatid exchanges.[⁶] Cells often have errors in chromosome segregation, forming a lagging chromosome that becomes lost during anaphase and is excluded from the reforming nuclei. These microscopically visible, round to oval cytoplasmic chromatin masses are observed as micronuclei (MN).[⁷]

MN count involves a rapid, efficient, and economical technique, providing a reliable quantitative analysis of the genotoxicity.[⁸] Significantly higher MN frequencies have been observed in people exposed to organic solvents, antineoplastic agents, DNA-damaging chemicals, and tobacco smoking. Overall, MN count is a valuable biomarker for oral cancer screening and diagnostic tool for identifying genetic lesions that develop over time.

Address for correspondence:
Dr. Kamini Kiran,
Department of Pathology, All India Institute of Medical Sciences(AIIMS), Rishikesh, Uttarakhand, India.
E mail: drkaminijha@gmail.com

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Materials and Methods

This study was undertaken to quantitatively analyze and compare MN counts in Papanicolaou (PAP) stained cytological smears. Sixty patients from the Out Patient Department of either gender, any age group, unaffected by diabetes or any other debilitating disease were included. Thirty patients with biopsy proven epithelial dysplasia (ED), and OSCC (15 patients each) comprised the study group. Controls consisted of 30 patients with normal buccal mucosa, reporting for minor surgical procedures. Ethical clearance was obtained for carrying out this study and written informed consent was taken from each participating patient after detailed explanation of procedure.

Exfoliated cells were collected from the affected buccal mucosa after rinsing mouth with tap water, using a clean premoistened wooden spatula and spread on two precleaned slides. Smears were fixed with 95% ethanol & 3% glacial acetic acid (Biofix spray), stained by PAP stain by modified rapid PAP method (Bio Lab Diagnostic) and subjected to microscopic examination. The most commonly used step ladder method was used for screening slides. Five hundred cells with intact nuclei and cell boundaries were counted on each slide. MN were identified and scored according to Tolbert et al. criteria.[9] Baseline frequency was 0.5–2.5 MN/1,000 cells.[10]

Statistical analysis was done using Statistical Package for the Social Science (SPSS 17.0) software using One Way Anova for carrying out this study and written informed consent was taken from each participating patient after detailed explanation of procedure.

Results

Maximum patients with ED were between 20 and 40 years (40%) and minimum above 60 (26.7%). Maximum patients with OSCC were between 40 and 60 years (53.3%) and minimum between 20 and 40 years (20%) [Table 1]. Maximum patients with ED and OSCC were males (93.3% and 80%, respectively).

The most commonly involved site of ED and OSCC was the buccal mucosa (66.7% and 46.7%, respectively). ED was also noted in the retromolar area (13.3%), labial mucosa (6.67%), palate, and tongue whereas OSCC on palate (33.3%), retromolar area (13.3%), labial mucosa (6.67%), and tongue [Table 1]. The maximum MN count/500 cells in OSCC group was 11.93, in ED was 4.0 and was minimum in the control group at 1.46. The mean of MN count/500 cells value of the three groups (control, ED, and OSCC) were, respectively, 0.51, 1.19, and 1.83, the difference being highly significant. The distribution of mean MN index ± SD was 1.46 ± 0.51, 4.0 ± 1.19 and 11.93 ± 1.83 in control, ED, and OSCC, respectively, the differences between them being highly significant [Table 2].

Maximum patients had mild ED (46.7%) and minimum had moderate ED (20%). Maximum patients of OSCC had well-differentiated OSCC (60%) and minimum had poorly differentiated OSCC (6.67%). The distribution of mean ± SD of MN count/500 cells in different grades of ED was maximum for moderate ED (4.6 ± 0.57) and minimum for mild ED (3.2 ± 0.83), showing significant difference between mild and moderate ED (P = 0.0369) [Table 3]. The distribution of mean MN count/500 cells in different grades of OSCC was maximum for moderately differentiated OSCC (13.2) and a high significance was found between well and moderately differentiated OSCC [Table 4].

Discussion

Oral carcinogenesis is a multi-step process of accumulated genetic damage. Cytogenetic markers like chromosomal aberrations, sister chromatid exchanges, and MN can be conveniently studied in the buccal mucosa in a minimally invasive manner, in easily

Table 1: Age and site distribution of epithelial dysplasia and oral squamous cell carcinoma

| Type of lesion | Age: 20-40 | Age: 40-60 | Age: >60 | Site of lesion: Buccal mucosa | Site of lesion: Labial mucosa | Site of lesion: Palate | Site of lesion: Tongue | Site of lesion: Retromolar area |
|----------------|------------|------------|----------|------------------------------|-----------------------------|-----------------------|------------------------|-----------------------------|
| Epithelial dysplasia | 6 (40) | 5 (33.3) | 4 (26.7) | 10 (66.7) | 1 (6.67) | 1 (6.67) | 5 (33.3) | 2 (13.3) |
| OSCC | 3 (20) | 8 (53.3) | 4 (26.7) | 7 (46.7) | 1 (6.67) | 1 (6.67) | 1 (6.67) | 1 (6.67) |

Table 2: Comparison of mean MN index between control, epithelial dysplasia, and oral squamous cell carcinoma

| Group   | MN count/500 cells | Mean | SD  | F      | P      | Post hoc t-value |
|---------|--------------------|------|-----|--------|--------|-----------------|
| Control | 22                 | 1.46 | 0.51|        |        | 5.375 (Control vs ED) |
| ED      | 60                 | 4.0  | 1.19| 260.09 | 0.000  | 16.780 (ED vs OSCC) |
| OSCC    | 179                | 11.93| 1.83|        |        | 22.155 (OSCC vs Control) |
Table 3: Mean MN count/500 cells grade wise in epithelial dysplasia

| ED Grade | n (%)  | MN count/500 cells (Mean ± SD) | P      | P between different grades |
|----------|--------|-------------------------------|--------|---------------------------|
| Mild     | 5 (33.3%) | 3.2±0.83                      | 0.1701 | NS                        |
| Moderate | 3 (20%)   | 4.6±0.57                      |        | 0.0369 (S- Mild vs Moderate) |
| Severe   | 7 (46.7%) | 4.2±1.38                      |        | 0.6578 (NS- Moderate vs Severe) |

*S-significant, NS: Not significant (P≥0.05=S)

Table 4: Mean MN count/500 cells in grades of oral squamous cell carcinoma

| OSCC Grade       | n (%)  | MN count/500 cells (Mean ± SD) | P      |
|------------------|--------|-------------------------------|--------|
| Well differentiated | 9 (60) | 10.8±1.16                     | 0.0040 |
| Poorly differentiated | 1 (6.67) | -                              |        |

- Since only 1 patient had Poorly differentiated OSCC, test values could not be applied; HS: Highly significant

Comparison between controls, ED, and OSCC of mean MN count/500 cells showed highly significant difference (P = 0.000). The mean MN count/500 cells in 20 cases was 83 ± 1.593. Mean MN% ± SD were 1.843 ± 0.467 in 30 cases of OSCC and 0.210 ± 0.168 in 20 cases of control group, in accordance with prior studies. MN count/500 in control, ED and OSCC groups were 22, 60 and 179 respectively, in accordance with that seen in literature. The MN frequency in controls was significant and similar to those in earlier studies with P < 0.001 and those in OSCC with P < 0.05.

The frequencies of MN were increased by almost twofold in the premalignant group and more than thrice in the malignant group, when compared to the control group. The mean MN in 13 controls was 21.38 + 6.09 and in 13 OSCC cases was 143.61 + 47.365.

The highly significant difference in mean MN count/500 cells between control and ED, control and OSCC and OSCC and ED (P = 0.000) indicates the importance of MN evaluation for prediction of genotoxicity. The mean ± SD of MN count/500 cells in different grades of ED showed statistical significance only between moderate (maximum mean MN count) and mild ED. The mean ± SD of MN count/500 cells in different grades of OSCC showed high significance between well and moderately differentiated OSCC (P = 0.004). The micronucleus frequencies have been found to increase from grade I to grades II (P < 0.001) and III.

PAP, a multichromatic cytological staining technique, was used for multiple comparisons among the different groups, being the easiest and most preferred method in field studies for scoring and detecting MN in the cells of the buccal mucosa for the diagnosis of malignant neoplasms.

The gradual increase in MN frequency from normal mucosa to precancerous lesion to carcinoma has suggested a link of this biomarker with neoplastic progression. Assessment of MN in exfoliated cells is a promising tool for the study of epithelial carcinogens. The minimal invasiveness of cell collection,
low cost, ease of storage, and slide preparation make the MN assay with buccal epithelial cells the ideal choice for molecular epidemiological studies. High reliability and low cost of MN technique contributes to its success in acknowledgment of genome damage and can be used for the early detection of carcinogenic effects in cells exposed to carcinogenic agents, occupational and lifestyle changes, and dietary factors. MN counts were uniformly elevated in all histologic grades of OSCC and ED, suggesting a strong correlation between cytogenetic damage of the oral epithelium with genotoxic and carcinogenic agents. Hence, the MN assay can be used as a biomarker of genotoxicity (acting as an internal dosimeter for carcinogenic damage)[11] in predicting the effects of cancer intervention and as an educational tool. However, further studies with large sample size should be carried out to support these findings.

Early detection of premalignant and malignant lesions would improve survival and also reduce the morbidity associated with the treatment considerably. MN assays in oral exfoliated cells represent a preferred target site for precocious genotoxic events during carcinogenesis and are indispensable early markers of chromosomal damage in oral premalignancies and squamous cell carcinoma with prediction of their histopathologic grade. The highly significant difference in mean MN count/500 cells between normal mucosa, epithelial dysplasia, and OSCC thus identifies a feasible and economical method for screening high-risk populations for oral cancer, to be able to timely introduce interventional strategy for the cancer epidemic.

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**Conflicts of interest**
There are no conflicts of interest.

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