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Original Research Article

Micronucleus Assay as Potential Biomarker – A Fluorescent Microscopic Study

Dr. Shruti Singh[1], Dr. Satish B.N.V.S[2], Dr. Prashant Kumar[3]
[1]Post-Graduate Student, HKES's S. Nijalingappa Institute of Dental Sciences & Research, Kalaburagi, Karnataka, India
[2]Professor, HKES's S. Nijalingappa Institute of Dental Sciences & Research, Kalaburagi, Karnataka, India
[3]Reader, HKES's S. Nijalingappa Institute of Dental Sciences & Research, Kalaburagi, Karnataka, India

***Corresponding author**
Dr. Shruti Singh
Email: singhshruti1009@gmail.com

Abstract: Biomonitoring of neoplasia has been one of the most challenging and upcoming issues in modern medicine. The relevance of biomarker in oral cancer has been extensively studied. Micronuclei are cytoplasmic chromatin masses with the appearance of small nucleus that arise from lagging chromosome at anaphase or from acentric chromosome fragments. Micronuclei can be a potential tool to reflect the cytogenotoxic damage in oral cancer and pre-malignant conditions such as oral submucous fibrosis. The present study aims at comparing the cytogenotoxicity in patients with oral cancer, oral sub mucous fibrosis and normal inhabitant by analyzing the frequency of micronucleated cells in the exfoliated buccal epithelial cells. The present study comprises of three groups: Group A: 20 Normal healthy individuals; Group B: 20 patients with Oral Submucous Fibrosis; Group C: 20 patients with Oral Squamous Cell Carcinoma. Smears made from exfoliated buccal epithelial cells and stained with Acridine-Orange stain and frequency of micronuclei were analyzed per 1000 cells. The statistical analysis of the resulting data was done using One-way Anova test and Kruskal-Wallis Analysis of variance. The mean micronuclei frequency in Group A was 0.70±0.733; Group B was 12.20±5.67 and in Group C was 26.30±6.07 cells per 1000 cells. The mean difference between the three groups was statistically significant ($p < 0.05$). The oral mucosal micronuclei frequency may be a marker of epithelial carcinogenic progression.
Keywords: Oral Cancers, Oral Submucous fibrosis and Micronuclei

INTRODUCTION

Cancer, even after decades, remains one of the leading causes of death in the world. Oral cancer is eleventh most common cancer as reported by World Health Organization (WHO). Every year 5,75,000 new cases are diagnosed and death rate of 3,20,000 occur worldwide [1]. The etiological agents such as chemical carcinogens or environmental pollutants causes genotoxic damage in the cells and exhibits altered expression of cancer cells and abnormal growth and abnormal disruptions of normal cells which leds to genomic instability and reflects in its primary stage as potentially malignant disorders and eventually into malignancy.

Human Biomonitoring requires accurate, sensitive and, possibly, easy and not time-consuming methodologies to assess mutations. Formation of micronuclei is an end point of chromosomal damage or segregation errors and reflects a genotoxic or carcinogenic exposure [2]. These damaged chromosomes appear either in the form of acentric chromatids or chromosome fragments, which lag behind in anaphase. These lagging elements are included in the daughter cells with the size smaller than the principal nucleus, hence, called micronuclei. Bigger micronuclei result from exclusion of whole chromosome after spindle apparatus damage (aneugenic effect), whereas the smaller micronuclei result from structural aberrations, resulting in chromosomal fragments (clastogenic effect). The micronuclei assay (MN) in exfoliated buccal cells is an innovative technique, which holds promise for the study of epithelial carcinogens [3].

The aim and objective of our study was to calculate and correlate the frequency of micronuclei in normal oral mucosa, oral submucous fibrosis and oral squamous cell carcinoma.

MATERIAL AND METHODS

This was a prospective observational study. Institutional ethical approval was duly obtained. The study was conducted at Department of Oral Medicine & Department of Oral Pathology & Microbiology of HKES's S. Nijalingappa Institute of Dental Sciences, Kalaburagi (Karnataka, India)

The study comprised of 60 subjects. Patient's demographic profile were noted, brief history was taken including history of relevant risk factors and habits. Clinical examination was performed. Biopsy in the required cases was taken.Subjects were divided in three groups: Group A: Healthy subjects (n=20) with no oral lesions and no habits; Group B: Patients with Oral Submucous Fibrosis (n=20); Group C: Patients with Oral squamous cell carcinoma (n=20).

Subjects with age above 18 years of either gender with no systemic illness were included in the study. The control group exhibits no morbidity and only established cases of Oral submucous fibrosis (OSMF) and Oral Squamous cell carcinoma (OSCC) cases were included. Patients with systemic disease, radiation therapy, those who were on long-term steroid therapy or occupational exposure were excluded from the study.

The subjects were asked to rinse their mouth with water before collecting specimens. A pre-moistened wooden spatula was used to collect sample cells from the oral mucosa. The spatula was then applied to a pre-cleaned microscope slide. Smears were immediately fixed in iso-propyl alcohol. Slides were stained by the Acridine orange stain. Staining was done as per the protocol of Von Bertalanaffy *et al* [4]. The slides were examined under microscope (Olympus BX 41). The MN frequency was then scored using the criteria described by Tolbert *et al.* with slight modifications [3, 5] [Table-1].

Table 1: MN frequency criteria

Cell Criteria:	**Micronucleus Identifiction Criteria:**
1. Intact cytoplasm and relatively flat cell position on the slide. 2. Little or no overlap with adjacent cell. 3. Little or no debris. 4. Nucleus normal and intact, nuclear membrane smooth and distinct.	1. Rounded smooth perimeter suggestive of a membrane. 2. $1/3^{rd}$ - $2/3^{rd}$ of diameter of associated nucleus, but large enough to discern shape and size. 3. Staining intensity similar to nucleus. 4. Texture similar to nucleus. 5. Same focal plane as nucleus. 6. Absence of overlaps or bridge to nucleus. 7. Feulgen positive

Statistical evaluation was done using Analysis of variance for scale and ordinal variance. One-way Anova test was used to compare the age and Kruskal-Wallis test was applied to compare the number of micronuclei between the groups. SPSS version 17 Software was used and significant value was considered when $p>0.05$.

RESULTS

Our study comprised of sixty individuals, which were divided into three groups. Group A having controls has 11 males and 9 females; Group B consists of 20 males and no females and Group C constitute 16 males and 4 females. (Figure-1) The control had an average age of 52.1 years (age range 38-66 years), Group B had mean age of 26.5 years (age range 20-35 years) while Group C had an average age of 42.3 year (age range 21-65 years) (Figure-2) The mean micronuclei frequency in Group A was 0.70 ± 0.733; Group B was 12.20 ± 5.67 and in Group C was 26.30 ± 6.07 cells per 1000 cells.

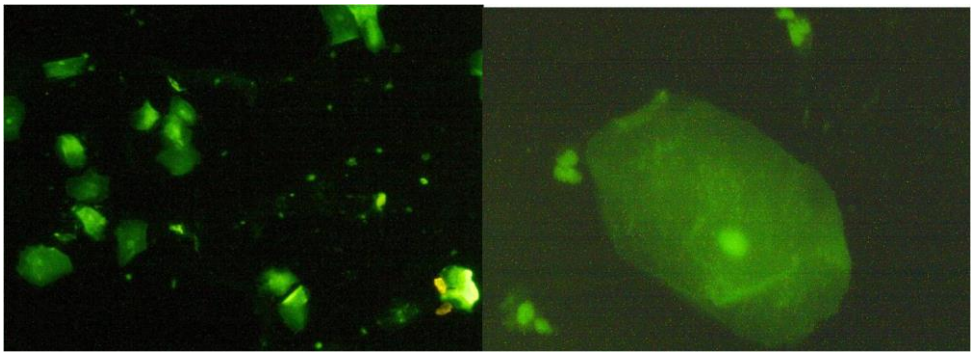

Fig-1A & B: Fluorescence microscopy images of micronuclei in normal Oral mucosa induced by Acridine-Orange stain B. Micronucleated cells (MN)

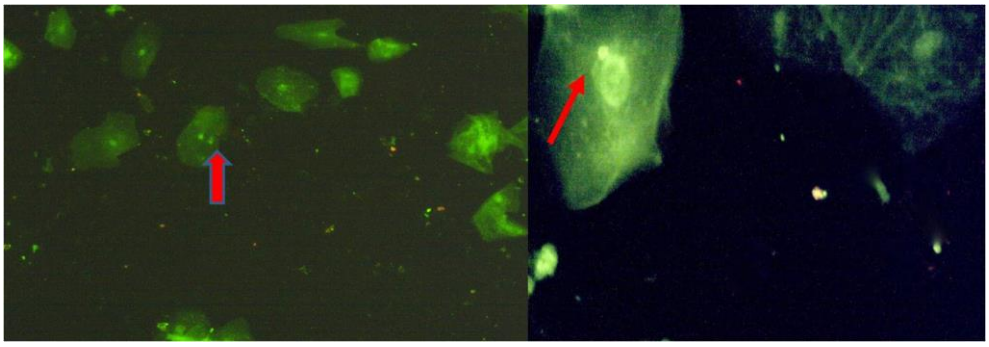

Fig-2A & B: Fluorescence microscopy images of micronuclei in Oral Sub Mucus Fibrosis (OSMF). B. Arrowhead shows Micronucleated cells (MN)

The mean MN frequency difference between the three group was statistically significant ($p<0.05$) [Figure-3]

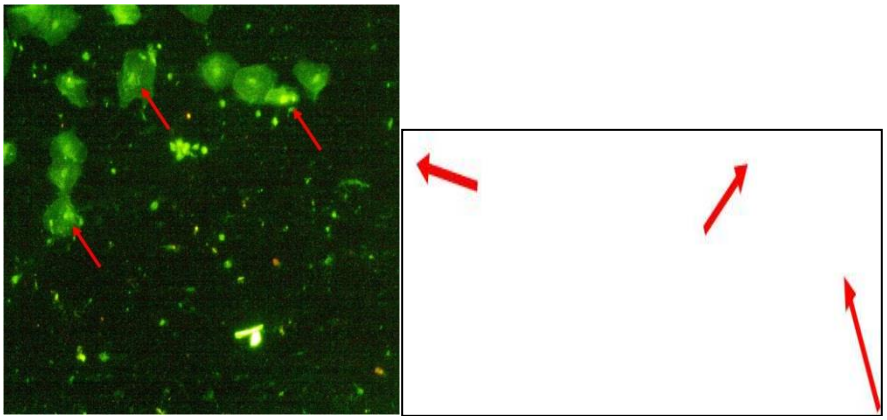

Fig-3A & B: Fluorescence microscopy images of micronuclei in Oral Squamous Cell Carcinoma (OSCC) show multiple Micronucleated cells (MN) (Arrowhead)

DISCUSSION

Genomic damage is considered to be the most important aspect, which reflect the association with carcinogenesis. Molecular epidemiology research focuses on three types of biomarkers: 1.Biomarkers of exposure (e.g., cytogenetic endpoints-chromosomal aberrations, micronuclei, and sister chromatid exchanges), 2. Biomarkers of susceptibility (e.g., genetic polymorphisms), 3. Biomarkers of disease (e.g., tumor biomarkers).

Micronuclei is a microscopically visible, round or oval cytoplasmic chromatin mass in the extra nuclear vicinity, originated from aberrant mitosis [6]. Unstable chromosome aberrations can be studied in epithelial cells by the detection of MN and other nuclear aberrations in exfoliated interphase cells [7]. Micronuclei are suitable internal dosimeters for revealing tissue-specific genotoxic damage in individuals exposed to carcinogenic mixtures [8].

Micronuclei have been used since 1937 as an indicator of genotoxic exposure, based on the radiation studies conducted by Brenneke and Mather [9]. Various groups have found analysis of MN in buccal cells to be a sensitive method for monitoring genetic damage in human populations [10-12].

Parvathi devi *et al* [13] found step wise increase in frequency of MN cells. Normal (0.06%), precancerous (0.12%) and cancerous (0.45%), which indicates cytogenetic damage to epithelium. Haldar *et al* [1] found MN frequency in precancerous (0.63%) and cancerous (1.36%). Palve and Tupkari [14] also concluded that gradual increase of MN from normal (0.21%) to cancerous lesions (1.84%). Casartelli *et al* [15] observed MN frequencies in exfoliated buccal cells in normal mucosa, precancerous lesions and squamous cell carcinoma. They concluded that the gradual increase in MN counts from normal mucosal to precancerous lesions to carcinoma suggested correlation of micronuclei as biomarker with neoplastic progression.

Kalita *et al* [16] observed increased micronuclei count (4.2 ±0.96 in females and 6.6±1.95 in males) in buccal cells of betel quid chewers. Jyoti *et al* [17] conducted a study on 25 OSMF patients (gutka chewers along with smoking) using acridine orange

stain found a significant increase in MN count when compared to gutka chewers and control group.

In our present study the oral mucosal MN frequency in the control population was 0.7%(males 0.39%, females 0.31%). In subjects with OSMF, the MN frequency was 1.22% (males). In OSCC patients, the MN frequency was 2.66% (males 2.11%, females 0.55%).

In the present study, we obtained higher level of micronuclei in all the three groups when compared with other studies. This could be attributed to the fact that we followed fluorescent acridine orange staining method and the analysis was done under fluorescent microscope, which increases the specificity to identify DNA containing structures and making micronuclei identification easier due to increased fluorescence. Our findings were consistent with previous studies and implicates that there is a gradual increase in MN count from control to OSMF and OSCC subjects.

CONCLUSION

Although our numbers are small, the oral mucosal MN frequency may be a marker of epithelial carcinogenic progression. Further studies with large sample size are required for determining its usefulness in this role.

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