Utility of DNA-Specific Stains in Micronuclei Assay as a Marker of Genotoxicity in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma

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Abstract

Background: The term oral potentially malignant disorder (OPMD) was recommended to refer to precancer as it conveys that not all disorders described under this term may transform into cancer. Oral squamous cell carcinoma (OSCC) arises through an accumulation of genetic alterations, deoxyribonucleic acid (DNA) changes, and epigenetic alterations. Thus, a simple yet a sensitive and specific test for early diagnosis is the need of an hour. The micronuclei (MN) assay in exfoliated epithelial cells is potentially an excellent biomarker to detect chromosome loss or malfunction of mitotic spindle. Aim of the Study: To compare the frequency of MN in exfoliated cells from oral mucosa exposed to genotoxic agents using different staining procedures and to observe the incidence of micronucleus in potentially malignant and malignant lesions. Materials and Methods: The study was undertaken to observe the cytogenetic damage in the exfoliated buccal cells of 75 cases of tobacco-related PMDs, OSCC and control subjects (25 cases from each group) and were evaluated with nonspecific May-Grünwald Giemsa stain and DNA-specific Feulgen stain. The results were statistically determined using SPSS version 17.0. Results: Correlation analyses in the present study depicted that MN frequency was significantly more in oral squamous cell carcinoma than OPMDs and normal group (P < 0.05). Giemsa-stained slides correlated significantly with karyorrhexis, karyolysis, condensed chromatin, and binucleates, whereas no such correlations were found with DNA-specific stains. Conclusion: Malignant transformation is accompanied by loss of cell capacity to evolve to death in situations of DNA damage. These findings indicate that nuclear anomalies may be misinterpreted as MN with nonspecific DNA stains and lead to false-positive results in studies with cells of epithelial origin.

Keywords: Feulgen stain, Giemsa stain, Oral potentially malignant disorders, Oral squamous cell carcinoma

Introduction

Genomic damage is considered to be the most important cause of developmental and degenerative diseases. It is also well known that genetic damage is produced by genotoxins, various medical procedures that include radiation and chemicals, micronutrient deficiency, lifestyle factors, and genetic factors such as inherited defects in DNA metabolism or repair.[1]

It is now well established that micronuclei (MN) mainly originate from a centric chromosome fragments, acentric chromatid fragments, or whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during mitosis because they did not attach properly with the spindle during the segregation process in anaphase. These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei after conventional nuclear staining.[2]

The proportion of basal cells and cells undergoing cell death in buccal mucosa is an indication of the regenerative capacity of this tissue. To evaluate the genotoxic risks, DNA damage can be assessed by cytogenetic markers such as chromosomal aberrations, sister chromatid exchanges, and MN assay which is employed to detect aneuploidy, chromosomal breaks, and changes in telomere length. MN are microscopically visible,

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round or oval cytoplasmic chromatin masses in the extranuclear vicinity that originate from aberrant mitosis.[1]

MN were originally identified and described in erythrocytes by the hematologists William Howell and Justin Jolly and were later found to be associated with deficiencies in vitamins, such as folic acid and vitamin B12.[2]

MN and other nuclear abnormalities are biomarkers of genotoxic events and chromosomal instability and are collectively measured in micronucleus cytome assay. The molecular mechanisms behind these events have been investigated using molecular probes and genetically engineered cells. The buccal cell micronucleus assay was proposed in 1983; thereafter it gained popularity as a biomarker of genetic damage in numerous applications. Peripheral blood lymphocytes have also been used for micronucleus approach in the past.[1]

There are numerous staining methods out of which DNA-specific stains are ideal for staining. Among them, the most widely used are Feulgen reaction followed by counterstaining with fast green to delineate cell cytoplasm. Acridine orange can also be applied with MN fluorescing bright green. The other stains include diamidino-2-phenylindole, propidium iodide, Papanicolaou stain, Hoescht, May-Grünwald Giemsa stain, and orcein.[1]

The turnover rate for the appearance of MN in exfoliated buccal cells in an otherwise normal cell after exposure to an acute genotoxic event was estimated to be a minimum of 5–7 days. As the intralaboratory and interlaboratory variations exist in studies for MN in exfoliated cells, efforts were made to standardize the assay. Because the first publications of Stich and Rosin are the basic criteria for identification of MN, a number of studies have been done by different authors to standardize the MN identification criteria.[1]

Oral carcinomas are characterized by complex karyotypes that involve many chromosomal deletions, translocations, and structural abnormalities. Cells often have errors in chromosome segregation that lead to the formation of a lagging chromosome or chromosome parts that become lost during the anaphase segregation that lead to the formation of a lagging chromosome. The laggards are observed in the cytoplasm as MN.[3]

The assessment of MN in exfoliated cells is a promising tool for the study of epithelial carcinogens and can be used to detect chromosome breakage or mitotic interference, thought to be relevant to carcinogenesis. The direct correlation between the MN formation and genomic damage makes the MN assay an efficient alteration to the metaphase analysis.[3,4] It has been used in the detection of oral squamous cell carcinoma (OSCC) with 94% sensitivity, 100% specificity, and an accuracy of 95%.[5]

It has been well established by researchers that virtually all oral cancers are preceded by visible clinical changes in the oral mucosa usually in the form of white or red patch. Prevention and early detection of such oral potentially malignant disorders (OPMDs) have the potential of not only decreasing the incidence but also improving the survival of those who develop oral cancer. OPMDs mainly include leukoplakia, erythroplakia, lichen planus, oral submucous fibrosis, which have the capability of transforming into malignancy if not caught at the right time.[4]

Exfoliated buccal cells have been used noninvasively to show the genotoxic effects of lifestyle factors, such as tobacco smoking, chewing of betel quids, radiotherapy, and occupational exposure to potentially mutagenic and/or carcinogenic chemicals. Hence the present study was designed to compare the frequency of MN in exfoliated cells from oral mucosa exposed to genotoxic agents using DNA-specific (Feulgen) and nonspecific (Giemsa) stains and to determine the qualitative efficacy of both the stains in assessment of MN in exfoliated oral mucosal cells and clinicopathological correlation of MN frequency in exfoliated oral mucosal cells.

Materials and Methods

This cross-sectional and observational study comprised 150 smears from 75 cases (25 smears each from potentially malignant disorders, oral cancer, and healthy subjects) and the smears were taken from the subjects with a habit of chewing and smoking tobacco for a minimum of past 10 years to observe the cytogenetic damage in the exfoliated buccal cells and is evaluated with nonspecific Giemsa stain and DNA-specific Feulgen stain.

Subjects were asked to rinse their mouth thoroughly with water before the smear was taken. A wet wooden spatula was used to scrape the buccal mucosa vigorously from the patient’s mouth. Scraped material was spread on the precleaned slides and fixed with Bio fix spray and air dried. Giemsa and Feulgen were used to stain the prepared slides and were viewed under light microscope at 100 × magnification (oil immersion) to count the MN, and 1000 cells were observed randomly at 100 × magnification using oil immersion.

MN were observed and recorded in exfoliated oral epithelial cells according to the criteria defined by Tolbert et al.[6] Only those MN were scored, which were round or oval in shape with a smooth perimeter suggestive of membrane, less than one-third of the diameter of the main nucleus, of the same texture, color, and rarefaction as the main nucleus with no overlap or bridge

| Table 1: Working parameters for qualitative analysis of staining under light microscope |
|---------------------------------------------|------------------|
| Parameter                          | Scoring characteristics |
| Background of the smear                | Clear            |
| Overall staining pattern               | Fair             |
| Nuclear morphology                    | Not preserved    |
| Nuclear characteristics                | Smudgy  

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Table 2: Clinicopathological profiling of the patients under study groups

| Study groups | Mean age (years) | Gender | Buccal mucosa | Tongue | Palate | Labial mucosa | Alveolar mucosa |
|--------------|-----------------|--------|---------------|--------|--------|---------------|----------------|
| Normal (n=25) | 18.68            | 11     | 14            | 16     | 4      | -             | 5              |
| OPMDs (n=25)  | 39.74            | 21     | 4             | 19     | 2      | 3             | 3              |
| OSCC (n=25)   | 55.58            | 19     | 6             | 15     | 3      | 1             | 4              |

OPMD: Oral potentially malignant disorder, OSCC: Oral squamous cell carcinoma

Table 3: Mean number of micronuclei and micronucleated cells using Feulgen stain

| Study group | Mean number of micronucleated cells | Mean number of micronuclei | Micronucleus index |
|-------------|-------------------------------------|---------------------------|--------------------|
| Normal (n=25) | 0.40±0.764                         | 0.44±0.821                | 1.00±0.534         |
| OPMDs (n=25)  | 4.36±2.361                         | 6.40±3.240                | 1.60±0.65          |
| OSCC (n=25)   | 13.3±4.742                         | 21.64±7.348               | 1.66±0.383         |
| P            | <0.05                               | <0.05                     | >0.05              |

OPMD: Oral potentially malignant disorder, OSCC: Oral squamous cell carcinoma

Table 4: Mean number of micronucleus and micronucleated cells using Giemsa stain

| Study group | Mean number of micronucleated cells | Mean number of micronuclei | Micronucleus index |
|-------------|-------------------------------------|---------------------------|--------------------|
| Normal (n=25) | 0.68±1.108                         | 1.08±1.631                | 1.608±0.926        |
| OPMDs (n=25)  | 7.00±2.661                         | 10.76±4.798               | 1.58±0.672         |
| OSCC (n=25)   | 17.52±6.138                        | 26.60±8.160               | 1.560±0.349        |
| P            | <0.05                               | <0.05                     | >0.05              |

OPMD: Oral potentially malignant disorder, OSCC: Oral squamous cell carcinoma

The results of MN index in study groups were statistically nonsignificant, P > 0.05 [Tables 3 and 4]. Additional nuclear features such as binucleation, condensed chromatin, and karyorrhexis were seen in smears of patients with OSCC and OPMD [Figure 1]. Qualitative assessment comparing DNA–specific (Feulgen) and nonspecific (Giemsa) stains, Feulgen stain proved to be superior as compared to Giemsa stain in terms of background staining, overall staining, and nuclear characteristics [Table 5]. Age, gender, and site did not significantly affect the MN frequencies with any of the staining methods, and these variables had also no effect on the differences between OSCC and OPMD.

**Discussion**

Despite commendable progress in the prevention, detection, and treatment of a wide variety of solid tumor types, OSCC remains a significant health burden across the globe. OSCC is often first diagnosed at late stages of the disease (advanced regional disease and/or metastasis). Delayed diagnosis precludes successful treatment and favorable outcomes. Complexity of the cancer could be attributed to its altered expression, abnormal growth, and invasion of tissues and disruption of normal functioning, which probably results from genomic instabilities caused by various chemicals or environmental carcinogens/mutants. It is also well known that genetic damage is produced by genotoxins, various medical procedures that include radiation and chemicals, micronutrient deficiency, lifestyle factors, and genetic factors such as inherited defects in DNA damage or repair. In clinical practice, opportunities exist to identify patients with OPMDs, which precede the development of cancer.

Among various chairside investigations of the lesion at early stage is MN assay using exfoliative cytology. MN assay is a sensitive, noninvasive, and inexpensive technique that offers a very simple method for obtaining information on status of the epithelial cells, particularly DNA damage, proliferative potential of basal cells, and cell death, which can be performed using exfoliative cytology.

Although it has been shown that increased MN frequency is observed in the buccal cells of populations exposed to occupational and environmental insults, various lifestyle factors, radiation and oral cancer, but the magnitude of changes is usually relatively small. Simplicity, accuracy, multipotentiality, and large tissue applicability of the MN
stain, hence along with MN, few keratin granules also take up the stain giving false positively high counts. These round cytoplasmic bodies, which are formed as a consequence of cell injury, do not contain DNA and may be classified as MN with nonspecific stains.

**Conclusion**

Assessment of MN assay in potentially malignant and OSCC subjects can be used as an efficient marker for genotoxic damage. Hence, MN estimation using exfoliative cytology of the lesion as a chairside investigation should be done routinely in the clinics, as it provides promising results when analyzed using DNA-specific stains. Our observations indicate that the results of earlier MN studies in cells of epithelial origin should be interpreted with caution when nonspecific DNA stains were used.

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**Conflicts of interest**

There are no conflicts of interest.

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