Micronucleus as Potential Biomarker of Oral Carcinogenesis

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INTRODUCTION

Oral Squamous cell carcinoma encompasses at least 90% of all oral malignancies.¹ It is considered the sixth most common malignancy, and is a major cause of cancer morbidity and mortality worldwide. Globally, about 5,00,000 new oral and pharyngeal cancers are diagnosed annually, and three quarters of these are seen in the developing world, including about 65,000 cases reported in India.² Oral squamous cell carcinomas (OSCC) continue to portend a poor prognosis, with an estimated 5-year overall survival of 56%.³ A fundamental factor in the poor prognosis of oral squamous cell carcinoma is that great proportions of oral cancers are still diagnosed in advanced stages and are treated late. Early detection of a premalignant or cancerous oral lesion would improve the survival to a greater extent and also will reduce the morbidity associated with the treatment to a considerable extent. Though histopathology of biopsied material is a gold standard in diagnosing cancers; biopsy being an invasive technique, it has limitations for some professionals and psychological implications for some patients. In the last few years the interest for oral cytology as a diagnostic, prognostic methodology, for monitoring patients in oral precancer and cancer has thus re-emerged substantially ⁴.

Genotoxic damage and Biomarkers

It is generally accepted that oral carcinogenesis is a multi-step process of accumulated genetic damage leading to cell dysregulation with disruption in cell signalling, DNA-repair and cell cycle which are fundamental to homeostasis.⁵ These events can be conveniently studied in the buccal mucosa, which is an easily accessible tissue for sampling cells in a minimally invasive manner and does not cause undue stress to study subjects.⁶,⁷,⁸,⁹,¹⁰ This method is increasingly being used in molecular epidemiological studies to investigate the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage and cell death.¹¹,¹²,¹³,¹⁴,¹⁵,¹⁶ Exfoliative cytology of buccal mucosa in cases of oral cancers has recently been successfully used to identify various mutations in important genes using

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ABSTRACT

In spite of several research breakthroughs, the outcome of oral cancers has not significantly improved despite recent advances in surgery, radiotherapy and chemotherapy. Yet oral cancer can be cured if treated early enough. Detection and quantification of certain 'biomarkers' in non-invasive and painless procedures such as oral exfoliative cytology can help detect high-risk patients and also help improve patient compliance to a larger extent. There has been a recent surge of interest generated in one such promising marker, the 'micronucleus'. The following review describes the various aspects of micronuclei and their importance as biomarkers in assessing the clinical course of oral precancers and early invasive cancer.

Key words: Micronuclei, Oral Cancer, Genotoxic damage
a polymerase chain reaction and other techniques and also to quantify formation of DNA-carcinogen adducts. The present goal in many research laboratories is to develop screening strategies indicating individual cancers with certain biomarkers. Biomarkers are instruments of individual tumor prevention and help to detect high-risk patients. They allow statements concerning environmental and occupational exposition and further give information on the status of susceptibility. Biomarkers are divided in three groups: the first to define the exposure to carcinogenic agents, the second to show biological effects on the target tissue and the third to give information about the individual susceptibility.

**Micronuclei: Formation & Identification**

Micronuclei (MN) are one of such biomarkers that are cytoplasmic chromatin masses with the appearance of small nuclei that arise from lagging chromosomes at anaphase or from acentric chromosome fragments. These are formed by chromosomal damage in the basal cells of the epithelium. When these cells divide, chromosomal fragments (or entire chromosomes which lack attachment to the spindle apparatus) lag behind and are excluded from the main nuclei in the daughter cells. These fragments form their own membranes and appear as feulgen-specific bodies, termed micronucleus in the cell cytoplasm. Criteria for identifing micronuclei as given by Heddle & Countryman (1976) are:

1. Diameter less than 1/3rd the main nucleus.
2. Non-refractility (to exclude small stain particles).
3. Colour same as or lighter than the nucleus (to exclude large stain particles).
4. Location within 3 or 4 nuclear diameters of a nucleus; and not touching the nucleus (to make frequency measurements meaningful).
5. No more than 2 micronuclei associated with one nucleus.

Baseline frequencies for micronucleated cells in the BM are usually within the 0.5-2.5 Micronuclei/1,000 cells range. Cells with multiple micronuclei are rare in healthy subjects but become more common in individuals exposed to radiation or other genotoxic agents.

The use of micronuclei as a measure of chromosomal damage in peripheral blood lymphocytes was first proposed by Countryman and Heddle in 1976 and subsequently improved with the development of the cytokinesis-block micronucleus (CBMN) method, which allowed micronuclei to be scored specifically in cells that had completed nuclear division. As a consequence the assay has been extensively used to evaluate the presence and the extent of chromosome damage in human populations exposed to genotoxic agents in various occupational settings, in the environment, or as a consequence of lifestyles. Subgroups of the general population are considered at risk because of their genetic make-up or because certain diseases affect them, have also been evaluated, to validate this biomarker as a predictor of adverse health effects.

The pattern of micronucleus formation in an individual is strongly dependent upon the type of carcinogen exposure he/she is receiving. A different
pattern will be produced in a tissue that receives a single, short-term exposure, as opposed to a chronic, uniform exposure. Alternatively, carcinogen exposure could be repetitive but erratic, with intervals of several days occurring between exposures. Micronucleus frequencies would vary significantly between samples. Such pattern would also be produced if large differences occurred in the dosage of the carcinogen in repeated exposures. As micronuclei tend to decrease in frequency with time as chromosomal damage leads to cell death or micronuclei are lost during cell division. It seems likely that cells with more chromosomal damage, and hence more micronuclei, would be lost at a higher frequency than those with less damage.

The assessment of micronuclei in exfoliated cells is a promising tool for the study of epithelial carcinogens. The technique involves examination of epithelial smears to determine the prevalence of cells containing micronuclei, extranuclear bodies composed of chromosomes or chromosomal fragments that failed to be incorporated into daughter nuclei at mitosis. The assay can be used to detect chromosome breakage or mitotic interference, thought to be relevant to carcinogenesis.

Other Nuclear Abnormalities

Exfoliated cell micronucleus assay also demonstrates certain background prevalence’s of nuclear anomalies. The prevalence of each of these anomalies is comparable to the prevalence of MN. Illustrates some of the pathways that are postulated to produce extranuclear DNA-containing bodies, only a subset of which is micronuclei formed by the classical mechanisms. The other processes that can give rise to objects difficult to distinguish from micronuclei are important in their own right as they may reflect events relevant to carcinogenesis. The following nuclear abnormalities can be observed in exfoliated smears (Figure 4) and (Figure 5).

1. Binucleated cells (BN), or the presence of two nuclei within a cell. They are probably indicative of failed cytokinesis following the last nuclear division in the basal cell layer. It has recently been shown that chromosomal non-disjunction occurs with a higher frequency in binucleated cells that fail to complete cytokinesis, rather than in cells that have completed cytokinesis. This mechanism identified recently is thought to be a cytokinesis checkpoint for aneuploid binucleated cells. The binucleate: mononucleate cell ratio may therefore prove to be an important biomarker for identifying individuals with a cytokinesis failure caused by higher-than-normal rates of aneuploidy, such as that observed in Down’s syndrome.

2. Broken-Eggs (BEN) or Cells with nuclear buds contain nuclei with an apparent sharp constriction at one end of the nucleus suggestive of elimination of nuclear material by budding. The mechanism leading to nuclear bud formation is not known but it may be related to the elimination of amplified DNA or DNA repair.

3. Pyknosis, or shrunken nuclei, are characterized by a small shrunken nucleus, with a high density of nuclear material that is uniformly but intensely stained. They may represent an alternative mechanism of nuclear disintegration that is distinct from the process leading to the condensed chromatin and karyorrhectic cell death stages.

4. Condensed chromatin, in which the cells show a roughly striated nuclear pattern in which the aggregated chromatin is intensely stained.

5. Karyorrhexis, or nuclear disintegration involving loss of integrity of the nucleus. Nuclei that are characterized by more extensive nuclear chromatin aggregation relative to condensed chromatin cells. They have a densely speckled nuclear pattern indicative of nuclear fragmentation leading to the eventual disintegration of the nucleus.

6. Karyolysis, or nuclear dissolution, in which a Feulgen-negative, ghost-like image of the nucleus remains.
When the target tissue of interest is epithelial tissue, the exfoliated cell micronucleus assay has advantages over the more widely used micronucleus test in lymphocytes. While lymphocytes must be stimulated to undergo mitosis, introducing certain problems of interpretation, epithelial cells do not need to be stimulated; micronuclei in exfoliated cells reflect genotoxic events that occur in the dividing basal cell layer 1 – 3 weeks earlier. Furthermore, at many sites, the technique is completely noninvasive and repeated sampling is acceptable. Sites that avail themselves for study using this assay include the oral cavity, the nasal cavity, the bronchi, the esophagus, the cervix, the bladder and the urinary tract.

Stains & Staining characteristics

The most widely used procedure for staining epithelial cell preparations for MN analysis involves a Feulgen reaction to identify the DNA of the nucleus and micronucleus, followed by a counterstain with Fast Green to delineate cell cytoplasm. The method of Feulgen & Rossenbeck (1924) is the standard technique for demonstrating deoxyribose. Acridine orange fluorescent staining can also be applied to micronucleus test with MN fluorescing bright green and are thus distinguished from MN-like inclusions or contaminants. Micronucleus assay can also be performed by using the fluorescence in situ hybridization (FISH) with a centromeric probe which is observed as very bright yellow-green spots in the red nucleus or MN. The main advantage of FISH modification of MN assay is that it allows for differentiation between two possible means by which a micronucleus can arise (i.e., clastogenic versus aneuploidogenic mechanism). Some authors have also used Giemsa staining method to detect micronuclei in exfoliated cells.

Some Studies Upon Micronucleus Assay

When the micronucleus test was applied to feulgen/fast green stained exfoliated buccal mucosa cells of 2 population groups at high risk of oral cancer in India, it was found that all raw betel nut eaters and all chewers of betel quids had significantly elevated frequencies of micronucleated mucosal cells over non-chewing controls of comparable ethnic backgrounds and dietary habits.
An attempt was made to correlate the percentage of micronuclei in different grades of OSCC with habits related to oral diseases and an increase in the percentage of micronuclei in grade III (mean 5.36%) than in grade II (4.61%) and grade I (2.93%) OSCC was found thus concluding that this could be due to chromosomal breakage associated with chromosomal translocation which may in turn lead to transposition and activation of oncogenes.

Buccal scrapings were collected from patients with cancer and pre-cancerous lesions, with non-malignant oral problems, and healthy controls for analysis of micronucleus formation. The analysis revealed that MN frequencies in cancer and pre-cancerous cases were 4-fold elevated ($p < 0.001$) and 3.87-fold ($p < 0.002$) elevated for other non-malignant pathologies.

**Conclusion**

Detection of micronuclei and their assay is an upcoming research domain in the field of cancer prevention and therapeutics. These miniature nuclear offshoots if properly identified can turn out to be important biomarkers with huge potential in screening and predicting patients with oral precancers and also can act as risk assessors in patient’s ongoing treatment for invasive cancers.

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