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Micronucleus as a Non-invasive Biomarker – A Review

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Authors' contributions

This work was carried out in collaboration among all authors. Author MSD designed the study and wrote the protocol. Authors MSD, SG, JB, RT, MJ and RSP did the literature search, collected all the data and wrote part of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Oral Cancer has a remarkably high incidence worldwide and a significant decrease in its mortality and morbidity rates has been established if it is diagnosed in early stages. There has been always a strong need to develop new, objective, non-invasive methods for its early detection. Micronucleus has come up in the recent past as non-invasive biomarker for diagnosis of not only malignant and pre-malignant lesions but also many other significant diseases. Micronucleus ("MN") is defined as microscopically visible, round or oval cytoplasmic chromatin mass next to the nucleus. Micronuclei

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("MNi") originate from aberrant mitoses and consist of eccentric chromosomes, chromatid fragments or whole chromosomes that have failed to be incorporated into the daughter nuclei during mitosis. The MN assay has been widely accepted as an in vitro genotoxicity test and a biomarker assay for genotoxic exposure and effect in humans. An attempt has been made to review the related studies, utilizing micronucleus assay of buccal cells as a novel marker of genotoxicity in head and neck region.

Keywords: Micronucleus; micronuclei; micronucleus assay genotoxicity marker; biomarker.

1. INTRODUCTION

The literal meaning of the word Micronucleus (MN) describes it as a small nucleus in a large cell, or the smaller nuclei in cells that have two or more such structures. Schmid (1975) defined MN as a microscopically visible, round or oval cytoplasmic chromatin mass next to the nucleus (Fig. 1). MN is the name given to the small extra-nucleus that forms whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division [1–4].

The two basic phenomena responsible for the formation of MNi in mitotic cells are dysfunction of the mitotic apparatus and chromosome breakage (Fig. 2). Micronuclei (MNi) are formed from the whole chromosomes or chromatid fragments that lag behind in anaphase and are separated out from the daughter nuclei in telophase. Additionally, some MNi gets originated from fragments derived from broken anaphase bridges formed due to chromosome rearrangements such as dicentric chromatids, intermingled ring chromosomes or union of sister chromatids [5–9]. In the course of telophase these chromosomal regions are included in the daughter cells where they can fuse with the main nucleus or can form one or more smaller secondary nuclei [8]. This smaller secondary nucleus is known as MN and its number can vary from one to many. Now the MN formed can either go with the daughter nucleus they derive from or the other daughter nucleus. In the former scenario, neither of the daughter cells is aneuploid, and in the latter case the micronucleated cell has gained a chromosome, while its daughter cell has lost it [5,10].

1.1 Micronucleus Assay

The Micronucleus assay is defined as an investigative procedure carried out to analyze micronuclei quantitatively. It is one of the standard cytogenetic tools implemented to assess micronuclei formation (signifying chromosomal damage) subsequent to exposure to genotoxic/cytotoxic agents [11]. In humans, MN assay can be easily employed in lymphocytes, erythrocytes, and exfoliated epithelial cells (e.g. oral, urothelial, nasal) to obtain a measure of genome damage induced *in vivo* [12].

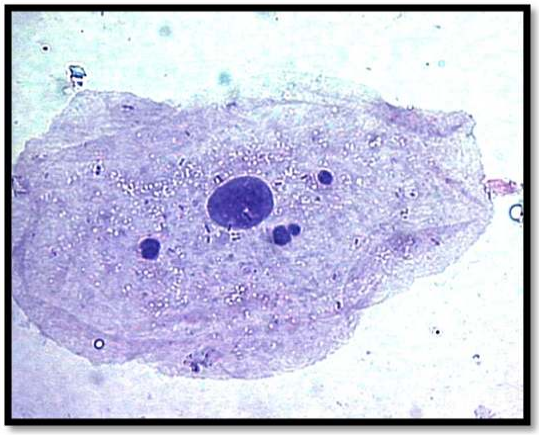

Fig. 1. Epithelial cell from buccal mucosa showing micronuclei (1000x, PAP stain) [4]

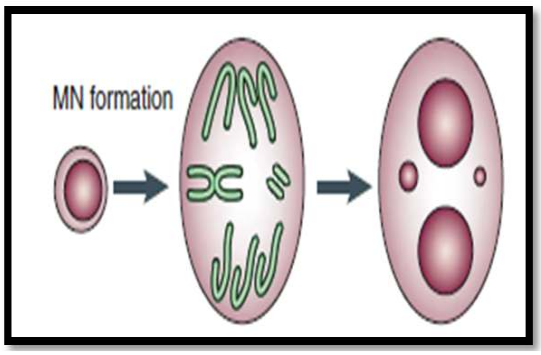

Fig. 2. Theories of origin of micronucleus
Chromosome breakage (Clastogenic Effect) and Dysfunction of the mitotic apparatus (Aneugenic Effect) [4]

In the earlier times, once-divided cultured cells (mostly peripheral lymphocytes) were considered

ideal for expressing MNi and the procedure used was known as cytokinesis-block micronucleus (CBMN) assay. In the CBMN assay, once-divided cells are identified by their BN appearance after blocking cytokinesis with cytochalasin-B (Cyt-B) [13]. In the last three decades, ever since the advent of MN assay on exfoliated cells, the buccal cells have completely replaced the usage of lymphocytes for the obvious reason of non-invasive collection of cells and simpler method for analyzing the MNi. The general genotoxicity results by both the methods of MN analysis are comparable [12].

The basal cells in the oral epithelium are the cells which possess the capability of mitosis, the new cells formed gets differentiated and mature as they migrate to the surface finally replacing the cells which are shed. Thus any genetic damage (expressed as MNi formation) instigated in these basal cells is reflected in the exfoliated cells; thus analysis of MNi in these cells can be correlated with the amount of genetic damage. As the oral epithelium acts as the first barrier for the inhalation or ingestion route and approximately 90% of cancers originate from epithelial cells, therefore, these cells represent a favored target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion [14 - 17]. The procedure for MN assay has been summarized in Tables 1-3.

Through this review, we intend to highlight the application of MN assay on buccal cells by reviewing significant studies from the English literature. We also aim to emphasize the pitfalls associated and the measures to overcome the same.

2. REVIEW OF LITERATURE

A comprehensive search of medical and dental databases including PubMed, Cochrane, Researchgate, and nonmedical search engines were utilized for the review. The search words included “Micronucleus”, “Micronuclei”, “Oral Cytology”, “Micronucelus Assay”, and “Non-invasive biomarkers”. Studies till 2014 were included in the review.

Table 1. The procedure for MN assay using a cytosmear [18]

- The subjects are asked to rinse their mouth with water and a cytobrush is used to obtain exfoliated cells from the oral mucosa.
- The samples are transferred to dry glass slides, to ensure an adequate harvest of cells.
- Smears are air dried and fixed with 95% ethanol spray.
- Smears are stained with either DNA specific or DNA non-specific stain.
- Smears are scored manually/digitally using a Tolbert (most accepted) criteria

Table 2. Stains commonly used for staining the cytosmears for MN Assay [18]

DNA specific stains:	DNA non-specific stains:
Feulgen Stain	Giemsa stain
Acridine orange	May Grunwald-Giemsa stain
DAPI (4’,6-diamidino-2-phenylindole)	PAP stain
	H & E stain

Table 3. For designating an extra nuclear body as micronucleus, the following criteria given by Tolbert [19] is applied

- Rounded smooth perimeter suggestive of a membrane.
- Less than a third the diameter of the associated nucleus, but large enough to discern shape and color.
- Staining intensity similar to that of the nucleus.
- Texture similar to that of nucleus.
- Same focal plane as nucleus.
- Absence of overlap with, or bridge to, the nucleus

In the early studies from the 1980s, exfoliated buccal mucosa cells were used to evaluate the genotoxic effects of betel nuts and quids and of chewing tobacco. Most studies showed higher MN frequencies at the site within the oral cavity where the quid or tobacco mixture was kept compared to the opposite, control site. The MN assay in buccal cells was also used to study cancerous and precancerous lesions and to monitor the effects of a number of chemopreventive agents. It is notable that the first studies of Stich and Rosin conducted between 1983 and 1984 had higher baseline MN frequencies than subsequent studies. This may have been due to a lack of defined scoring criteria and a relatively small number of scored cells (in some cases less than 500). Since then, published biomonitoring studies using the MN assay in buccal mucosa cells have investigated the effects of multiple factors including environmental and occupational exposures, radiotherapy, chemoprevention, vitamin supplementation trials, lifestyle habits, cancer, and other diseases [12].

2.1 Micronuclei as a Novel Biomarker

2.1.1 Occupational and environmental exposures

In the last 15–20 years the MN assay has been applied to evaluate chromosomal damage for biological monitoring of human populations exposed to a variety of mutagenic and carcinogenic chemical or physical agents. Significantly higher frequencies of MN have also been observed in exfoliated buccal cells from people exposed to organic solvents, antineoplastic agents, diesel derivatives, polycyclic aromatic hydrocarbons, lead-containing paints and solvents, and drinking water contaminated with arsenic [12,20,21,19].

2.2 MN and Radiation

Ionizing radiation plays an important role in the treatment of many neoplasias, but it also produces genetic damage. As a consequence, secondary tumors may develop years after the primary tumor treatment. Several studies evaluated MNi in buccal cells of patients undergoing radiotherapy in the head and neck region. The most striking increase in cytogenetic damage (150– 300 MN/1000 cells) was observed in an early study of three patients exposed to a cumulative dose of 3400–4000 cGy [12].

2.3 MN Frequencies in Buccal Cells of Patients with Some Specific Diseases

A site-specificity was observed for Xeroderma pigmentosum patients, with a higher MN frequency in cells from the dorsal tip of tongue, possibly due to greater light exposure. Down syndrome was associated with a 733% increase in MNi in comparison to younger healthy controls, and the MN frequency was 78.5% higher than in older controls. An increase in MN frequency in buccal cells was reported for Diabetes mellitus with the patients having double the level of genetic damage in comparison to matched controls and for treated pediatric patients with ulcerative colitis in comparison with controls or children with Crohn's disease [12]. Findings of the study carried out at Genetics Research Unit, National Council Research Institute of Clinical Physiology, Italy supported the hypothesis that CBMN assay may provide an useful tool for screening of the obesity and metabolic syndrome and its progression to diabetes and cardiovascular disease in adults as well in children [22]. Results of the study carried out in Mexico demonstrated a strong association between HPV type infection and MN frequency [23].

2.4 Lifestyle and Host Factors

Lifestyle factors that are associated with genetic damage include smoking, alcohol consumption, and diet, especially vitamin deficiencies and supplementation. The majority of the studies reporting a significant increase in MN in buccal mucosa cells related to a risk of oral cancer were performed in subgroups of subjects with specific lifestyle habits, i.e. chewers of betel quids (areca nut, betel leaves, slaked lime and tobacco) from India, Taiwan and Philippines; reverse smokers (who hold the lit end of the cigarette inside their mouths) from India and Philippines; snuff dippers from Canada; users of Khaini tobacco (tobacco mixed with slaked lime) from India, and other similar practices [12]. The HUMN project is an effective vehicle for the development and implementation of an international collaborative validation effort to bring together the various buccal MN databases, and to identify and quantify the key variables affecting this biomarker [12].

3. EXFOLIATIVE CYTOLOGY AND MICRONUCLEI RELATED STUDIES

3.1 Studies in Relation to Oral Cancer & Potentially Malignant Lesions

Biomonitoring of the changes in patients with diagnosed diseases or pathological changes that may lead to the development of cancer and other illnesses is becoming increasingly popular, and may be the most rapidly growing area of application of the MN assay to epithelial cells

A study carried out in France, in the year 1987, found out a baseline of 1.30 Micronucleated cells/1000 Cells in patients with carcinoma of upper digestive tract, using Feulgen Stain [12]. A revised protocol for the exfoliated cell micronucleus assay was field-tested in a population exposed to a genotoxic agent, snuff, at levels associated with a significant increase in cancer risk in North Carolina. The assay was revised to increase specificity and to include separate scoring of other nuclear anomalies associated with cytotoxicity and genotoxicity. The prevalence of micronucleation was elevated in the snuff users as compared with the nonusers (prevalence ratio = 2.4, 95% confidence interval 1.1–5.2) and, to a lesser extent, at the usual contact site as compared with a distal buccal site in the snuff users (prevalence ratio = 1.5, 95% confidence interval 0.9–2.5) [24]. In a study carried out in India, frequencies of micronucleated cells (MNCs) were analyzed in the exfoliated buccal mucosa of normal healthy individuals from different parts of India who were regularly using either areca nut alone, mava, tamol, tobacco with lime, dry snuff or masheri. The analyses were also carried out among oral submucous fibrosis patients who had the habit of chewing either mava or areca nut. Compared with 'no habit' healthy individuals, all the groups, irrespective of their type of habit, had significantly higher frequencies of MNCs [25].

A study carried out in Texas, using Feulgen stain, found out an increase in Micronuclei frequency at the site of lesion in Leukoplakia, which got decreased after the administration of α – tocopherol [26]. A study done in India in year 1996, found out an increased micronuclei count in oral exfoliated cells of patients suffering from Leukoplakia, Lichen Planus and Oral Submucous Fibrosis, using Giemsa stain. A baseline of 1.9 Micronucleated cells/1000 cells was found in each of the lesions as compared to normal subjects [12]. A study done in Germany in year 2000, found out a baseline of 9.0 Micronucleated cell/1000 cells each in patients suffering from Squamous Cell Carcinoma of upper digestive tract and Leukoplakia, using Giemsa stain [12]. A study done in Brazil in the year 2002 found out a baseline of 1.13 Micronucleated cell/1000 cells in patients diagnosed with oral cancer, using Feulgen stain [12]. In another study carried out in West Bengal, India, 50 patients with precancerous or malignant oral epithelial lesions were compared with 50 age and sex matched healthy controls without any oral lesions, using Giemsa stain. The MN frequency was increased in preoperative cancer cases and decreased in postoperative cases, while in pre-cancerous cases it was higher than in the controls [27]. A group of researchers from Italy, studied Micronuclei and p53 accumulations in preneoplastic and malignant lesions of head and neck and concluded that both these biomarkers were found in precancerous lesions, suggesting that they are early event in head and neck squamous cell carcinoma progression. The two biomarkers were not related to each other, indeed micronuclei frequency was found out to be higher in p53 -negative than in p53 – positive cells [28]. Another study done in India, in year 2007, found out a baseline of 1.6 Micronucleated cell/1000 cells each in patients diagnosed with Oral Cancer and pre malignant lesions of the oral cavity, using Giemsa stain [1]. In an infield study carried out on smokers in Egypt, Papanicolaou (PAP) stain was compared with May Grunwald Giemsa (MGG) for staining of micronuclei in exfoliated buccal cells. Researchers found out that PAP stain was faster and easier to process and transport in the field study than was MGG stain. Regarding MGG smears, bacteria and cell debris masked the MN as compared to PAP smears, in which the fixative destroyed the bacteria and made the cell boundaries clearly demarcated. Using PAP stain, MNi were seen easily in transparent cytoplasm. Finally they concluded that PAP stain is the preferred method infield studies for scoring and detecting MN in cells of buccal mucosa [29].

A study carried out in JIPMER, Pondicherry, India screened 25 patients in varying stages of squamous cell carcinoma of oral cavity and 25 patients with premalignant lesions for the presence of Micronuclei in the epithelial scrapings obtained from the site of the lesion. Highest Micronucleus Index was found in carcinoma and erythroplakia among the premalignant lesions, using the MayGrunwald

Giemsa and Giemsa stain [3]. In a study carried out in Thailand on patients with Oral Lichen Planus (OLP), it was found out that the frequency of Micronucleated Epithelial Cells (MEC) in OLP patients was 3.79% and 0.37% in the lesions and normal-appearing mucosa, respectively. Using a paired *t*-test, it was found that the MEC frequency in the OLP lesions was significantly elevated ($p < 0.01$) as compared to that in normal-appearing mucosa adjacent to lesions and that in normal individuals. The results indicated genotoxic damage in atrophic and erosive OLP [18]. In another study carried out in India, Micronucleus frequencies in oral exfoliated cells stained with Papanicolaou stain were counted and correlated with the histopathological grades and clinical stages of squamous cell carcinoma patients. They were also compared with the healthy control subjects. Micronuclei (MN) frequencies were found higher in squamous cell carcinoma patients than in control subjects. MN frequencies were also found to be raised with increasing histological grades of squamous cell carcinoma [30]. Grover et al. [4,31] observed a significantly raised micronuclei count in potentially malignant disorders like Leukoplakia, Lichen planus and OSMF, when compared to normal healthy mucosa. In another study, Grover et al. [32] applied Hematoxylin & Eosin stain, the most commonly used stain for histopathological examination, for staining the cytosmears and found statistically significant results.

3.2 Studies Not in Relation to Potentially Malignant Lesions

The micronucleus test was applied to exfoliated cells of the buccal mucosa of four population groups: (A) non-smokers and non-drinkers of alcoholic beverages, (B) non-smokers but alcohol drinkers, (C) smokers but non-drinkers, and (D) smokers and drinkers. An elevated frequency of micronucleated buccal mucosa cells was observed only in group D (smokers and alcohol drinkers). Neither smoking alone of up to and over 60 cigarettes per day nor ethanol drinking alone of up to 1.21 per day led to a detectable elevation of micronucleated buccal mucosa cells [33]. A study carried out in Amsterdam, Netherlands in an attempt to define a standardized protocol for counting micronuclei to assess the genotoxic damage in human exfoliated cells, concluded that atleast 10,000 exfoliated cells should be screened to monitor a significant reduction of 50% in the number of micronuclei (for a patient with an initial frequency in the micronuclei frequency range given). Since it takes ~7 h to evaluate this number of cells, it was also concluded that counting of micronuclei requires automation [2]. A structured literature review done on smoking and smokeless tobacco associated changes concluded that the assay used most frequently for tobacco-associated buccal cell changes was the micronucleus assay. The biological significance of the micronuclei in buccal cells of the oral mucosa is that the micronuclei are a manifestation of a readily identifiable clastogenic event that, has been associated with smoke and smokeless tobacco [34]. A study carried out on Gas Station Attendants in Brazil, found out an increased Micronuclei frequency in exfoliated buccal cells of the exposed as compared to the controls, using Feulgen stain [35]. A group of researchers from West Bengal, India, in an attempt to study the genotoxic effects of combustion fumes on targeted sites carried out Micronucleus assay using Feulgen stain in buccal epithelial cells of 47 firefighters and they found a 3 fold increase in Micronuclei frequency as compared to the controls [36]. To study the effects of occupational exposure to petroleum derivatives such as benzene, exfoliated buccal cells from 50 petrol station attendants and 50 age- and sex-matched control subjects were examined for micronucleus (MN) frequency by a group of researchers in Turkey. Frequencies of nuclear abnormalities (NA) other than micronuclei, such as binucleates, karyorrhexis and karyolysis, were also evaluated, using Feulgen stain. Analysis of buccal cells revealed that MN and NA frequencies in petrol station workers were significantly higher than in control subjects ($P < 0.01$) and also significantly related to smoking habit ($P < 0.01$) [37].

Another study in Brazil was carried out to comparatively evaluate the DNA damage (micronucleus) and cellular death (pyknosis, karyolysis and karyorrhexis) of exfoliated buccal mucosa cells from children and adults following dental X-ray exposure. The results indicated no statistically significant differences ($P > 0.05$) in children's as well as in adult's micronucleated oral mucosa cells before and after dental X-ray exposure [38]. In a study conducted in Europe, a total of 239 agricultural workers and 231 unexposed controls were examined for cytogenetic effects in lymphocytes of peripheral blood and exfoliated cells of the oral mucosa. The frequency of micronuclei (MN) was evaluated in both cell types and their relationship to different confounding factors (e.g. sex, country, smoking habit, etc.) was determined,

using Giemsa stain. The results obtained indicated that there are no increases in MN frequencies in the agricultural workers when compared with the controls for either lymphocytes or buccal cells [39].

4. CONCLUSION

- MN formation is generally considered as a manifestation of genetic damage or chromosomal breakage.
- Many investigators already and unequivocally have called MN as an upcoming marker of tumorogenesis.
- MN is thus a potential biomarker to screen genotoxicity, biomonitoring of various diseases, detection of malignancies and preneoplastic conditions and also a lot of other diseases.
- Since MN is a manifestation of day to day exposure to environmental pollutants, infections, nutrition, radiation, foods, and the genetic make-up or ethnicity which again varies around the globe. So there must be an upper limit of the base-line MN frequency only beyond which we can label it as increased MN frequency.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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