Buccal Cell Micronuclei Assay: A Non-Invasive Genotoxic Marker

Vipul Jain[1], Parul Lohra[1], Bhanu Priya[2], Deepak Sindhu[3]

ABSTRACT

The introduction of non-invasive and presumptive diagnostic techniques for oral premalignant and malignant lesions in an early stage gave rise to the need for Buccal Cell Micronuclei assay. Formation of micronuclei due to carcinogenic agents depicting cytogenic damage forms the basis of this assay. During nuclear division in cell replication cycle, the chromosomal fragments which fall short from participation in new daughter cell nuclei formation, give rise to micronuclei. These may be clastogenic and aneugenic depending on presence or absence of centromere. Similarly, other nuclear abnormalities like pyknotic cells, binucleated cells, karyorrhectic cells, condensed chromatin may indicate towards pending genomic instability leading to major or minor degenerative or developmental diseases. The knowledge regarding cytotoxicity of several genotoxins and their affliction with micronuclei formation has been discussed in detail. This review summarise the methodology and result analysis of the test including the various stains that can be utilised for buccal cell micronuclei assay. It also highlights various studies done on BCMN assay in various lesions and subjects exposed to various genotoxic agents and also the futuristic aspects in this assay.

Keywords: Micronuclei, genotoxic, nuclear, cytogenic, biomarker

BACKGROUND

Oral cancer is most common in India and 11th most common cancer worldwide.[1] An early detection and identification of predisposing factors may be helpful in curbing the menace of the disease and its risk factors. It is well accepted that carcinogenesis is a multiple step process of accumulation of genetic damage leading to cell dysregulation and disruption.[2] Genotoxic effects on buccal mucosa can be assessed by deoxyribonucleic acid (DNA) damages which are manifested as chromosomal aberrations, exchange of sister chromatid and formation of micronuclei. Micronuclei have been defined as a round to oval cytoplasmic chromatin mass which is visible only under a microscope and is located next to the nucleus.[3] The (Buccal Cell Micronuclei) BCMN assay is an excellent tool to serve as a biomarker which primarily involves examination of exfoliated cells to determine the presence of micronuclei(MN) which are either composed of a complete chromosome or chromosomal fragments that fail to incorporate into the daughter nuclei during mitosis.[4] BCMN assay has an advantage of minimal invasiveness for the collection of cells along with low cost and ease of storage and thus making it a good choice for epidemiological studies.[5]

REVIEW OF LITERATURE

Boller, Schmidt and Heddle in the early 1970s suggested the term micronucleus test for the first time. They utilised bone marrow erythrocytes after in vivo exposing animals to show that this assay provides a simpler and easier method for detecting the genotoxic nature of mutagens. A few years later Countryman and Heddle showed that peripheral blood lymphocytes could be used for approaching micronuclei and thus they recommended testing of micronuclei as a biomarker.[6]

Studies done by Stich and Rosin between 1983 and 1984 exhibit higher baseline MN frequencies in comparison to the subsequent studies. Since then studies using the MN assay in buccal exfoliated cells have confounded the effects of multiple factors including occupational and environmental exposures, vitamin supplementation trials, radiotherapy, chemoprevention, lifestyle habits, cancer, and other diseases.[7] In 1997 an international synergistic program was done in the peripheral blood lymphocytes to standardise the micronucleus assay named Human Micronucleus (HUMN) project. The program also included the effects that protocol may have. The values so obtained were used as the scoring criteria.[5]

BCMN assay can be said to be one of the most suitable approach for the detection of increased cancer risk in humans.[3,5] The limited DNA repair capacity of buccal cells in comparison to peripheral blood lymphocytes can reflect the efficacy of buccal cells in determining age-related genomic instability.[8] Buccal cells are in immediate contact with various chemical metabolites and other ingested and inhaled genotoxic agents.[9] So they are the first to exhibit the genotoxicity of these agents. Buccal cells have high turnover rate so genotoxic changes are generally seen in the dividing basal layer 1-3 weeks' prior.[7]

BCMN assay has given a new direction to exfoliative cytological studies by analysing biomarkers for oral cancers and thus helping in early detection of tumorigenesis.

MICRONUCLEI FORMATION

MN are formed during the transition phase from metaphase to anaphase of the mitosis cycle and they can either be complete chromosomes (aneuploidogenic effect) or fragments of chromosomes without centromere (clastogenic effect). These events may occur spontaneously or due to certain endogenous or exogenous factors.[10]

The pattern of micronucleus formation is primarily dependent upon the type of carcinogen exposure to the cells. Tissues receiving single, short-term exposure will exhibit a different pattern compared to tissues receiving chronic, uniform exposure.

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The micronuclei decrease in number with time either because of cell death by chromosomal damage or by the loss of micronuclei during cell division. It has been appreciated that those cells will be lost at a higher frequency which has more chromosomal damage and thus more no of micronuclei than those with less damage.[11]

MICRONUCLEI IDENTIFICATION

Micronuclei (MN) are cytoplasmic chromatin mass biomarkers that contain small nuclei which arise from chromosomes lagging at anaphase or from the fragments of acentric chromosomes.[4] They form a membrane of their own and are Feulgen-specific bodies in the cell cytoplasm, later these cells mature and exfoliate.[8]

Micronuclei can be identified depending upon following criteria:

1. Cell containing one or more nuclear like substance along with the main nucleus.
2. Each MN will have the diameter less than 1/3rd of the nucleus.
3. MN will have oval or circular shape along with membrane.
4. MN will be located within 3 or 4 nuclear diameters around a nucleus and will not be in contact with the nucleus (this will make the count of MN meaningful).
5. MN will exhibit similar focal plane, texture and even almost similar staining intensity as that of the main nucleus.[7]

Collection of exfoliated buccal cells

Exfoliated buccal cells can be easily collected from buccal mucosa of both the cheeks utilising a small headed toothbrush, toothpick, wooden spatula or metal spatula or with water moistened cytobrush.[12] Small-headed brushes or cytobrushes are most commonly used and are also an effective tool for the collection of buccal cells. Casartelli et al. stated that more number of MN are seen when vigorous scraping is done. This suggests that MN frequency decreases from basal to the superficial layers of buccal mucosa.[7]

Preparation of Slide

After collection of the buccal cell sample, the buccal smears are prepared by spreading the sample on a clean slide. But in most of the studies, before smear preparation, the buccal cells were transferred into a test tube containing Tris/ EDTA buffer (pH=7) and is centrifuged.[12,13] The buffer inactivates the endogenous DNAase and also aids in removing bacteria that can interfere with the scoring.[13] Cell suspensions are now fixed and then transferred to slides and finally air dried at the room temperature. Commonly used fixatives include 80% methanol, or 80% ethanol, or methanol- glacial acetic acid mixture (3:1) or methanol-ethanol mixture (3:1).[7]

Staining of the slide

To assess the MN Feulgen reaction is the most widely used for staining buccal cell followed by counterstaining with Fast Green to delineate the cell cytoplasm. Acridine orange fluorescent staining can help in distinguishing from MN like inclusions or contaminants. Fluorescence In Situ Hybridization (FISH) with a centromeric probe can help in differentiation between the clastogenic and aneuploidogenic mechanism of micronuclei formation. Giemsa stain is avoided because it increases false positive scoring by scoring of non- nuclear bodies, keratohyalin bodies or bacteria leading to overestimation of micronuclei.[7]

Slide analysis

Most widely used criteria for identification and classification of the nuclear anomalies was given by Tolbert et al. which suggested scoring of at least 1000 cells per slide. They also proposed to score 2000-3000 cells if less than 5 micronucleated cells were observed after counting 1000 cells. 3000-4000 cells evaluation suggested by Ceppi et al.[14] Most of the published studies have 1000-3000 cells scoring. The same observer should analyse to eliminate inter-observer bias in the results.

VARIOUS OTHER NUCLEAR ABNORMALITIES

Beside micronuclei, following nuclear abnormalities can be appreciated in smears of exfoliated buccal cells:

1. **Binucleated cells (BN)** i.e. presence of two nuclei. The formation of BN cell is related to the failure of cytokinesis either due to cell cycle arrest due to dysjunction of telomere or aneuploidy or defects in the formation of microfilament ring. The ratio of binucleate: mononucleate cell can prove to be an important biomarker for identifying cytokinesis failure caused by higher rates of aneuploidy, like one seen in Down's syndrome.[15]

2. Nuclear Buds (NBUDs) or Broken Eggs (BE)

Amplification of DNA, elimination of DNA repair complexes in aneuploid cells is responsible for the formation of NBUDs.[16] Misrepair of DNA breaks and telomere end fusions leads to the formation of dicentric chromosomes which suggests the formation of the Nucleoplasmic bridge. NRUDs can also be formed because of failure of decatenation leading to defective separation of sister chromatid at anaphase[17]

3. Pyknosis or shrunken nuclei exhibit a small shrunken nucleus which has a high density of nuclear material which is intensely stained. It may represent an alternative pathway of nuclear disintegration[18]

4. **Condensed chromatin** is formed due to rapid proteolysis of matrix proteins of the nucleus and it represents a stage of apoptosis where a roughly striated nuclear pattern is seen.[19]

5. Karyorrhexis or nuclear disintegration involves the loss of integrity of the nucleus. It is characterised by the extensive aggregation of nuclear chromatin. It exhibits a densely speckled nuclear pattern which indicates nuclear fragmentation and can eventually lead to the disintegration of the nucleus.[7]

6. Karyolysis or nuclear dissolution represent an advanced stage of apoptosis and necrosis. It is angular and flat in shape and is Feulgen and aceto-orcein negative. It has a cytoplasmic area that of the size of terminally differentiated cell and only ghost-like image of nucleus remains.[20]

Ionizing radiation

Ionising radiation like X-rays causes breakage of crosslinks and DNA strands due to which DNA and proteins tend to adduct. It primarily has Clastogenic MN formation but aneugenic MN may be formed due to the mutation in certain cell cycle and repair genes.[21]

Anti-mitotic agents

The agents like vinca alkaloids cause disruption of Mitotic spindle and malsegregation of chromosomes.[22] They chiefly form aneugenic MN.

Heavy Metals and their compounds

They bind to DNA and proteins causing damage to DNA, thus gene expressions are altered leading to mutations and altered cell cycle and finally dysfunction of the cytoskeleton. Depending on the metal they can give rise to both clastogenic and aneugenic MN.[23]

DNA methylating agents

They cause loss of methylation in paracentromeric and centromeric regions and decondensation of sister chromatid leading to improper segregation of chromosomes. If hypomethylation in centromeric regions occurs, they lead to the formation of aneugenic MN and if global hypomethylation leading to DNA strand breaks is seen it leads to clastogenic MN formation.[24]

Anthracycline drugs

It leads to damage of DNA by disruption of DNA replication and DNA repair. It primarily exhibits clastogenic MN.[25]

Some Studies Upon Micronucleus Assay

Table 1 Micronucleus frequency in exfoliated oral mucosa cells of patients with oral cancer and precancerous conditions

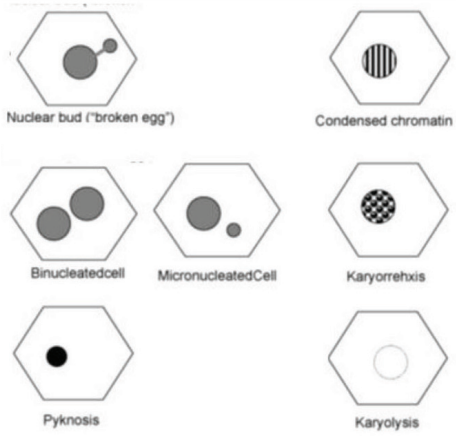

Figure-4: Nuclear anomalies seen in exfoliated buccal cells

FUTURE PERSPECTIVE

BCMN assay is an excellent marker for human genotoxicity studies in biomonitoring of malignant and premalignant lesions, so there seems to be an urgent requirement for the automation of MN and other nuclear anomalies because such analysis will help in rapid and relatively more reliable detection of nuclear anomalies, thus allowing the applicability on the large scale. The frequency of MN in buccal exfoliated cells that are specific targets of genotoxic carcinogens, can be utilised to act as an "endogenous dosimeter" where carcinomas can develop. But before all this we must have to carry out a systematic investigation of key variables like age, diet, gender, genotype, lifestyle, oral hygiene and dental health (e.g. more number of missing or decayed teeth, periodontal status, etc.), smoking, alcohol and other drugs to identify the variables that might affect the reliability of test. Also possibility of formation of MN in buccal cells from systemic exposure should be considered in addition to a direct contact exposure in the oral cavity. Only then we will be able to exhaust BCMN assay as an effective, reliable and early diagnostic tool for oral carcinomatous lesions.

CONCLUSION

Our review indicates the potential useful aspects of the BCMN assay in the pre-screening phase of patients who are at the risk for oral cancer. We have compared various studies done using this assay and calculated the fold difference vs control values in table 1. These values allow us to see the efficacy of the assay as field reality in various types of carcinomatous lesions. Not only for detection of cancer but this assay can be utilised in estimating the genotoxicity of several agents. It can also justify the impact of lifestyle factors on mutagenesis. Thus this assay can prove to be a good epidemiological tool. But there are certain shortcomings in the assay that we need to focus on. First is the

Type of cancer	Study Subjects/ Control	Fold- Difference Vs Control	No of Cells Scored per Subject	Staining Method	Scoring Criteria	Year	Country	Reference
Head and neck cancer	59/65	2.3	1500	1	3/4	2011	Turkey	26
Oral Leukoplakia	18/20	5.7	1000	2	3	1996	India	27
	16/98	2.2	1000	2	1	2000	Germany	28
Oral Lichen Planus	14/20	6.2	1000	2	3	1996	India	27
Oral Submucous Fibrosis	68/20	6.1	1000	2	3	1996	India	27
	30/30	5.3	1000	3	NR	2015	India	29
Oral squamous cell carcinoma	30/30	4.5	2000	1	4	2002	Brazil	30
	24/60	1.6	2000	2	NR	2007	India	31
	20/40	4.9	1000	4	4	2011	Brazil	32
	81/136	8.7	1000	3/2	2	2015	India	33
	30/30	10.6	1000	3	NR	2015	India	29
	20/20	6.9	500	4	4	2015	India	34
	30/30	9.3	500	3	4	2016	India	35
Precancerous oral lesions	29/60	1.2	2000	2	NR	2007	India	31
	55/136	3.3	1000	3/2	2	2015	India	33
	30/30	6.1	500	3	4	2016	India	35
Upper aerodigestive tract carcinoma	38/37	2.0	1000	1	2	1987	France	36
	44/98	2.3	1000	2	1	2000	Germany	28

A. Staining techniques: (1) Feulgen/Fast Green, (2) Giemsa, (3) Papanicolaou (4) Periodic acid Schiff
B. Scoring criteria (1) Basic (2) Stich and Rosen (3) Sarto et al (4) Tolbert et al NR not reported

Table-1. Micronucleus frequency in exfoliated oral mucosa cells of patients with oral cancer and precancerous conditions

large variability in the MN frequency across the laboratories. This variability should be eliminated by standardisation of a protocol which should be followed by all laboratories worldwide. This variability arises due to the different scoring criteria and various experimental protocols which are used for this assay. Efforts are already being done in same direction. The detailed description of the criteria and specific guidelines for scoring are being developed. The inter-laboratory and intra-laboratory variability was recently evaluated by a scoring exercise which showed a significant reduction in variability. The need of the hour is to develop so called "range of normal values" for the BCMN assay. Also we need to address the role of BCMN assay as a biomarker for susceptibility for other carcinomatous, precancerous and degenerative diseases. It's no denying the fact that if these shortcomings are met, then this assay is going to be an indispensable futuristic tool for early and easy detection of precancerous and carcinomatous lesions justifying itself to be a reliable non-invasive genotoxic marker.

LIST OF ABBREVIATIONS

BCMN: Buccal Cell Micronuclei Assay; DNA: Deoxyribo nucleic acid; MN: Micronuclei; HUMN: Human Micronucleus; EDTA: Ethylene Diamine Tetra acetic acid; FISH: Fluorescence In Situ Hybridization; BN: Binucleated cells; NBUSs: Nuclear Buds; BE: Broken Eggs; DAPI: 4',6-Diamidino-2-Phenylindole.

AVAILAIBILITY OF DATA AND MATERIALS

An extensive search of medical and dental databases using engines like PubMed, Cochrane, Research gate was done to gather the knowledge for this review. "Micronucleus", "Genotoxic biomarker", "Micronucleus Assay" and "Cytogenic markers" were the main keywords utilised in scrutinizing the online database. Studies till 2016 were included in the review.

AUTHORS' CONTRIBUTIONS

VJ wrote the review. BP and DS collected the references for the review to be cited. PL checked scientific contents, revised the paper for important intellectual content and designed the review. All authors read and approved the final manuscript.

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