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ORIGINAL ARTICLE

Cytogenetic biomonitoring of premalignant and malignant oral lesions by micronuclei assessment: A screening evaluation

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ABSTRACT

Background: Micronuclei (MN) are extracytoplasmic nuclear bodies, which are induced in cells by numerous genotoxic agents that damage the chromosomes. The damaged chromosomes in the form of acentric chromatids or chromosome fragments lag behind in anaphase when centric elements move towards the spindle poles, and thus leads to the formation of secondary nuclei called MN, which are also transmitted to the daughter cells. In oral exfoliated cells these MN are induced by variety of carcinogenic compounds like tobacco, betel nut, and alcohol; which have been suggested to be the most common cause of premalignant lesion conditions and oral cancer, and thus can be used as a biomarker for cytogenetic damage. **Objectives:** To evaluate and compare the frequency of MN in Papanicolaou (PAP) stained smears of oral exfoliated cells from healthy control, leukoplakia, and squamous cell carcinoma (SCC) group in a small population of eastern Uttar Pradesh. **Materials and Methods:** Cytological smears were prepared from buccal mucosa of healthy control, leukoplakia patients, and SCC group and stained with PAP stain. Slides were screened, and micronucleated cells were counted out of thousand and compared in different groups. **Results:** Mean MN count was highest for the SCC group (10.13), followed by leukoplakia group (6.15), and lowest for healthy controls (3.28); with count ranging from 7-14, 4-8, and 2-4, respectively. Tukey's HSD and ROC analysis showed the intergroup differences were significant statistically ($P < 0.05$) and thus, mean MN density seems to be a useful tool for differential diagnosis with high accuracy. **Conclusion:** MN were higher in SCC than in leukoplakia and healthy control, moreover. Hence, MN assay can be used as an important biomarker for cytogenetic damage in oral leukoplakia and SCC.

Key words
Leukoplakia, micronuclei, squamous cell carcinoma

INTRODUCTION

The World Health Organization (WHO) and other agencies have produced a considerable amount of epidemiological data, suggesting that the incidence of oral cancer is increasing in several countries.[1] Oral and pharyngeal cancer, when grouped together is the sixth leading cancer in the world and ranks in the top three in high incidence areas. The annual estimated incidence is around 275,000 for oral and 130,300 for pharyngeal cancers excluding nasopharynx; two-thirds of these cases occurring in developing countries such as countries in south Asia and Pacific islands in Melanesia.[2] Among the ten most common incident cancers worldwide, 90% of head and neck cancer is squamous cell carcinoma (SCC) and each year there are approximately 300,000 deaths due to this.[3]

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The risk factors known could be grouped as nonmodifiable and modifiable, and the latter group relates to risky lifestyles. The most important being tobacco, excess consumption of alcohol, and betel quid usage; these factors acting separately and synergistically together.[1] Other factors could be radiation, chronic irritation, nutritional deficiencies, and viruses. Predominantly in the Asian-Pacific region, smokeless tobacco and areca nut used either singly or in various combinations of "betel-quid" or "pan", consists of betel leaf, areca nut, and slaked lime, to which tobacco is often added; accounts for the vast majority of the most common oral potentially malignant lesions and malignant disorders.[4,5]

The development of oral cancer is a multistep process

arising from preexisting potentially malignant lesions, leukoplakia being the most common precancer representing 85% of such lesions.[6] Finding of such multiple premalignant lesions in the upper aerodigestive tract has led to the concept of field cancerization, as proposed by Slaughter *et al.*, in 1953, stating that the entire epithelial surface of the upper aerodigestive tract is exposed to carcinogens (genotoxic agents), which results in an increased risk of cancer development by causing multiple genetic abnormalities in the whole tissue region.[7]

Oral SCC is characterized by complex karyotypes that involve many chromosomal deletions, translocations, and structural abnormalities; due to genotoxic exposure. Cells of this type of tumor often have errors in chromosome segregation that lead to the formation of a lagging chromosome (acentric chromatids) or chromosome parts that become lost during the anaphase stage of cell separation and are excluded from the reforming nuclei. The laggards are observed in the cytoplasm of daughter cells as several secondary nuclei, which are smaller than principal nucleus and are therefore called micronuclei (MN).[8,9] Bigger MN result from exclusion of whole chromosome following damage to spindle apparatus of the cell (aneugenic effect), whereas smaller MN result from structural aberrations; causing chromosomal fragments (clastogenic effect).[9]

The direct correlation between the MN formation and genotoxic exposure makes the MN assessment as an indicator of genomic damage.[10] Hence, MN analysis in oral exfoliated cells can be used as a biomarker to study genomic changes directly in target site affected by cancerous changes.[11]

AIMS AND OBJECTIVES

1. To evaluate the frequency of MN in Papanicolaou (PAP) stained smears of oral exfoliated cells from healthy control, leukoplakia, and SCC groups in a small population of eastern Uttar Pradesh
2. To compare the frequency of MN in healthy control, leukoplakia, and SCC groups.

MATERIALS AND METHODS

The study was conducted in Department of Oral Pathology and Microbiology at Purvanchal Institute of Dental Sciences, Gorakhpur (UP) in collaboration with Department of Oral Pathology and Microbiology at Kothiwal Dental College and Research Centre, Moradabad (U.P), India.

Subject selection

Total 90 subjects of almost similar age group were involved after thorough history of possible risk factors and proper clinical examination, and divided into following groups:

1. Healthy control group (n = 30) with no clinical oral lesion and without deleterious oral habit
2. Leukoplakia group (n = 30), clinically and histopathologically proven and with or without deleterious oral habits
3. SCC group (n = 30), clinically and histopathologically proven and with or without deleterious oral habits.

Cytological smear preparation and staining

After thorough oral rinse, cytological smears were obtained on microscopic slides using wooden spatula from buccal mucosa of the study subjects. Just prior to drying, smears were fixed with commercially available spray fixative (available with the RAPIDPAP™ KIT) for 15 min. Staining of fixed smears was done by PAP technique using a commercially available staining kit RAPIDPAP™ (Biolab Diagnostics, Tarapur, Maharashtra).[9,10]

MN counting

Slides were viewed under light microscope (Olympus) at high magnification (×400) in a zig-zag method. One thousand cells were included in each slide following criteria developed by Tolbert *et al.*[12]

(a) Intact cytoplasm and relatively flat cell position on the slide
(b) Little or no overlap with adjacent cells
(c) Little or no debris and
(d) Nucleus normal and intact, nuclear perimeter smooth and distinct.

Cells positive for MN were counted out of thousand cells following Tolbert *et al.*, criteria and compared among different study groups[12]

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

The frequency of MN was expressed as mean count for a particular group and comparison was done statistically.

RESULTS

Papaniculaou stained cytological smears of different study groups were obtained, then screened for the presence of MN positive exfoliated cells [Figures 1-3] and following findings were obtained.

The mean MN count was highest for the SCC group (10.13), followed by leukoplakia group (6.15), and lowest for healthy controls (3.28); with count ranging from 7-14, 4-8, and 2-4, respectively [Table 1 and Figure 4].

Table 1: Micronuclei frequency in different study groups

Group	*n*	Mean	Standard deviation	Standard error	95% confidence interval for mean		Minimum	Maximum
					Lower	Upper		
Control	40	3.28	0.68	0.11	3.06	3.49	2	4
Leukoplakia	40	6.15	1.10	0.17	5.80	6.50	4	8
SCC	40	10.13	2.52	0.40	9.32	10.93	7	14
Total	120	6.52	3.25	0.30	5.93	7.10	2	14

SCC – Squamous cell carcinoma

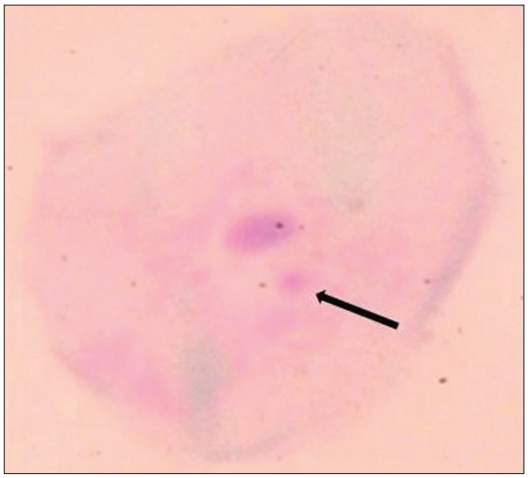

Figure 1: Exfoliated buccal mucosal cell showing one micronucleus (Papanicolaou stain (PAP), ×400)

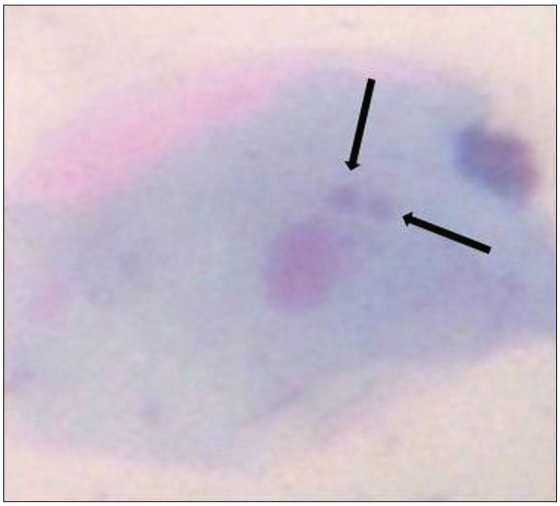

Figure 2: Exfoliated buccal mucosal cell showing two micronuclei (PAP, ×400)

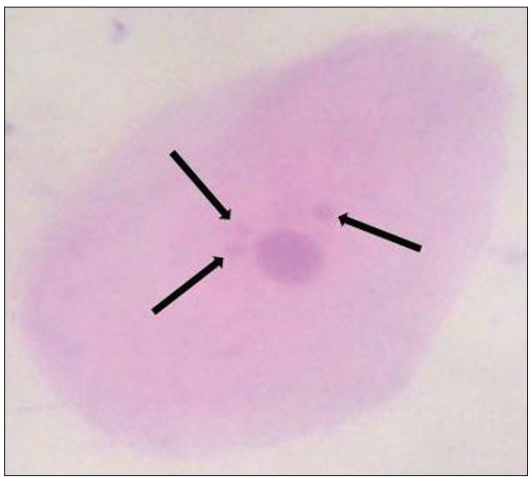

Figure 3: Exfoliated buccal mucosal cell showing three micronuclei (PAP, ×400)

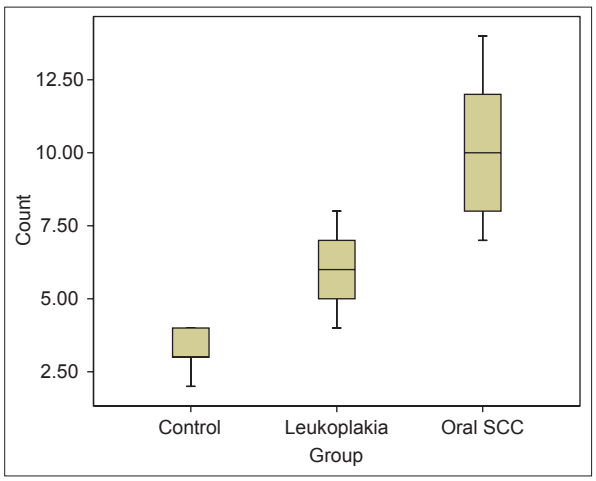

Figure 4: Box plot for comparison of micronuclei frequency in different study groups

Intergroup comparison was done using analysis of variance (ANOVA) and there was statistically significant difference in the mean MN count in different groups, with healthy control group showing minimum and SCC group showing maximum value ($F = 38.505$; $P < 0.001$) [Table 2].

On multiple comparisons using Tukey's honestly significant difference (HSD), it was seen that all the intergroup differences were significant statistically ($P < 0.05$). Thus, mean MN density seems to be a useful tool for differential diagnosis [Table 3].

Receiver operator curve (ROC) analysis for differentiation of SCC from leukoplakia and healthy controls have shown area under curve = 0.973, thereby indicating a very high accuracy of the MN assay. A cut off value of ≥7 was regressed to be 100% sensitive and 80% specific in diagnosis of SCC from leukoplakia and healthy control group [Table 4 and Figure 5].

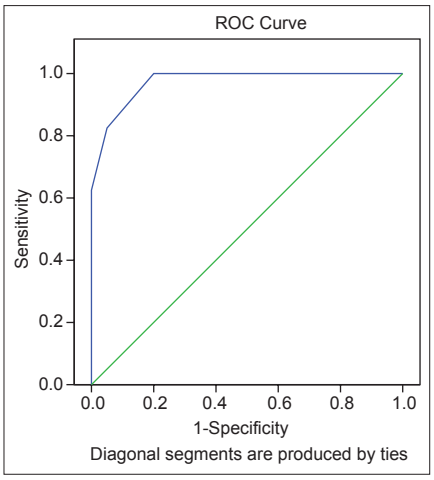

Figure 5: Receiver operator curve analysis for differentiation of squamous cell carcinoma from leukoplakia and healthy controls

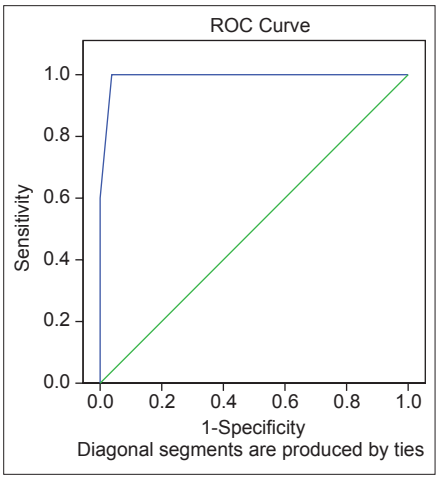

Figure 6: ROC analysis for differentiation of healthy controls from leukoplakia and SCC groups

Table 2: Comparison of micronuclei between and within groups by ANOVA

	Sum of squares	df	Mean square	*F*	Sig.
Between groups	233.600	2	116.800	38.505	0.000
Within groups	81.900	27	3.033		
Total	315.500	29			

ANOVA – Analysis of variance; df – Degrees of freedom; F – Frequency; Sig. – Significance

ROC analysis for differentiation of healthy controls from leukoplakia and SCC groups have shown area under curve = 0.993. Thus, indicating near perfect diagnostic ability of the MN assay in differentiation of healthy controls from diseased groups. A cut-off value less than 5 was regressed to be 100% sensitive and 96.2% specific [Table 5 and Figure 6].

DISCUSSION

The induction *in vivo* and *in vitro*, of micronucleated cells by carcinogens and mutagens is a sign of the genotoxic effect of such substances. Due to its association with chromosomal aberrations, MN have been used since 1937 as an indicator of genotoxic exposure, based on the radiation studies conducted by Brenneke and Mather.[10] For the first time, MN assay was systematically used to analyze cytosmears from smokers and betel chewers in India by Stich, and showed that the MN frequency in the superficial layers of the oral mucosa is higher in smoking and betel-chewing individuals than in probands not exposed.[13] The International Collaborative Project on Micronucleus Frequency in Human Populations (HUMN) was organized to collect data on MN frequencies in different human populations and different cell types to determine the extent to which MN frequency is a valid biomarker of ageing and risk for diseases such as cancer.[14]

In humans, MN can be easily assessed in erythrocytes, lymphocytes, and exfoliated epithelial cells (e.g., oral, urothelial, nasal) to obtain a measure of genome damage induced *in vivo*. Buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products. Approximately, 90% of human cancers originate from epithelial cells. Therefore, oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion. Thus MN assay can be performed in buccal cells without the need for *ex vivo* nuclear division, so that the cell cultures required for cytogenetic assays based on analysis of metaphase chromosomes, such as chromosome aberrations and sister chromatid exchanges, are not needed. The collection of buccal cells is arguably the least invasive method available for measuring DNA

Table 3: Multiple comparison of micronuclei by Tukey' honestly significant difference

(I) Group	(J) Group	Mean difference (I-J)	Standard error	Sig.	95% confidence interval	
Control	Leukoplakia	–2.87500 (*)	0.36600	0	–3.7438	–2.0062
	Oral SCC	–6.85000 (*)	0.36600	0	–7.7188	–5.9812
Leukoplakia	Control	2.87500 (*)	0.36600	0	2.0062	3.7438
	Oral SCC	–3.97500 (*)	0.36600	0	–4.8438	–3.1062
Oral SCC	Control	6.85000 (*)	0.36600	0	5.9812	7.7188
	Leukoplakia	3.97500 (*)	0.36600	0	3.1062	4.8438

SCC – Squamous cell carcinoma

Table 4: ROC analysis for differentiation of SCC from leukoplakia and healthy controls

Area	Standard error (a)	Asymptotic Sig. (b)	Asymptotic 95% confidence interval	
			Lower	Upper
0.973	0.011	0.000	0.951	0.995

SCC – Squamous cell carcinoma; ROC – Receiver operator curve

Table 5: Receiver operator curve analysis for differentiation of healthy controls from leukoplakia and squamous cell carcinoma groups

Area	Standard error (a)	Asymptotic Sig. (b)	Asymptotic 95% confidence interval	
			Lower	Upper
0.993	0.005	0.000	0.982	1.003

damage in humans, sample can be easily and repetitively obtained especially in comparison to obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies and final results can be obtained in 2 h. The buccal cell MN assay was first proposed in 1982 and continues to gain popularity as a biomarker of genetic damage in numerous applications.[10,15,16]

Conventional MN assays performed in peripheral blood lymphocytes apply a chemical to block cytokinesis after a single cell division, and MN are manually counted and scored using microscopy. Offer *et al.*, combined immunomagnetic separation technique and single-laser flow cytometry to isolate and analyze immature reticulocytes in the peripheral blood for the presence of MN before these cells are removed by the spleen. The method enables rapid analysis of large number of cells and will help monitor human populations for genetic damage.[17]

Several investigations have shown significant correlation between the level of chromosomal aberrations in lymphocytes and MN in exfoliated buccal mucosa cells of subjects exposed to environmental mutagens. In cancer patients' lymphocytes the MN level is two-fold higher than in corresponding healthy both males and females.[11,18] Nersesyan *et al.*, evaluated the MN level in exfoliated buccal mucosa cells of nonsmoking primary cancer patients (breast cancer, Hodgkin's disease, cancer of cervix uteri, lung cancer) and concluded that the evaluation of MN number in oral mucosa cells can show genomic instability in somatic cells of organism.[11]

MN shows the degree of field cancerization in the upper aerodigestive tract depending on the smoking and drinking habits of the examined person. The dimension of cytogenetic damage of oral mucosa in smokers is dependent on the amount and duration of tobacco abuse.[11,19] These facts also have been observed in the present study. Neither alcohol nor smoking, alone, increase the MN frequency in buccal cells, but a synergistic effect of smoking and alcohol was evident, with up to a 5.5-fold increase relative to nonsmoker and nondrinker controls.[15]

In present study, the tobacco chewers, betel quid chewers, and smokers have shown greater number of MN as compared to the healthy control group. This finding relates with that of Sellappa *et al.*[5] Smokers on an average had almost triple the MN frequency compared to nonsmokers.[20] Naderi *et al.*, in their study showed higher prevalence of MN in individuals with smoking history of more than 10 years than in persons having smoking history of less than 10 years.[21] On the other hand Bansal *et al.*, in their work showed higher level of mean number of MN in smokeless tobacco users as compared with smokers, thus indicating higher carcinogenic potential of smokeless tobacco.[22]

There is two-fold higher MN rate per cell in the group of tumor and leukoplakia patients than in the control group and number of studies have shown a gradual increase in MN frequency from normal to precancerous to cancerous lesions.[9,10] The present work have shown the similar pattern in MN level. Also, the mean MN frequency significantly increases from histopathological grade I to grade II to grade III SCC.[9] It is interesting that in two studies, men had a slightly higher MN frequency in buccal cells than women. These results are in contrast to data showing higher MN frequencies in lymphocytes of women and older subjects.[23]

MN assessment can strongly be used to differentiate between healthy control from leukoplakia and SCC, and other way round, since ROC analysis has revealed very high sensitivity and specificity of MN assay.

In human cytogenetic studies, it is important to consider some confounding factors. Viruses, alterations in the immune system, failures in DNA repair system and interindividual variations have already been associated with increased frequencies of chromosome aberrations. Moreover, the influence of tobacco smoke has usually been considered as a relevant confounding factor.[24] Bloching *et al.*, assessed patients with oral cancer and did not find any relation between daily alcohol intake and MN rates. Because tobacco is usually associated with increased MN and most patients with oral cancer smoke and drink, it seems that any subtle changes in alcohol induced MN would get overshadowed by cofounding influences.[19,25]

The MN assay in buccal cells has also been used to monitor the effects of a number of chemopreventive agents. A number of micronutrients, including beta-carotene and other vitamins, have been shown to significantly decrease MN levels (1.4-4-fold) in healthy tobacco users,

as well as in individuals with precancerous lesions. Other micronutrients, such as retinol, riboflavin, zinc, and selenium, however, failed to reduce the MN frequency in a study carried out in China in areas with a high incidence of esophageal cancer.[15,26]

Methodological factors that can affect the levels of MN in buccal cells include differences in cell collection (timing and implements used), fixation and staining techniques, selection and number of cells counted, and the scoring criteria for MN, and other nuclear anomalies in normal and degenerated cells.

Different diagnostic methods, such as routine histopathology, exfoliative cytology, and immunohistochemistry are available today. Of these, oral exfoliative cytology is particularly valuable for mass screening purpose. It has been shown to have a sensitivity of 94%, specificity of 100% and accuracy of 95%. Exfoliated buccal mucosal cells can be collected using a wooden tongue depressor, a metal spatula, toothpicks, or toothbrushes, or a cytobrush moistened with water or buffer to swab or gently scrape the mucosa of the inner lining of one or both cheeks.[27] Cytobrushes appear to be most effective for collecting large numbers of buccal cells. Casartelli *et al.*, observed that MN frequencies were higher when cells were collected by vigorous, rather than by light scraping, suggesting a decreasing MN frequency gradient from basal to superficial layers of mucosa.[28]

DNA specific stains are preferred for staining nuclei, MN, and other nuclear anomalies in buccal exfoliated cells. Feulgen-Fast Green is favored by many investigators because of its DNA specificity and a clear transparent appearance of the cytoplasm which enables easy identification of MN; however method is relatively lengthy and may lead to the underscoring of MN. Other stains include fluorescent dyes, such as diamidino-2-phenylindole (DAPI), acridine orange, Hoechst, and propidium iodide and nonspecific stains like May-Grunwald Giemsa (Giemsa), PAP, hematoxylin and eosin, and Orcien.[29] Some of the studies reported increased frequencies of MN with Giemsa staining and suggest the possibility that cellular structures resembling MN, such as keratohyaline granules or bacteria, can lead to false positive results.[30] Bacteria can be differentiated from MN by their characteristic shape, smaller size, color, staining intensity, and their presence upon and between buccal cells on the slide.[31] Misinterpretation of nuclear anomalies like karyorrhexis, karyolysis, condensed chromatin, and binucleates as MN sometimes may occur, as significant correlation was observed between these anomalies and MN count using DNA nonspecific stains in a study carried out by Nersesyan *et al.*[11,30]

Ayyad *et al.*, compared Giemsa with the PAP stain for buccal cell analysis under field conditions and concluded that the PAP stain was preferred method of detecting MN in oral epithelia.[23,31] We agree to this finding since the Papaniculaou stain has resulted good clarity and transparency of epithelial cells which enables to identify MN easily.

Further, in a study Feulgen-Fast Green staining was compared with propidium iodide, which also served as a counterstain for pancentromeric fluorescent probes following *in situ* hybridization (FISH). In this study the fluorescent dye propidium iodide performed equally well as Feulgen-Fast Green, but provided an additional advantage of identifying the mechanism of MN formation through centromeric FISH labeling.[31]

The first publication of Stich and Rosin referred to the well-established basic criteria for MN that was initially described by Heddle, but the criteria for identifying cells for inclusion into the MN frequency count were not provided. Some authors refer to the Heddle criteria as such, or with minor modifications. However, the criteria developed by Tolbert *et al.*, for choosing the cells is the most widely used.[12] Tolbert *et al.*, recommended the scoring of at least 1,000 cells, with an increase to 2,000-3,000 if fewer than five micronucleated cells were observed after counting 1,000 cells. The majority of the published studies have scored between 1,000 and 3,000 cells, although it has been suggested that 10,000 cells may be needed to observe a statistically significant, 50% increase, in the MN frequency.[32]

CONCLUSION

Specific biomarkers on cytogenetic end points may help in establishing preventive measures to reduce cancer risks but they will not allow any statement, as to whether or when a malignant change may happen. Biological monitoring cannot replace the medical check-up and histopathological diagnosis when cancer is suspected. However, it becomes more significant for prescreening programs of high-risk groups, especially for those cancers such as head and neck SCC and lung cancers, whose etiology can be strongly influenced by environmental genotoxic exposures.[18]

The present study demonstrated that frequency of MN was higher in SCC than in leukoplakia and healthy control, moreover frequency of MN increases with increased duration of genotoxic exposure, suggesting increase in cytogenetic damage. Hence, MN assay can be used as an important biomarker for cytogenetic damage in oral leukoplakia and SCC.

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