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ORIGINAL ARTICLE

Micronucleus—an upcoming marker of genotoxic damage

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Abstract This study was conceived for the early detection of oral precancer and cancer lesions using a noninvasive reliable technique. Micronucleus assay was performed on oral exfoliated cells of chosen subjects having leukoplakia and squamous cell carcinoma (SCC) using fluorescent (Acridine Orange) and conventional (Feulgen) stainings. The results were analyzed using Mann–Whitney *U* test, Kruskal–Wallis test, Spearman's Correlation and SPSS statistical package. The frequency of mean percentage occurrence of micronucleated cells increased significantly in comparison to controls with leukoplakia and squamous cell carcinoma. Subjects with synergism of abnormal oral habits also showed increased micronucleated cells. Fluorescent staining was found to be more sensitive than the conventional one for micronucleus detection. The results clearly demonstrate that micronucleus assay in oral exfoliated cells can be used as a simple reliable marker to assess the genotoxicity and for the early diagnosis of premalignant and malignant lesions. Micronucleus assay is, thus, an easy tool for early detection of cancer.

Keywords Micronucleus assay · Oral exfoliated cells · Leukoplakia · Squamous cell carcinoma · Conventional · Fluorescent stainings

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Introduction

Cancer is one of the most life threatening diseases afflicting mankind. The word "cancer", in itself, generates fear amongst all human beings, to whichever strata of the society they may belong. It is often diagnosed at an advanced stage because of the lack of early diagnostic markers, and therefore, the survival rate is markedly reduced despite the best available treatment options.

Oral cancer mostly occurs as a result of malignant transformation of a preexisting lesion. Leukoplakia is a premalignant lesion commonly affecting the oral cavity. It has been associated with smoking, tobacco chewing and alcohol consumption. Pindborg, too, emphasized smoking as one of the strongest etiological factors associated with the production of leukoplakia [9]. Therefore, cases having leukoplakia were included in the study.

As tobacco and alcohol are also said to be important etiological factors for cancerous lesions, and if truly, premalignant lesions like leukoplakia are left untreated they will progress to malignancy, so the second group chosen for the study were cases of squamous cell carcinoma. These two groups were matched with a control cohort group of ten patients with no abnormal habits and normal appearing mucosa.

Analysis of chromosomal alterations in malignant cells from patients has provided diagnostic and prognostic information [19, 21]. However easy and reliable techniques for the detection of precancerous lesions are needed [11, 12].

Micronucleus assay could be used to assess chromosome damage as they are examined in routine cytopathological preparations [15]. Their frequency of occurrence is a measure of chromosome breakage in early cell divisions, and the number of micronuclei is known to increase with carcinogenic stimuli, long before the development of

clinical symptoms [15]. The advantage of micronucleus assay lies in its simplicity, as the scoring of micronuclei is rapid and does not require much expertise.

Thus, micronucleus in a cell represents an "internal dosimeter" to estimate exposure to genotoxic and carcinogenic agents [16]. The use of fluorescence dye in the screening of micronuclei enhances the demonstration of nuclei and micronuclei [5, 14, 20].

The aim of the present study was to define micronucleus as an early diagnostic tool of leukoplakia and SCC. It also compared the relative advantage of fluorescent staining over the conventional one in micronucleus detection.

Materials and methods

Selection of subjects

Exfoliated oral mucosal cells were taken from clinically established and histologically confirmed patients, 25 each of leukoplakia (group II) and SCC (group III). The complete case history and clinical findings of subjects were recorded. A control cohort (group I) consisted of ten subjects with no abnormal oral habits and normal appearing mucosa between the age group of 15–20 years.

Smear procedure

All the patients were asked to rinse their mouth thoroughly for 2 min with Chlohex mouthwash before taking the smear. The site of the smear was located and wiped with cotton and moistened in normal saline, to remove slough/surface coatings. In hyperkeratotic lesions such as leukoplakia, surface keratin layer was scraped off by a curette. The scrapings of each case were smeared onto two grease-free slides. This was followed by a biopsy from the affected site, and only those patients were considered into the groups, which were histologically diagnosed as leukoplakia and SCC consistent with clinical findings.

Micronucleus assay

The smeared slides were fixed in cytology fixative. The slides were stained with two parts 0.1% Acridine orange (Loba Chemie) solution in 30 parts phosphate buffer (pH 6.8). The buffer mounted slides were observed under a fluorescence microscope (Leitz, Aristoplan, Germany) equipped with 450 to 490 nm BP filter set with excitation at 453 nm using a ×40 objective for the presence of micronuclei in the oral exfoliated cells. Then, the same slides were destained using 50% ethyl alcohol for 3 min. The destained slides were restained using conventional Feulgen method employing de Thomasi Schiff reagent.

The criterion for scoring micronuclei was based on the study by Schmid, which states that the micronuclei resemble the nuclei of the cell but are smaller in size. They are round to ovoid with distinct outlines, non-refractile and have the same color as that of the main nucleus and the diameter is less than one-third of the main nucleus. A total of 500 cells were counted in the smear from the affected and unaffected site of each patient; thus 1,000 cells per individual were counted.

The statistical significance was determined using Mann–Whitney *U* test, Kruskal–Wallis test and statistical package SPSS.

Results

Micronucleus assay is a simple and rapid screening test applied for an early detection of cancer [18]. The test is used on exfoliated cells to identify the genotoxic damage in human tissues, which are targets for carcinogens and from which carcinomas develop.

The study showed a significant increase in the mean percentage micronucleated cells both in group II (2.30; 1.70) and group III (2.71; 2.05) when compared with group I (0.64; 0.45) in fluorescent and conventional stainings. The difference among the three groups was found to be very highly significant (H=21.88, p<0.001; H=15.29, p<0.001) for both fluorescent and conventional stainings using Kruskall–Wallis test (Table 1, Figs. 1, 2, 3, 4 and 5).

Table 1 Mean percentage occurrence of micronucleated cells and its comparison between fluorescent and conventional stainings

Group	Staining (mean % MN±SD)		Comparison	
	Fluorescent (F)	Conventional (C)	F vs C	Remarks
I (Control)	0.64 (±0.24)	0.45 (± 0.15)	p>0.05	NS
II (Leukoplakia) (H=21.88)	2.30 (±0.95)	1.70 (±0.77)	p<0.001	VHS
III (SCC) (H=15.29)	2.71 (±1.03)	2.05 (±1.00)	p<0.001	VHS

SCC Squamous cell carcinoma, *MN* micronucleated, *SD* standard deviation, *NS* not significant, *VHS* very high significance, *H* Kruskal–Wallis test

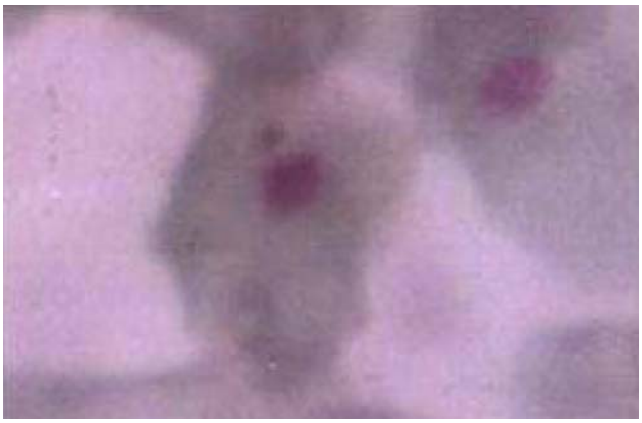

Fig. 1 Photomicrograph of a single cell with a single micronucleus (×100) using conventional Feulgen staining

Variations in the percentage micronucleated cells were seen between fluorescent and conventional stainings in different groups. Fluorescent staining showed significantly more ($p<0.001$) than conventional staining in both leukoplakia and squamous cell carcinoma patients, respectively. (Table 1). The mean micronucleated cell count was seen to be higher in cases with abnormal oral habits 3.93±0.696 than those with no habits (1.06) (Table 2). The results showed that premalignant lesions occur a decade and a half (age 43.64) before the occurrence of carcinoma (age 58.76), and the most commonly affected site in both the conditions was the buccal mucosa (group I, 64%; group II, 40%), respectively.

A comparison was also made between the mean percentages of micronucleated cells in affected and unaffected mucosa of the two affected groups. The micronucleated cell counts were found to be higher, and a very high significance was seen between the affected mucosa as compared to the unaffected mucosa in both the groups ($p<0.01$). The presence of micronucleated cells in the unaffected site could be due to the distribution of genotoxic agents through saliva (Table 3).

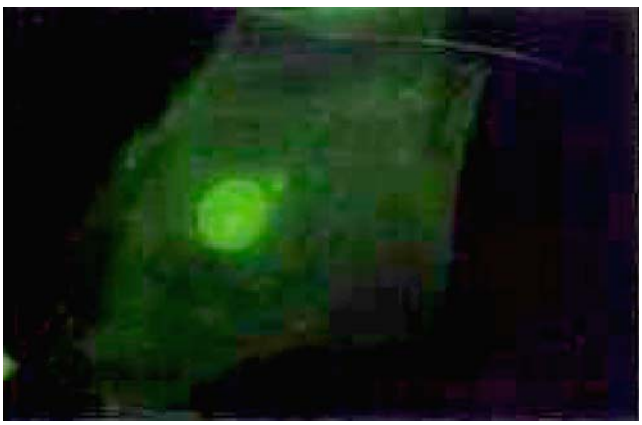

Fig. 2 Photomicrograph of a single cell with a single micronucleus (×100) using fluorescent staining

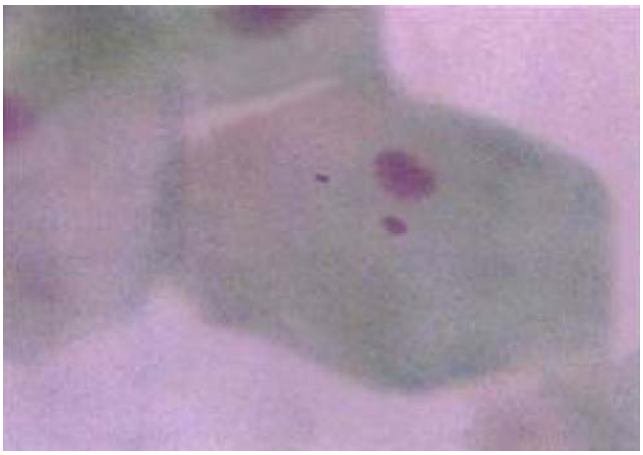

Fig. 3 Photomicrograph of a single cell with a two micronuclei (×100) using conventional Feulgen staining

Discussion

The majority of human cancers are caused by tobacco, synthetic and natural chemicals of occupational, environmental, medical and dietary origin. The chemical carcinogens cause structural alterations in the DNA of target cells leading to genomic instability in the form of chromosomal abnormalities [3].

A micronucleus is a small extra nucleus separated from the main nucleus and is generated during cellular division when a chromosome fragments or divides late [10]. Micronucleus assay has been used to determine the genotoxic and mutagenic potentials of various physical and chemical agents which could lead to the production of micronuclei [3, 18].

An early diagnostic test would be highly beneficial to check the progress of leukoplakia to squamous cell carcinoma followed by its early treatment, as shown by this study that pre-malignancy occurs much before malignancy. Buccal mucosa is seen to be the most affected, as it is the

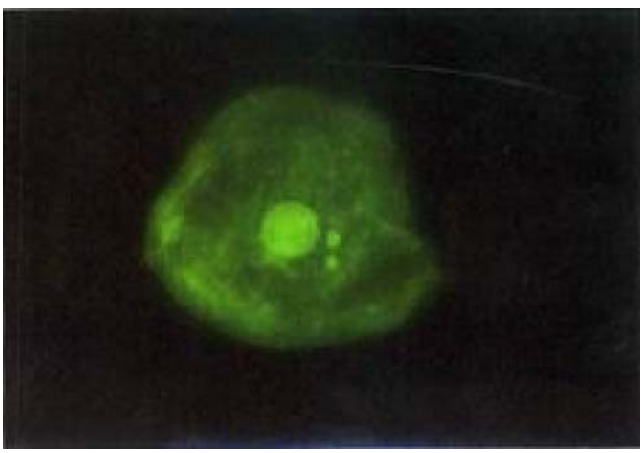

Fig. 4 Photomicrograph of a single cell with a two micronuclei (×100) using fluorescent staining

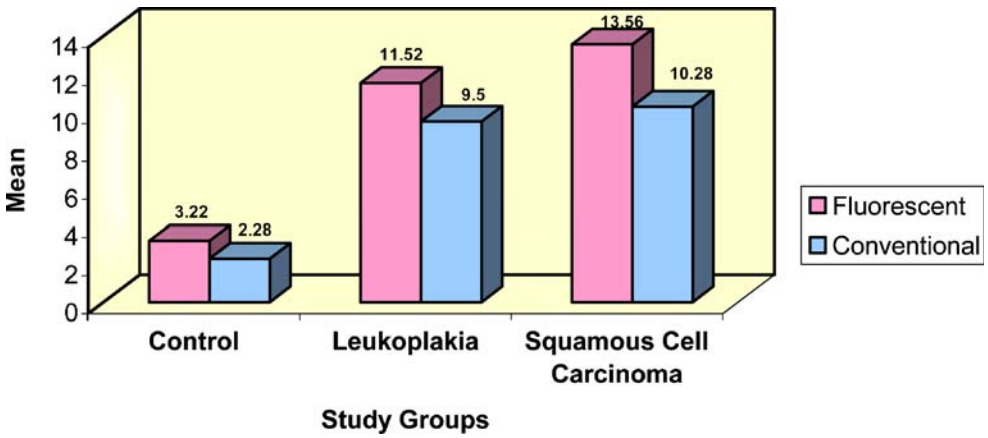

Fig. 5 Mean micronucleated cells in different study groups using fluorescence and conventional stainings

most accessible site in the oral cavity, and its covering epithelium is non-keratinised. In addition, more surface area of the mucosa is exposed to the insults in the oral cavity, making it more vulnerable to changes.

In the present study, a mean percentage occurrence of 0.64% micronucleated cells was obtained for the control group (Table 1). Regional variation is seen in the mean percentage of micronucleated cells in normal populations as shown by the different values in different studies. Stich, Stich and Parida [16] (1982) showed the mean percentage of micronucleated cells in the buccal mucosa of controls in Orissa population as 0.39 and 0.44% in the populations of Meghalaya. Stich and Rosin [15] (1984) quoted the mean percentage occurrence of micronucleated cells in the control populations of different countries as 0.44% ranging from 0.0 to 0.9%. The difference in the frequencies of micronucleated cells in the control group could be due to the different food habits of the population groups studied. Nishioka and Nishi et al. [7] stated that man ingests various types of mutagens and/or carcinogens in his daily diet, and that could be the reason for the variable level of micronucleated cells.

In this study, group II of leukoplakia showed a highly significant increase of micronucleated cells (2.30%) when compared with the control group (Table 1). The study also showed the mean micronucleated cells in leukoplakia patients as 11.52 as compared to the control group (3.22). Due to the increase in the number of micronucleated cells, a correlation of 0.954 and $P<0.001$ was seen between exfoliated cells of leukoplakia and controls.

Desai et al. [1] in 1996 also reported similar results in a study conducted on the exfoliated cells of patients with precancerous oral lesions, like oral submucous fibrosis, leukoplakia and lichen planus. They showed a highly significant increase in the mean micronucleated cells in leukoplakia (10.8) as compared to their control group (1.9). Sun Z. et al. [17] also compared the micronucleated buccal mucosal cells of leukoplakia and controls to find a similar correlation as the present study of 0.997, which was very highly significant ($P<0.001$).

Group III included cases with squamous cell carcinoma, and the mean percentage occurrence of micronucleated cells in them was 2.71% that is highly significant when compared with the controls (0.64%) [4, 6] (Table 1). None of the samples obtained showed poorly differentiated squamous cell carcinoma in histopathology. Elevated frequencies of micronucleated cells reveal the genotoxic action of carcinogens and may indicate an elevated probability for the formation of particular chromosome changes, which in turn, via the effect of such alterations on

Table 2 Mean percentage micronucleated cells in habit patterns of combined study groups in fluorescent staining

Number	Habit patterns	*N*	Mean % MN cells	SD
1	S	5	2.57	0.91
2	TC	8	2.48	0.632
3	A	1	1	–
4	S+TC	9	2.5	0.952
5	S+A	16	2.6	0.911
6	TC+A	1	3.4	–
7	S+TC+A	7	3.93	0.696
8	NH	3	1.06	–

S Smoking, *TC* tobacco chewing, *A* alcohol, *NH* no habits, *MN* micronucleated, *SD* standard deviation

Table 3 Mean percentage occurrence of micronucleated cells in the affected and unaffected mucosa in fluorescent and conventional staining

Group	Staining			
	Fluorescent		Conventional	
	Affected	Unaffected	Affected	Unaffected
I (Control)	–	0.64	–	0.45
II (Leukoplakia)	2.30	0.44	1.70	0.40
III (SCC)	2.71	0.44	2.05	0.30

SCC Squamous cell carcinoma

oncogene expression, could be associated with neoplastic transformation. Thus, micronucleus assay can be a viable tool to show genotoxic damage in the healthy mucosa of people having a high risk to develop squamous cell carcinoma, but it cannot predict when such carcinomas will arise [16].

The mean percentage occurrence of micronucleated cells with smoking as the only habit is 2.57% (Table 2). Stich and Rosin [15] (1984) reported a much higher value of 4.62% micronucleated cells from scrapings of palatal mucosa of inverted smokers. Variations in the mean percentage micronucleated cells may be attributed to many factors. P.N. Wahi et al. pointed out that heat application on the mucosa has a definite effect in enhancing the action of tobacco smoke on the mucosal cells. The quantum of consumption of cigarettes, the quality and volume of tobacco also act as factors responsible for the increased genotoxicity in the affected mucosa, thereby, causing an increase in the micronucleated cell counts of an individual.

The patients with the habit of only tobacco chewing in this study had mean percentage occurrence of 2.48% micronucleated cells (Table 2). Stich, Stich and Parida [16] (1982) studied the percentage occurrence of micronucleated cells in quid chewers of Orissa and reported a value of 5.89%. They also reported a percentage occurrence of micronucleated cells in raw betel nut chewers of Meghalaya as 4.68%. Stich and Rosin [15] in 1984 mentioned the percentage micronucleated cells in tobacco chewers in the range of 2.18 to 7.25%.

These variations in the micronucleated cells may be attributed to the ingredients in the quid, the number of quids per day and to the different lifestyles and food habits of people as these studies were conducted in different populations of Orissa and Meghalaya tribes. The mean percentage occurrence of micronucleated cells in cases having habits of both smoking and alcohol was 2.6% (Table 2), which was quite similar to the study done by Stich and Rosin in 1984, who reported a value of 2.29% micronucleated cells in smokers and alcohol drinkers [15].

The mean percentage occurrence of micronucleated cells in patients having habits of tobacco chewing and alcohol consumption was 3.4% (Table 2), and cases with the habits of smoking, tobacco chewing and alcohol consumption showed the mean percentage micronucleated cells as 3.93% (Table 2). Addition of tobacco is said to enhance the occurrence of chromosomal aberrations, thereby, causing micronucleus formation, which explains the increasing count in the affected tissues.

The synergistic effect of more than one habit is proved by the higher values of mean percentage micronucleated cells in the mixed habit groups. Ghose and Parida [2] have also reported similar results in the tribal populations of Orissa stating the mean percentage micronucleated cells as 5.90–7.34% who had habits of smoking, tobacco chewing and alcohol consumption. Obe [8] stated that alcohol inhibits salivation, which leads to a higher concentration of carcinogens from tobacco smoke in the mouth. The frequency of micronucleated cells will reflect the capacity of target tissues to activate procarcinogens into reactive species or to inactivate or trap ultimate carcinogens. It also reveals additive, synergistic and antagonistic interactions between the myriad compounds to which a cell is exposed [16].

The variations in the micronucleated cell counts in various studies could possibly be due to the fact that before the cells exfoliate they must migrate from the basal layer where the micronuclei are formed to the surface of the epithelium [13]. This migration may require a few days or weeks as different tissues have different turn over rates. During this time span, micronucleated cells could conceivably lyse and disappear from the epithelium before reaching the surface layers. Thus, on analyzing exfoliated surface cells, the actual frequency of micronucleated cells may be underestimated [15].

A comparison was also made between the mean percentage occurrence of micronucleated cells in affected and unaffected mucosa of group II and group III patients (Table 3). The micronucleated cell counts were found to be higher, and a very high significance was seen between the affected mucosa as compared to the unaffected mucosa in both the groups. The involvement of tobacco in the induction of micronucleated cells can also be deduced from the higher frequencies of mean percentage micronucleated mucosal cells at the site at which tobacco product is placed as compared to the opposite unaffected site (Table 3) in the oral cavity. This presence of micronucleated cells in the unaffected site could be due to the distribution of genotoxic agents through saliva [4, 16].

Thus, in conclusion, micronucleus can be stated as an early indicator and an upcoming marker for diagnosing oral precancer and cancer. Also, the fluorescent staining was more sensitive than the conventional one for micronucleus detection. Abnormal oral habits too significantly increase the counts of micronucleus.

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