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Research Article

Comparative Study of Exfoliated Oral Mucosal Cell Micronucleus Frequency in Potentially Malignant and Malignant Lesions

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Abstract

Objectives: 1. To observe and compare the micronuclei (MN) index in potentially malignant lesions / conditions and malignant cases. 2. To compare Papanicolaou (Pap) and May-Grunwald Giemsa (MGG) stain as two techniques to detect micronuclei in potentially malignant and malignant cases. **Study design:** Cytological smear of ten patients with habit of tobacco using in any form without any lesion, 20 smokers with potentially malignant lesions / conditions and 20 smokers with oral squamous cell carcinoma were studied using Pap and MGG staining method. The frequency of MN was determined under 40X. The mean MN count was compared using the ANOVA with Bonferroni test for statistical analysis. **Results:** Average MN frequencies were increased in potentially malignant lesions / conditions as compared to tobacco users without any lesion and further increased in oral squamous cell carcinomas. Pap stain is the preferred method in the field studies for scoring and detecting MN compared to MGG. **Conclusion:** The MN count can be used as a noninvasive early detection tool, for mass screening, patient education and to check for the efficacy of treatment.

Keywords: Micronuclei;Cytology;Papanicolaou;May-Grunwald Giemsa Stain;Preneoplastic Conditions; Oral Squamous Cell Carcinoma.

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Introduction

Cancer, modern epidemic among non-communicable diseases is the second commonest cause of mortality in developed countries and remains one of the ten commonest causes of mortality in developing countries like India, is a complex disease with altered expression, abnormal growth and disruption of normal function of cells caused by genotoxic effects of chemical carcinogens or environmental pollutants resulting in genomic instability at an early stages of cancer, which is reflected often as leukoplakia, erythroplakia, Lichen planus, and submucous fibrosis. Oral cancer is the 11th most common cancer worldwide and the most common in India.[1]

To evaluate the genotoxic risks / effects in the tobacco users on buccal mucosa, deoxyribo nucleic acid (DNA) damages can be assessed by chromosomal aberrations, sister chromatid exchanges and micronuclei (MN) test.[2] Exposure of a tissue to genotoxic carcinogens leads to an increase in chromosomal aberrations.[3-6] Consistent with this hypothesis, karyotypic anomalies and elevated DNA content have been observed in various preneoplastic lesions. Before particular karyotypic anomalies can be established, a long period of breakage and translocation of chromatid must occur. Such chromatid anomalies or chromosomal deletions can lead to formation of MN, which are DNA containing bodies in the cytoplasm without any structural connection to the main nucleus.[7] MN are small, extra-nuclear bodies separated from the main one, generated during cellular division by late chromosomal fragments because of their association with chromosomal aberrations.[6-8] The MN test is one of the current rapid, efficient and economical techniques used as a indicator of genotoxicity, as it provides a quantitative measures of the genotoxic action of carcinogens and mutagens.[9-14]

Micronuclei have been defined as a microscopically visible; round to oval cytoplasmic chromatin mass next to the nucleus.[15] MN formation is the result of segregation defects due to chromosomal instability causing chromatin to be excluded from the reforming nucleus.[16] MN count has been proven to be a reliable biomarker for oral cancer risk.[17-19]

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Materials and Methods

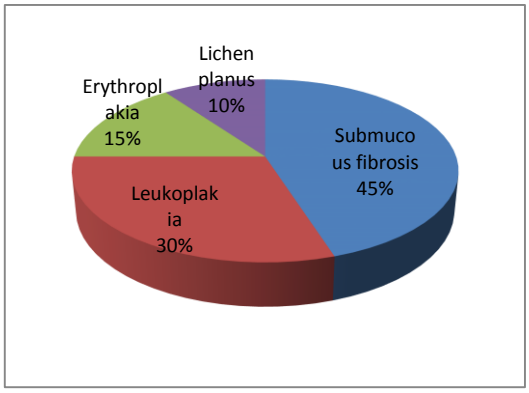
Study sample: The study sample was collected from the outpatient department. All individuals gave a written consent for participation. All individuals were interviewed for type of habit, duration and intensity of habit, dietary habits, systemic and local disease history and family history.

The study sample was divided into three categories as follows:

Group I: Ten patients of tobacco users in any form without any lesion.

Group II: Twenty patients of potentially malignant lesions / conditions like submucous fibrosis, lichen planus, leukoplakia and erythroplakia. Out of 20 potentially malignant lesions / conditions, 9 (45%) patients had submucous fibrosis, 6 (30%) had leukoplakia, 3 (15%) had erythroplakia and the rest 2 (10%) had lichen planus. (Graph 1)

Group III: Twenty patients suffering from different stages of oral squamous cell carcinoma confirmed by histopathological examination were included in our study.



Graph 1: Distribution of cases among potentially malignant lesions/conditions.

Procedure: Before sampling all individuals were asked to rinse the mouth thoroughly with tap water. Exfoliated cells were collected from the buccal mucosa on affected side using a clean premoistened wooden spatula. Scraped material was spread on the precleaned slides and smeared for each individual, two slides were prepared by smearing the oral scraping on the slides for a total of 100 (50 slides stained with Pap and 50 with MGG stain). For Pap stain, the smears were allowed to air dry and fixed with Biofix spray and then stained with Pap stain (Fig 1) and for MGG stain the slides were kept in freshly prepared fixative in the proportion of 3 parts of methanol and 1 part of glacial acetic acid for 20 minutes and then stained with MGG stain (Fig 2). 500 cells per slide were counted under high power magnification (40X). Only cells which were not fragmented and not overlapping were counted. Five blind examiners carried out the count. Observations were recorded and tabulated. Collected data was subjected to ANOVA with Bonferroni test (Multiple comparison tests).

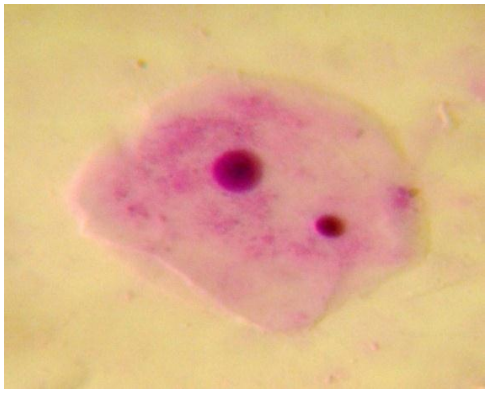


Figure 1: PAP stain showing MN (40X)

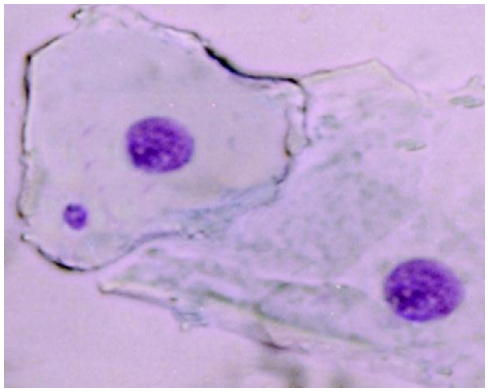


Figure 2: MGG stain showing MN (40X)

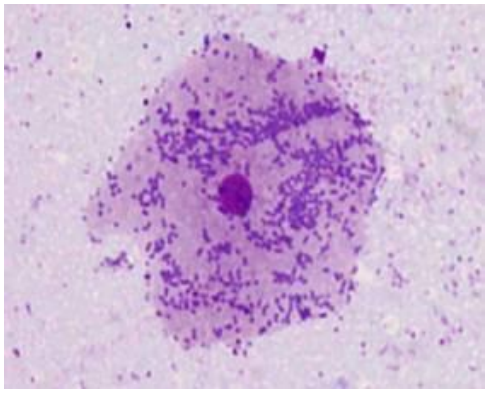


Figure 3: MGG stain showing bacterial colonies and cell debris masking the MN completely (40X).

Results

Out of 40 referred cases of lesions, 20 were in the age group of 10-60 years presenting with various potentially malignant lesions / conditions and the rest 20 were in the similar

age group, presenting with oral squamous cell carcinoma. (Table 1) Mean MN index was 8.66 in erythroplakia cases and it was more in comparison with other potentially malignant lesions / conditions. (Table 2) There was a statistically significant difference in the mean MN count between the tobacco users without any lesion to potentially malignant lesions/ conditions to malignant cases. (Table 3)

Age group	Potentially malignant lesions / conditions		Malignant cases	
	Male	Female	Male	Female
10-20	1	-	-	-
21-30	3	-	3	-
31-40	4	2	4	2
41-50	5	3	4	1
51-60	2	-	6	-
Total	15(75%)	5(25%)	17(85%)	3(15%)

Table1: Age and Sex wise distribution of potentially malignant and malignant cases.

Premalignant cases	No.	MN count / 500 cells	Mean	%
Submucous Fibrosis	9	46	5.1	25.17
Leukoplakia	6	18	3	14.80
Erythroplakia	3	26	**8.66**	42.74
Lichen planus	2	7	3.5	17.27
Total	2	97	20.26	100

Table 2: The comparison of mean micronuclei index in potentially malignant lesion.

	No.	MN count / 500 cells	Mean (x)	Std. deviation	P - value
Tobacco users without any lesion	10	28	2.8	1.229	< 0.05
Potentially malignant lesions/ conditions	20	97	4.85	2.330	
Malignant lesions	20	166	8.3	1.592	

Table 3: Comparison of mean MN index between premalignant and malignant cases.

There was statistically significant difference between group I and group II, group II and III and highly significant difference between group I and III. (Table 4) Difference between mean MN count among group I with premalignant lesion (PML) and premalignant condition (PMC) was statistically non-significant. (Table 5) Difference between mean MN count between group III with premalignant lesion and premalignant condition was statistically significant. (Table 6) There was statistically significant difference between group III with PML and PMC, but in difference between PML and PMC is non- significant. (Table 7)

Group I and II	P = 0.001	Significant
Group II and III	P = 0.017	Significant
Group I and III	P = 0.000	Highly Significant

Table 4: The intercomparison analysis between different groups.

Group	N	Mean	S.D.	ANOVA	
				F	p-value
Group I	10	2.8	1.229	3.501	0.058
PML	9	4.89	3.1		
PMC	11	4.82	1.328		

Table 5: Comparison between Group I with PML and PMC.

Group	N	Mean	S.D.	ANOVA	
				F	P - Value
Malignant	20	8.3	1.593	15.431	0.001
PML	9	4.89	3.1		
PMC	11	4.82	1.328		

Table 6: Comparison between Group III with PML and PMC.

(I)	(J)	P - value	Significance
Group III	PML	0.001	Significant
Group III	PMC	0.001	Significant
PML	PMC	1	Non - Significant

Table 7: Statistical analysis between Group II and Group III.

Discussion

Squamous cell carcinoma of the oral mucosa accounts for 90 - 95% of all oral malignancies.[20] Oral exfoliative cytology has been used extensively for screening cellular alteration. An accuracy of 95% and a reliability of more than 96% in detection of squamous cell carcinoma in mass screening

have been reported in the literature.[21] Oral exfoliative cytology can reveal various cellular alterations in squamous cell carcinoma. It includes karyorrhexis, karyolysis, micronucleus formation, pyknosis, binucleation, broken-egg nucleus, anucleation, etc.[22,23]

Micronuclei in oral exfoliated cells is a marker of chromosomal damage caused by genotoxic agents from tobacco and tobacco-related substances, alcohol, etc.[24] The micronucleus assay has been used to assess the genotoxic damage in oral squamous cell carcinoma and oral premalignancies.[19,25]

The present study was undertaken to identify a feasible and economical method which could be used as a screening test in high risk population for identifying the effects of genomic instabilities and to introduce timely interventional strategy in order to treat and control the epidemic. The present study shows both premalignant and malignant cases were found to be more prevalent in males which are in order with the findings given by Butterworth[26] in 2000. Maximum numbers among the males were in the age group of 30-50 years and in the females in the age group of 31-40 years. In the present study, among patients with oral carcinoma 70% had consumed tobacco either in the form of chewing/ smoking. Our findings are in accordance with the study conducted by Scully et al[27] in 2000 who reported that 75% of the patients of oral carcinoma were tobacco users. Similar results were also observed by Matsui[28] in 2006. Hence, tobacco can be considered as a leading carcinogenic agent for causing DNA damage by its genotoxicity which leads to cancerous proliferation.

In the present study amongst all premalignant conditions, the erythroplakia cases had highest number of micronuclei because it has highest degree of epithelial dysplasia which, probably explains the observation made by many researchers as to why majority of erythroplakic lesions finally progressed to malignancy.

Mean micronuclei index shows count in tobacco users without any lesion was 2.230, in potentially malignant cases its 4.85 which to a large extent confirms the findings given by Buajeeb et al[29] in 2007 and in malignant cases it is 8.3. It suggest mean MN index was less in potentially malignant cases compared with malignant cases, this might be due to the minimal dysplastic changes occurring in SMF and lichen planus.

In the present study comparison between Pap stain and MGG stain was done. The study showed that under field conditions Pap stain is more practical than MGG stain for staining MN. Regarding Pap stain, it is easier to read, easier to process and transport in the field, it is more practical, and the 95% ethyl alcohol used for fixation which is bactericidal effect and it also maintains the morphological integrity of cell. PAP consists of nuclear stain i.e. Haemotoxylin that stains all nuclear DNA, both intranuclear and extra-nuclear. It also consists of two counter stains that make the cytoplasm transparent and cell boundaries well demarcated.[30]

However, there are certain disadvantages of MGG stain over PAP stain i. e. it is difficult to read, had bacterial colonies and cell debris masking the oral cells completely, so mean MN could not be detected properly (Fig 3). MGG stain requires slides to be air dried and then placed in methanol (80%) at 0^0C, this was difficult to apply in the field and resulted in difficulty in transport. So this study showed PAP stain is better than MGG stain for staining MN, this conclusion is consistent with studies by Roberts[31] and Guzman et al.[32]

Conclusion

1. Micronucleus index was observed to be two folds more in malignant cases when compared with potentially malignant cases.
2. Obtained data shows significant number of micronuclei in potentially malignant lesions / conditions which can be used in high risk populations as a screening test.
3. Pap stain is the preferred method in the field studies for scoring and detecting MN as compared to MGG.

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