

## Role of Micronucleus Scoring in Buccal Smears of Carcinoma Breast Cases - A Study in Rural Central India

### Abstract

**Background:** Micronucleus (MN), a small additional nucleus represents a sensitive indicator of chromosomal damage. It can be detected in buccal cells with the use of Giemsa stain by light microscopy and acridine orange (AO) stain by fluorescent microscopy. **Objective:** The aim of this study was to analyze the MN score in buccal smears of infiltrating duct carcinoma (IDC) cases and fibroadenoma (FA) cases as a control group. **Materials and Methods:** It was a prospective observational study, which included 78 IDC cases and 82 FA cases (as controls). The Giemsa and AO stained buccal smears were analyzed, and MN scoring was compared between IDC and FA cases. **Results:** The mean MN score of FA and IDC was  $0.10 \pm 0.31$  and  $1.97 \pm 0.73$  in AO and  $0.10 \pm 0.31$  and  $1.58 \pm 0.74$  in Giemsa stained smears ( $P = 0.0001$  and  $0.0003$ ) respectively. The MN score increased in a stepwise manner from Grade I to II, II to III of IDC in Giemsa-stained smears. The comparison between FA and three different grades of the IDC ( $P < 0.001$  each) and mean MN score between Grade II and III IDC ( $P = 0.007$ ) was statistically significant. While the comparison of mean MN score between Grade I and Grade II IDC was not statistically significant ( $P = 0.940$ ). The mean MN score with AO stain was higher than the mean MN score with Giemsa, and this difference was statistically significant ( $P = 0.001$ ). **Conclusions:** MN scoring in buccal smears in IDC cases was significantly increased than in FA cases. MN assay in buccal smears of a breast lump can be used as a potential biomarker for screening for breast carcinoma as it represents generalized chromosomal damage.

**Keywords:** Acridine orange, breast carcinoma, buccal, fine-needle aspiration cytology, Giemsa

### Introduction

Micronucleus (MN) is a small additional nucleus, which is morphologically identical but is smaller than the main nucleus (1/16 to 1/3 of the diameter of the main nucleus). It can be easily identified in the cytoplasm of the interphase nucleus on light microscopy.<sup>[1]</sup> These are formed when the acentric chromosome fragments, chromatid fragments or whole chromosomes fail to be incorporated in the daughter nuclei at the completion of telophase during mitotic cell division and thus, represents a measure of chromosome breakage and loss and is one of the sensitive indicators of chromosomal damage.<sup>[2]</sup>

The term chromosomal instability (CI) comprises aneuploidy and breakage. It is observed in approximately 85% of all solid tumors.<sup>[2]</sup> It can be estimated by high

resolution techniques such as cytokinesis block, immunostaining centromeres, telomeres, and DNA double stranded breaks and fluorescence in situ hybridization (FISH) based techniques.<sup>[3,4]</sup> There are few studies that have proven that a simpler technique like MN scoring is also a sensitive indicator of CI.<sup>[5]</sup>

Breast carcinoma is the second most common malignancy of females in India<sup>[6]</sup> and, like other cancers, is associated with CI.<sup>[7]</sup> The mutations in patients with breast carcinoma lead to CI, which results in an increase in MN, and thus, it may be helpful in breast carcinoma screening, diagnosis, and grading.<sup>[8]</sup> There are isolated studies in which MN score has been done in lymphocytes<sup>[9]</sup> and in buccal mucosa cells<sup>[10]</sup> to assess the generalized genetic damage in breast carcinoma.

The present study was carried out to compare the MN score in buccal smears in benign and malignant breast epithelial

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Submitted: 24-Apr-2020

Revised: 20-Jun-2020

Accepted: 15-Jul-2020

Published: 12-Oct-2020

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### Access this article online

Website: [www.cci-j-online.org](http://www.cci-j-online.org)

DOI: 10.4103/ccij.cci\_j\_57\_20

### Quick Response Code:



**How to cite this article:** Mangam SN, Deshmukh AV, Shivkumar VB. Role of micronucleus scoring in buccal smears of carcinoma breast cases - A study in rural central India. Clin Cancer Investig J 2020;9:193-7.

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cases with the help of Giemsa and acridine orange (AO) stains.

## Material and Methods

### Study design

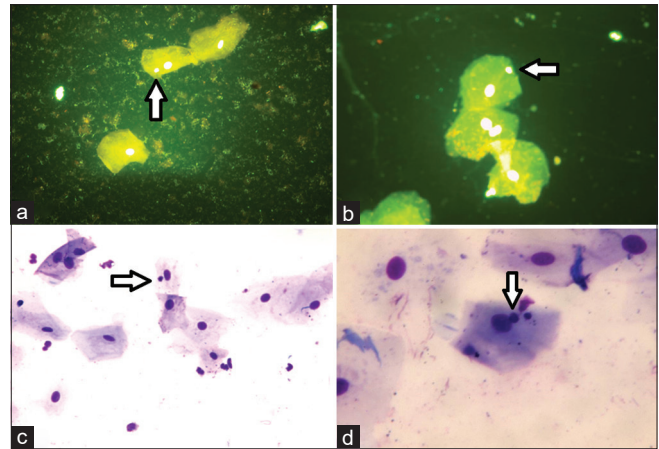
This was a prospective observational study conducted in the Department of Pathology at Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, Wardha, a rural tertiary care hospital in Central India for 24 months (November 2016–October 2018) after clearance from the Ethics Committee of the Institute. A total of 1751 breast fine-needle aspiration cytologies (FNACs) was done during this period in our institute. Of these, 532 cases were infiltrating duct carcinoma (IDC), 822 as fibroadenoma (FA), and 397 cases had a diagnosis other than above two.

FNAC was performed in all patients with a breast lump by making multiple passes by using 24G needle with an attached 10 ml syringe for applying constant negative pressure. The smears were prepared from the obtained material. Simultaneously, in the same sitting, buccal smears of the same patients were taken by a clean wooden spatula. Two buccal smears were prepared for each patient in the study. One air-dried smear from buccal mucosa was stained with Giemsa,<sup>[11]</sup> and the other 95% alcohol fixed smears were stained with 0.01% AO stain.<sup>[12]</sup>

### Inclusion criteria

The smears of patients diagnosed with IDC on cytology were considered as study participants, while those diagnosed with FA were taken as controls. Two observers separately and independently carried out MN scoring per 2000 epithelial cells under light microscope-*Olympus CX21i* in an oil immersion for Giemsa stained smears ( $\times 1000$  objective) according to the criteria described by Thomas and Fenech.<sup>[13]</sup> For, AO-stained smears, scoring was carried out under the fluorescent microscope-*Olympus BX41* ( $\times 400$  objective) according to the criteria described by Patino-Garcia *et al.*<sup>[14]</sup> Almost 30 min were required for MN scoring in each case. A minimum of 2000 cells were observed for MN scoring.

In AO-stained smears, MN was identified as bright green, round to oval in shape with a smooth perimeter having similar intensity and color as the main nucleus [Figure 1a and 1b]. In Giemsa-stained smears, MN was noted as nonrefractile, round to oval in shape with smooth perimeters suggesting a membrane. The diameter of micronucleus varied from 1/16 to 1/3 of the diameter of the main nucleus, and the color and texture were similar or slightly darker to the main nucleus [Figure 1c and 1d]. The only histopathology confirmed cases were included in the study.



**Figure 1: Buccal smear showing micronucleus (arrow) in acridine orange stain in case of (a) Fibroadenoma ( $\times 400$ ) (b) Infiltrating duct carcinoma ( $\times 400$ ); While micronucleus (arrow) in Giemsa stain in case of (c) Fibroadenoma ( $\times 400$ ) (d) Infiltrating duct carcinoma ( $\times 400$ )**

### Exclusion criteria

The cases having smears with scant cellularity ( $< 2000$  cells), severely obscured background due to dense inflammation, and other artefactual changes, clumps of cells with obscured nuclear and cytoplasmic boundaries and overlapped cells were excluded from the study.

Finally, a total of 160 cases were selected, which included 78 cases of IDC and 82 cases of FA for the study. The cytological grading of IDC cases was done according to the criteria given by Robinson *et al.*<sup>[15]</sup> MN score in buccal mucosa cells of IDC and FA cases were compared in both Giemsa and in AO-stained smears.

### Ethics

The study protocol was approved by the Institute Ethics committee of MGIMS, Sevagram, letter number MGIMS/IEC/PATH/191/2012, dated December 30, 2012. Informed consent was obtained from the patients before proceeding for the procedure. Patient confidentiality was maintained during all research procedures.

### Statistical analysis

Statistical analysis was performed using descriptive and inferential statistics using measures of central tendency (mean and standard deviation [SD]), independent sample *t*-test, Chi-square test, one-way analysis of variance (ANOVA) test and multiple comparisons: Tukey test. The software used in the analysis of the present study was IBM Corp. Released 2011. IBM Statistics for Windows, Version 20.0 SPSS (Statistical presentation system software) 22.0 version and Graph Pad Prism 6.0 version, and  $P < 0.05$  was considered as a level of significance.

### Results

The comparison of mean MN scores of FA and IDC was

made. In the case of AO stained smears, it was  $0.10 \pm 0.31$  and  $1.97 \pm 0.73$  respectively, while;  $0.10 \pm 0.31$  and  $1.58 \pm 0.74$ , respectively, in Giemsa-stained smears. The *P* value was significant in both cases ( $P = 0.0001$  and  $0.0003$ ) [Table 1-Upper half]. The comparison of mean MN scores of FA with cytological grades in Giemsa-stained buccal smears showed that the mean MN score ( $\pm$ SD) of FA and Grade I, II and III IDC were  $0.10 \pm 0.31$ ,  $1.34 \pm 0.62$ ,  $1.43 \pm 0.72$ , and  $1.93 \pm 0.75$ , respectively. The MN score increased in a stepwise manner from Grade I to II, II, to III of IDC [Table 1-Lower half].

An ANOVA test was applied to compare mean MN scores between FA and three different grades of IDC as well as MN score among three different grades of IDC in buccal smears. It indicated that there is a significant difference in the group means ( $P < 0.001$ ). Multiple comparison Tukey test showed that there was a significant difference of mean MN score between FA and Grade I, FA and Grade II, FA and Grade III ( $P < 0.001$  each), between Grade I and Grade III ( $P = 0.001$ ) and between Grade II and Grade III (0.007). No significant difference was found between Grade I and Grade II ( $P = 0.940$ ) [Table 2].

The mean age ( $\pm$ SD) of FA and IDC cases was  $28.74 \pm 8.44$  and  $49.84 \pm 12.62$ , respectively. To rule out increasing age as a confounding factor in the increase in MN scores of IDC than FA cases, the mean MN scores in different age groups in cases of IDC were noted. It was observed that increasing age was not a confounding factor for the increase in mean MN scores in IDC as there is no

relationship between the mean MN scores and increasing age group. This was noted in both AO as well as Giemsa stained buccal smears of IDC [Table 3].

Comparison of mean MN score in AO and Giemsa stain in IDC showed MN score of  $1.97 \pm 0.73$  and  $1.58 \pm 0.74$  in AO and Giemsa stains, respectively. The difference between these was statistically significant ( $P = 0.001$ ) [Table 4].

## Discussion

MN is a small additional nucleus lying within the cytoplasm. It is formed whenever a chromosome or its fragment is not incorporated into one of the daughter nuclei during cell division, and it is considered as one of the sensitive markers of CI.<sup>[16]</sup> Thus, MN scoring determines the chromosome breakage in a tumor.<sup>[17]</sup> FISH based techniques can detect CI, but these are not cost effective. Thus, there is a need to search for more economical and reasonable methods to assess CI in cancer patients,<sup>[3]</sup> and MN assay is one of them.

In our study, the baseline MN score was provided by the MN frequency seen in the FA cases. In 73 out of 82 cases of FA, no MN was seen, whereas; it was present in the smears of all the cases of IDC. There was some minor overlapping of MN scores in FA and IDC cases, but generally, a higher frequency of MN was observed in the IDC cases. We found significant differences in MN scores of FA and IDC cases in both AO- and Giemsa-stained smears ( $P = 0.0001$  and  $0.0003$ , respectively) [Table 1]. Our findings are consistent with Dey *et al.*<sup>[10]</sup> and Rajeswari *et al.*<sup>[18]</sup> who also noted a highly significant difference in MN scores of IDC and FA cases in their individual studies. In the present study, MN scoring was done with both Giemsa as well as AO stains in buccal cells. The increase in the mean MN score in the buccal cells of IDC cases compared to the FA cases indicates that there is more chromosomal damage in the buccal cells of the breast carcinoma patients. This points the finger to the probability that the chromosomal damage in breast cancer patients is generalized.

In this study, of 78 cases of IDC, 26 were Grade I, 23 were Grade II, and 29 were Grade III on cytology [Table 1]. The increase in the mean MN score from FA cases to Grade I of IDC cases, from Grade I to II and from Grade II to III of IDC cases denotes that there is an increase in the chromosomal damage from the baseline status (FA cases) to increasing grades of IDC cases [Table 2]. Similar findings were observed in by Verma and Dey<sup>[19]</sup> and Goel *et al.*<sup>[20]</sup> in breast fine-needle aspirates. We could not find any specific study which has compared the MN scores in the buccal smears with different grades of IDC and also with FA cases till date. Although there is an increase in the mean MN score in the buccal smears from FA cases to Grade I, from Grade I to Grade II and from Grade II to Grade III the increase in MN score between Grade I and Grade II was not statistically significant. This implies that there is

**Table 1: Comparison of mean micronucleus score of fibroadenoma and infiltrating duct carcinoma in both stains and comparison of mean micronucleus score of fibroadenoma and cytological grades of infiltrating duct carcinoma in giemsa stained buccal smears**

Comparison of mean MN score of FA and IDC in acridine orange stain					
Type of lesion	n	Mean	Standard deviation	t*	P**
FA	82	0.10	0.31	20.96	0.0001
IDC	78	1.97	0.73		
Comparison of mean MN score of FA and IDC in giemsa stain					
FA	82	0.10	0.31	16.48	0.0003
IDC	78	1.58	0.74		
Comparison of MN score of FA and cytological grades of IDC in Giemsa stain					
Type of lesion	n	Mean $\pm$ SD			
FA	82	0.10 $\pm$ 0.31			
IDC					
Grade I	26	1.34 $\pm$ 0.62			
Grade II	23	1.43 $\pm$ 0.72			
Grade III	29	1.93 $\pm$ 0.75			

\*Independent sample *t*-test, \*\*Chi-square test. FA: Fibroadenoma, IDC: Infiltrating duct carcinoma, SD: Standard deviation, MN: Micronucleus

**Table 2: Buccal smears: One-way ANOVA test for difference in the group means and Tukey test for multiple comparisons for difference in mean micronucleus scores in fibroadenoma and infiltrating duct carcinoma and difference within different grades of infiltrating duct carcinoma**

One-way ANOVA test						
Source of variation	Sum of squares	df*	Mean squares	F	P**	
Between groups	93.03	3	31.01	106.53	<0.001	
Within groups	45.41	156	0.29			
Total	138.44	159	-			
Multiple comparison: Tukey test						
Type of lesion	Mean difference	SE	P**	95% CI		
				Lower bound	Upper bound	
FA						
Grade I	1.23	0.12	<0.001	0.92	1.55	
Grade II	1.32	0.12	<0.001	0.99	1.65	
Grade III	1.82	0.11	<0.001	1.51	2.12	
Grade I						
Grade II	0.08	0.15	0.940	0.31	0.48	
Grade III	0.58	0.14	0.001	0.20	0.96	
Grade II						
Grade III	0.49	0.15	0.007	0.10	0.88	

\*Df: Degree of freedom, \*\*Chi-square test. FA: Fibroadenoma, SE: Standard error, CI: Confidence interval

**Table 3: Mean micronucleus score±SD in different age groups in acridine orange and giemsa stained buccal smears**

Age group	Number of IDC cases (%)	Mean MN score±SD	
		AO	Giemsa
14-23	0	0	0
24-33	8 (10.26)	1.75±0.46	1.50±0.53
34-43	19 (24.36)	1.89±0.73	1.63±0.95
44-53	20 (25.64)	2.15±0.74	1.65±0.67
>53	31 (39.74)	1.96±0.79	1.54±0.72

AO: Acridine orange, IDC: Infiltrating duct carcinoma, SD: Standard deviation

**Table 4: Comparison between acridine orange and giemsa staining in infiltrating duct carcinoma**

Stain	n	Mean±SD	t*	P**
MN-AO	78	1.97±0.73	3.23	<0.001
MN-Giemsa	78	1.58±0.74		

\*Independent sample t-test, \*\*Chi-square test. AO: Acridine orange, MN: Micronucleus, SD: Standard deviation

no proportionate increase in the generalized chromosomal damage with the increasing grades of IDC.

We noted that MN scores were present uniformly in both stains in all the age groups, which excluded increasing age as a confounding factor [Table 3]. We found that the mean MN score of AO stained smears in IDC was higher than the mean MN score of Giemsa-stained smears and this difference was statistically significant ( $P = < 0.001$ ) [Table 4]. Hence, AO is a better stain for doing MN scoring. Our findings are consistent with Polard *et al.*<sup>[21]</sup> who conducted a study on fine-needle aspirate of the breast. AO is a DNA specific stain, whereas

Giemsa is a nonspecific for DNA and thus can give false results.<sup>[21]</sup>

## Conclusions

We conclude that MN score in buccal smears is significantly increased in breast carcinoma as compared to FA cases, which show that there is a presence of increased chromosomal damage in malignancy. Since it represents a generalized chromosomal damage in the body, MN assay in buccal smears of a breast lump can be used as a potential biomarker for screening for breast carcinoma. AO is a comparatively better stain than Giemsa stain for scoring MN score in exfoliated buccal epithelial cells.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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