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Original Article

Role of Micronucleus Assay as an Indicator of Chromosomal Instability in Aspirates of Breast Carcinoma

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Abstract

Background: A micronucleus (MN) is a small additional nucleus, morphologically identical to but smaller than the main nucleus. It is a sensitive indicator of chromosomal instability, and it can be detected in fine-needle aspiration (FNA) smears with the Giemsa stain by light microscopy and the acridine orange (AO) stain by fluorescent microscopy. The objective of this study was to analyze the MN score in FNA smears of patients with breast carcinoma and fibroadenoma (FA). **Materials and Methods:** This was a prospective observational study which included 78 cases of infiltrating duct carcinoma (IDC) and 82 of FA (as controls). Giemsa- and AO-stained FNA smears were analyzed and MN scores were compared between the IDC and FA cases. **Results:** The mean MN scores of the FA and IDC groups were 0.28 ± 0.45 and 11.28 ± 7.22 in the AO-stained smears and 0.13 ± 0.34 and 9.79 ± 6.5 in the Giemsa-stained smears (P = 0.0002 and 0.0001), respectively. The MN score increased in a stepwise manner from FA to Grade I, II, and III of IDC in Giemsa-stained smears. Comparisons of mean MN score between FA and the three different grades of IDC and between Grade I and II and Grade III were statistically significant (<0.001 in each category). Although the mean MN score with AO stain was higher than the mean MN score with Giemsa stain, this difference was not statistically significant (P = 0.17). **Conclusion:** The MN score in FNA smears in the IDC group was significantly higher than in the FA group, suggesting that it can be used as a potential additional surrogate marker for diagnosing and grading breast carcinoma. Both AO and Giemsa stains were equally good for MN scoring of the FNA smears.

Keywords: Acridine orange, breast carcinoma, fibroadenoma, fine needle aspiration, Giemsa

Introduction

Micronucleus (MN) is a small additional nucleus which is usually found in the cytoplasm of interphase nuclei on light microscopy.[1] It is round to oval and identical to but smaller than the main nucleus. The diameter of the MN varies between 1/16 and 1/3 that of the main nucleus.[2] MNs are formed when 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.[3] The formation of MNs also represents a measure of chromosome breakage and loss and is thus a sensitive indicator of chromosomal damage.[3]

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Most solid tumors are aneuploid and have a chromosome number that is not a multiple of the haploid number. Thus, when chromosomes frequently mis-segregate, the phenomenon is called chromosomal instability (CI).[4] CI indicates that reduced mitotic fidelity has contributed to cancer progression by increasing genetic diversity among tumour cells; which indirectly indicates a poor patient prognosis.[4] CI can be estimated using high-resolution techniques such as immunostaining centromeres, telomere cytokinesis block and fluorescent *in situ* hybridization-based techniques.[5] Previous studies have suggested that MNs are a sensitive indicator for the detection of CI.[5] MN scoring can be done in low resource settings.

Breast cancer is one of the most frequently diagnosed cancers in females in India, with an estimated number of cases of 1,45,000 with an age-standardized incidence rate of 25.8/1,00,000 women.[6] As with other cancers, breast cancer is also associated with CI.[7] Most cases of breast cancer in high-risk families are attributed to mutations in the BRCA I and BRCA II genes.[8] These genes are needed for the repair of the double-stranded DNA breakup and mutation, and the loss of this function may cause CI. This may manifest as an increase in MN score, which may be helpful in breast carcinoma screening, diagnosis and grading.[9] Several studies have evaluated the MN score in lymphocytes[10] and in buccal mucosa cells[11] to assess the generalized genetic damage in breast carcinoma, and a few studies have compared MN score in primary epithelial cells between benign and malignant breast lesions.[9]

The present study was carried out to evaluate the role of MN score as a morphological indicator of CI in fine-needle aspiration (FNA) smears of breast epithelial cells using Giemsa and acridine orange (AO) stains.

Materials and Methods

This prospective observational study was approved on October 30, 2012, by the Ethics Committee of the Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, Wardha, Maharashtra, India (approval number: MGIMS/IEC/PATH/191/2012). The study was conducted at the cytopathology section of the Department of Pathology at MGIMS, a rural tertiary care hospital in Central India over a period of 24 months (November 2016 to October 2018). Informed consent from the patients was not required in this approved study. A total of 1751 cases of breast FNA cytology were performed during this period in our department.

FNA cytology was performed in all of these patients from breast lumps after the patient had provided informed consent and as per a clinician's request by making multiple passes using a 24-gauge needle with an attached 10 ml syringe. Smears were prepared from the obtained material. Two smears were prepared for each patient for the study. The air-dried smears were stained with Giemsa stain as per routine procedure in the cytopathology section[12] and the other 95% alcohol-fixed smears were stained with 0.01% AO.[13]

The patients who were diagnosed on cytology with infiltrating duct carcinoma (IDC) were enrolled as the study subjects, and those diagnosed with fibroadenoma (FA) were enrolled as controls. Two observers, blinded to the diagnosis, separately and independently carried out MN scoring per 2000 epithelial cells in an oil-immersion field for the Giemsa-stained smears (×1000 objective) under a microscope (Olympus CX21i) according to the criteria described by Thomas and Fenech.[14] For the AO-stained smears, scoring was carried out under a fluorescent microscope (Olympus BX41, ×400 objective) according to criteria described by Patino-Garcia *et al*.[15]

Almost 30 min was required for MN scoring in each case. In the Giemsa-stained smears, MNs were noted to be nonrefractile, round to oval in shape with smooth perimeters suggesting a membrane. The diameter of the MNs varied from 1/16 to 1/3 that of the main nucleus, and the colour and texture were similar or slightly darker than the main nucleus [Figure 1a-d]. The AO-stained smears were identified as bright green, round to oval in shape with a smooth perimeter and a similar intensity and colour as the main nucleus [Figure 2a-d]. Only histopathology-confirmed cases were included in the study. We excluded cases with scant cellularity (<2000 cells), those showing clumps of cells with obscured nuclear and cytoplasmic boundaries, overlapped cells, with a recent history of alcohol consumption, smoking or tobacco addiction. In addition, smears with a severely obscured background due to necrosis, dense inflammation and other artefactual changes were also excluded. Finally, 160 cases were selected, including 78 cases of IDC and 82 cases of FA. We further graded the IDC cases according to the cytology as Grade I ($n = 26$), Grade II ($n = 23$) and Grade III ($n = 29$) according to the criteria by Robinson *et al*.[16]

MN scores were recorded in both Giemsa and in AO stains in the FA and IDC groups. MN scores were also compared

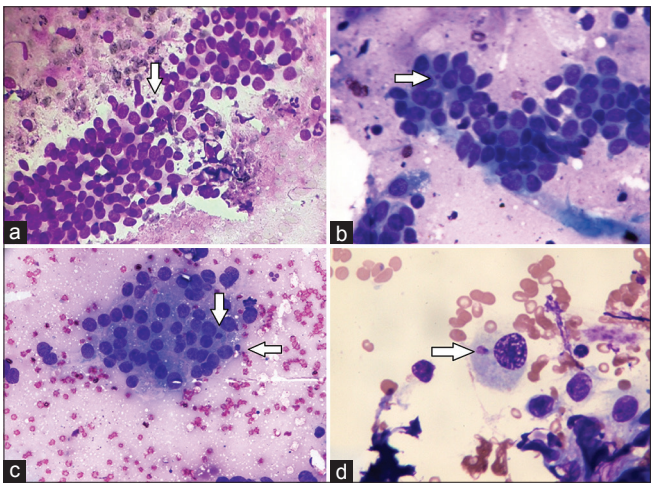

Figure 1: Fine needle aspiration smears of breast tissue showing micronuclei (arrow) with Giemsa staining in; (a) fibroadenoma (×400), (b) infiltrating duct carcinoma Grade I (×400), (c) infiltrating duct carcinoma Grade II (×400), (d) infiltrating duct carcinoma Grade III (×1000)

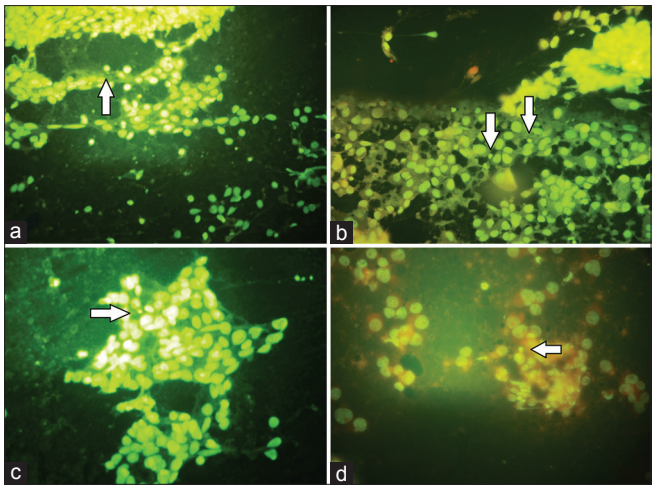

Figure 2: Fine needle aspiration smears of breast tissue showing micronuclei (arrow) with acridine orange staining in; (a) fibroadenoma (×400), (b) infiltrating duct carcinoma Grade I (×400), (c) infiltrating duct carcinoma Grade II (×400), (d) infiltrating duct carcinoma Grade III (×400)

among different cytological grades of breast carcinoma. Most FA cases (71 and 59 out of a total of 82 cases in AO-and Giemsa-stained smears, respectively) had an MN score of zero, and only a few cases (11 and 23 cases in AO-and Giemsa-stained smears, respectively) had an MN score of 1. In comparison, the MN score was > 2 in all cases with breast carcinoma.

Statistical analysis

Statistical analysis was done using descriptive and inferential statistics using measures of central tendency (mean and standard deviation), independent sample *t*-test, Chi-square test, one-way analysis of variance (ANOVA) and multiple comparisons (Tukey test). SPSS software version 22.0 (IBM Corp., Armonk, New York, USA) and GraphPad Prism version 6.0 (GraphPad, San Diego, California, USA) were used to conduct the statistical analyses, and $P < 0.05$ was considered to indicate a significant difference.

Results

Comparisons of mean MN scores in the FA and IDC groups were done. The MN scores in the AO-stained smears were 0.28 ± 0.45 and 11.28 ± 7.22, respectively, compared to 0.13 ± 0.34 and 9.79 ± 6.51, respectively, in the Giemsa-stained smears. The *P* value was significant in both cases ($P = 0.0002$ and 0.0001) [Table 1 - upper half]. Comparisons of mean MN scores of FA with cytological grades in the Giemsa-stained smears showed that the mean MN scores (± standard deviation) of FA and Grades I, II and III IDC were 0.13 ± 0.34, 3.80 ± 1.57, 9.34 ± 3.93 and 15.51 ± 5.90, respectively. The MN score increased significantly in a stepwise manner from Grades I to II, II to III of IDC [Table 1 - lower half] [Table 1].

The mean ages (± standard deviation) of the FA and IDC groups were 28.74 ± 8.44 and 49.84 ± 12.62 years, respectively. To rule out increasing age as a confounding factor in the increase in MN scores between the IDC and FA groups, the mean MN scores in different age groups in the cases with IDC were analyzed. The results showed that increasing age was not a confounding factor for the increase in mean MN scores in the IDC group, as there were no relationships between the mean MN scores and increasing age groups. This was noted in both AO-and Giemsa-stained FNA smears of IDC [Table 2].

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 fine-needle aspiration smears

Type of lesion	*n*	Mean±SD	*t**	*P***
Comparison of mean MN score of FA and IDC in AO stain				
FA	82	0.28±0.45	13.75	0.0002
IDC	78	11.28±7.22		
Comparison of mean MN score of FA and IDC in Giemsa stain				
FA	82	0.13±0.34	13.41	0.0001
IDC	78	9.79±6.51		

Comparison of MN score of FA and cytological grades of IDC in Giemsa stain

Type of lesion	*n*	Mean±SD
FA	82	0.13±0.34
IDC		
Grade I	26	3.80±1.57
Grade II	23	9.34±3.93
Grade III	29	15.51±5.90

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

ANOVA was applied to compare mean MN scores between FA and three different grades of IDC as well as MN scores among the three different grades of IDC in the FNA smears. The results showed a significant difference in group means (<0.001) in each group. Multiple comparison Tukey test showed that there were statistically significant differences in mean MN scores between FA and different grades of IDC as well as Grade I and Grade II, between Grade II and Grade III, and between Grade II and Grade III of IDC in the FNA smears (<0.001) [Table 3].

The mean MN scores in AO and Giemsa stains of IDC in the FNA smears were 11.28 ± 7.22 and 9.79 ± 6.51, respectively. Although the mean MN score of the AO-stained smears was higher than that of the Giemsa-stained smears, it was not statistically significant (independent sample *t*-test, $P = 0.17$) [Table 4].

Discussion

MN score is a sensitive marker of CI.[1] MN scoring can be done on blood cells, including lymphocytes,[11] breast epithelial cells[12] and also buccal mucosa cells.[9] CI can be detected using

many modern techniques,[5] but all the modern techniques are costly and unaffordable[17] for a resource constraint country such as India, where there is a huge burden of breast cancer patients.[6] Thus, there is a need to look for more economical and cost-effective methods to detect CI, and MN scoring is one of them.

The mean ages (± standard deviation) in the FA and IDC groups were 28.74 ± 8.44 and 49.84 ± 12.62 years, respectively, in our study, which are similar to the studies by Samanta *et al*.[9] and Goel *et al*.[18] MNs were not seen in 71 cases with FA in the present study, while all IDC cases showed variable numbers of MNs.

Our study showed statistically significant differences in mean scores of MN in both AO and Giemsa smears in the FA and IDC groups (P = 0.0002 and 0.0001, respectively) [Table 1]. Our findings are consistent with the studies by Samanta *et al*.,[9] Goel *et al*.[18] and Hemlatha *et al*.[19] The only difference between our study and the study by Hemalatha *et al*.[19] was the presence of higher mean MN scores in all categories of breast carcinoma grades in the latter study. This could be because of a higher baseline MN frequency of the study population in that specific area. Out of 78 cases with IDC, 26 were Grade I, 23 were Grade II, and 29 were Grade III on cytology in our study. We found an increase in MN score in a stepwise manner from Grades II to III of IDC [Table 1], similar to the findings observed by Samanta *et al*.,[9] Goel *et al*.,[18] Sylvia *et al*.[20] and Verma and Dey.[21] These findings showed that cancer is associated with the accumulation of chromosomal mutations which are present in all epithelial cells and that MN score can definitely help to detect it. In addition, breast carcinoma, as with any other malignancy, is associated with several types of chromosomal abnormalities and mutations.[22] Germ-line mutations in BRCA1 and BRCA2 genes are an important part of genetic and hereditary factors for breast and ovarian cancers.[23] These genes are necessary for the repair of double-stranded DNA breaks, and these chromosomal breaks manifest as MNs.

We also found that MN scores were present uniformly in both stains in all age groups, which excluded increasing age as a confounding factor [Table 2]. The increase in mean MN score from FA cases to different grades of IDC i.e., FA to Grade I, FA to Grade II and FA to Grade III, as well as from Grade I to II, from Grade I to III and from Grade II to III were statistically significant difference (P < 0.001 in each category) [Table 3]. Our findings are consistent with Goel *et al*.[18] and Verma and Dey.[21] This indicated that there was an increase in chromosomal damage from the baseline status (FA cases) to increasing grades of IDC and that the increase in chromosomal damage was proportionate to the tumour grade. It is well accepted that cancer is associated with increased

Table 2: Mean micronucleus score±standard deviation in different age groups in acridine orange and Giemsa stained fine-needle aspiration smears

Age group	Number of IDC cases (%)	Mean MN score±SD	
		AO	Giemsa
14- 23	0 (0)	0	0
24- 33	8 (10.26)	10.75±6.86	8.87±7.12
34- 43	19 (24.36)	9.74±7.01	8.78±6.52
44- 53	20 (25.64)	14.05±8.33	12.2±7.24
>53	31 (39.74)	10.58±6.50	9.10±5.71

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

Table 3: Fine-needle aspiration cytology smears: One way analysis of variance 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	5617.07	3	1872.35	210.13	<0.001
Within groups	1390.02	156	8.91		
Total	7007.09	159			

Multiple comparison: Tukey test

Type of lesion	Mean difference	SE	*P***	95% CI	
				Lower bound	Upper bound
FA					
Grade I	3.67	0.67	<0.001	1.92	5.41
Grade II	9.21	0.70	<0.001	7.38	11.04
Grade III	15.38	0.64	<0.001	13.70	17.05
Grade I					
Grade II	5.54	0.85	<0.001	3.32	7.75
Grade III	11.70	0.80	<0.001	9.61	13.80
Grade II					
Grade III	6.16	0.83	<0.001	4.00	8.33

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

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

Stain	*n*	Mean±SD	*t**	*P***
MN-AO	78	11.28±7.22	1.35	0.17
MN-Giemsa	78	9.79±6.51		

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

incidence and accumulation of chromosomal mutations. These mutations lead to chromosomal instabilities which manifest as structural abnormalities of the chromosomes or alterations in the chromosome number.[7,8] As the cytological grading increases in a stepwise manner, the higher the MN score, the higher the histological grading of the tumour. This, in turn, reflects the poor prognosis considering the other prognostic factors of the patient. Sylvia *et al*.[20] also reported low MN scores in benign and adenosis groups, borderline MN score in the hyperplasia group and higher MN score in malignant cases increasing with the grade of breast carcinoma. As MN score detects the gradual increase in genetic damage, it can serve as an additional tool in the classification of breast lesions on cytology, especially the borderline gray zone categories of ductal hyperplasias.[20] Thus, it gives a preoperative clue according to the grading of breast lesions to the clinician so that he/she can make an appropriate decision before surgery, and it also suggests the prognosis of the patient.

We found that although the mean MN score of AO-stained smears of IDC was higher than the mean MN score of Giemsa-stained smears, the difference between them was not statistically significant ($P = 0.17$) [Table 3]. All previous studies have tried to evaluate the effect of staining on the MN score in exfoliated buccal smears, and the interpretations have been varied. Nersesyan *et al*.[24] reported higher MN scores in Giemsa stain compared to AO stain. However, the sample size in their study was very small (20 smokers and 10 nonsmokers as controls).

Liu *et al*.[25] reported that AO stain can be used to identify circulating tumour cells in renal cell carcinoma, and also for screening high-risk patients to assist clinical treatment and diagnosis. We could not find any study which tried to explore the use of AO stain in breast cancer patients, and to the best of our knowledge, this is the first report to date in the literature. As data regarding the treatment plan and prognosis according to MN score are lacking, a cut-off point to differentiate between benign and malignant cases based on MN score and correlations between MN score and prognosis as well as its efficacy for several precision medicine strategies, including patient and risk stratification are unclear. Therefore, further studies with a larger sample size or meta-analysis of such studies where sensitivity and specificity are calculated to provide these data are warranted. This will also indicate the clinical applicability of the MN score.

Conclusion

Mean MN score was significantly increased in patients with breast carcinoma compared to those with FA in FNA smears, indicating that there is increased chromosomal damage in breast carcinoma and that this damage is proportional to the increasing grade of breast carcinoma. MN assays should be used as a potential additional surrogate marker for diagnosing and grading breast carcinoma. Both AO and Giemsa stains are equally good for MN scoring in FNA smears.

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Conflicts of interest

There are no conflicts of interest.

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