

Micronuclei and Ag NOR as Biomarker in Fine Needle Aspiration Cytology of Breast Tumor

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ABSTRACT

Background: Micronuclei and Ag NOR scoring can be used as a bio- marker of genotoxic and chromosomal damage.

Aim: The aim of this study is to assess the difference in the silver-stained nuclear organizer regions (Ag NOR) and Micronuclei (MN) scores on aspiration smears from benign and malignant breast tumors, to determine the feasibility of Ag NOR staining as part of the cytological investigation of breast tumors. To evaluate the role of MN and Ag NOR as biomarker in breast tumor screening.

Materials and Methods: Smears from 100 breast tumor cases (each case two smears) were examined. Counting was performed on a small number of tumor cells (50 cells) in Ag NOR smear, and 1000 tumor cells examined for MN, the score is assessed for each specimen.

Statistical Analysis: Statistical evaluation was carried out using the Student's'-test. The result was considered significant when $P < 0.000$.

Results: The Ag NOR score and MN score were significantly higher in smears from malignant tumors than in those from benign tumors the mean score of Ag NOR(2.74-8.62)($P=0.000$.) and the mean score of MN (0.75,3.70) for benign and malignant respectively.

Conclusion: An increase in micronuclei and Ag NOR values was seen in benign tumors and even infiltrating ductal carcinoma. Micronuclei and NOR scoring can be used as a biomarker on fine needle aspiration cytology smears of breast carcinoma.

Key words: micronuclei-Ag NOR- Biomarker - Cytology - Breast Tumor

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INTRODUCTION

Cancer is a genomic disease associated with genetic damage accumulation. Majority of solid tumors show large number of chromosomal aberrations.[1]

These aberrations are not always shared by cells of the same tumor and may not necessarily linked to a particular tumor type.[2] It was Theodor Boveri who first observed that cells with supernumerary centromeres missegregated their chromosomes through the assembly of multipolar spindles and hypothesized that those abnormal chromosomes might contribute towards carcinogenesis[.3]

Breast carcinoma is the most common malignancy in the female population, worldwide and among Sudanese women. Incidence of breast carcinoma in Sudan reported is approximately 34% among total malignancies seen in females.[4] Informational access to epidemiology, etiology, risk factors and treatment of breast cancer can assist in diagnosis and management.

Fine needle aspiration cytology (FNAC) is applied as the primary tool for diagnosis in breast masses because of its ease and rapidity.

Application of ancillary techniques like Ag NOR stain and MN are useful in providing an objective and reproducible diagnosis, especially in borderline and malignant lesions.

Micronuclei are fragments of a chromosomes or whole chromosomes that are left out of daughter nuclei during division.[5] These micronuclei are round to oval in shape with a diameter range from 1/3 to 1/16th of main nucleus. Their intensity and texture is similar to that of the main nucleus. Further these micronuclei must be located within the cytoplasm of the cell.[1] Micronuclei scoring in routinely stained smears has been applied to study the different epithelial pre-neoplastic and neoplastic conditions of the head and neck, cervical intraepithelial lesions, cervical carcinomas and also in liver carcinogenesis[6,7,8,9].

(Ag NOR) Nucleolar Organizer Regions

Nucleolar Organizer Regions (NORs) -segments of DNA, closely associated with nucleoli containing coding gene for Ribosomal RNA and contribute to the regulation of the cellular synthesis.

Recent modification of a silver staining technique allows NORs to be visualized in conventional histopathological sections where they are called as “ArgyrophilicNucleolar Organizer Regions (Ag NORs)”.

In keeping with the current interest in cell proliferation markers, Silver-stained nucleolar organizer regions (AgNOR) scores have been evaluated in a variety of lesions. Studies have shown a significant difference in the Ag NOR scores for benign and malignant breast tumors.^[10-13] The utility of Ag NOR scores lies in their assistance in the assessment of tumor proliferation rates and in understanding cell and tumor kinetics. In recent times, Ag NOR analysis is carried out through standardized morphometry. However, such techniques are not widely available, especially in developing countries.

The manual evaluation of AgNOR scores is a cost effective alternative to automated methods of evaluation. The staining technique^[14] is a relatively simple and rapid one and can be applied to both aspiration smears and tissue sections. On the other hand, it is beset with certain difficulties which preclude its use in routine diagnostic work.^[15,16] Over/under

staining of sections and variability in section thickness often lead to difficulties in counting the dots.^[15,16] Another significant hurdle is the tedious process of counting a large number of cells.

Cytology offers a medium for easy application of AgNOR scoring with a reduction in some of these difficulties. Therefore, in this study, we have tried to simplify the problems in counting a large number of tumor cells in tissue sections by applying the process to aspiration smears and by determining AgNOR values in a limited number of cells.

MATERIALS AND METHODS

The study was conducted on 100 patients who presented in the Surgical Outpatient Department with breast lumps or associated complaints. All cases were subjected to fine needle aspiration (FNA).

The selection of the patient was on the basis of admission in Ribat Teaching Hospital, Khartoum Teaching Hospital, and Total Lab Care (TLC) in Sudan during the period from 1st January 2015 to 30 September 2015.

AgNOR Method

Principal: Acidic proteins are present on the active ribosomal gene of interphase nucleoli as well as on the NORs of metaphase chromosomes. These proteins are selectively stained with silver.

Technical Method

AgNOR staining solution:

Solution A: 50 % Aqueous Silver nitrate solution was prepared by dissolving 5 gms of aqueous silver nitrate in 10 cc of double glass distilled water. It was filtered through 0.22 micron Millipore Filter and placed in dark before it was used to prepare working solution.

Solution B: Gelatin 2 gm% w/v was prepared by dissolving 2 gms gelatin in 99 ml of double glass distilled water in a water bath at 60 to 70°C. One ml pure formic acid was added to above prepared gelatin solution.

Working Solution: Finally the working solution was prepared by mixing both the solution in Coplin Jar in the following proportion Solution A: 2 parts and Solution B: 1 part. This mixture was kept in dark till light brown colour developed.

The smear were then incubated in working solution for 30 to 35 minutes in dark. Then smears were washed with running double glass distilled water for 10 to 15 minutes, dehydrated and mounted with DPX.

Finally the AgNOR count was done counting nuclei of 100 cells under 100x lens. In this clusters of black dots within nucleoli were counted as one AgNOR and dispersed dots throughout the nucleus were counted as discrete AgNORs.

In each case, the mean number of total AgNOR dots per case was calculated. The results were assessed by analysis^[17, 18] to decide the significance

MN method

Inclusion and exclusion criteria

Clumps of cells with obscured nuclear or cytoplasmic boundaries and overlapping of cells were avoided and separated or cells lying singly were preferred for counting of MNC. Degenerated cells, apoptotic cell and cytoplasmic fragments were exempted from counting and scoring. The zigzag method was followed for screening of slides.

Criteria for MN

Diameter of MN was variable from 1/16 to 1/3 the diameter of the main nucleus. The shape, colour and texture of MN were similar to those of nucleus. Staining intensity was similar to, or slightly weaker than that of the nucleus. MN were round to oval in shape having close proximity but no actual contact with the nucleus. Plane of focus was same as that of the main nucleus.[19]

Micronuclei scoring were done on 1,000 cells on PAP stained smear under oil immersion ($\times 1,000$). Two observers independently scored each slide and a mean of value was taken. Scoring was done according to Fenechet *al.*[20] Care was taken to differentiate micronuclei from condensed chromatic pyknotic cells, stain deposits, apoptotic bodies, nuclear debris and bacterial colonies. Degenerated cells with unclear nuclear morphology, cells that had indistinct nuclear morphology were not considered for micronuclei scoring

RESULTS

This prospective study assessed breast tumor by tow cytological methods(AgNOR and MN) in 100 study subjects.

Independent Samples Test (Student *t* test) showed significant difference between benign and malignant groups ($P < 0.000$)

The study examined (74) benign breast tumors and (26) malignant tumors (Infiltrating ductal carcinoma, no otherwise specified). All patients were female. The peak incidence of benign tumors was in the third decade of life while malignancy was more frequent in the fifth decade.

Ag NORs were visible as dark brown or black dots in the nucleus, either singly or in clusters. The dots were fine, round, discrete, and singly dispersed in benign tumors [Figure 1a], whereas the dots were larger, coarse, irregularly distributed, and tended to form clusters in malignancy [Figure 1b].

Benign lesions yielded a significantly lower AgNOR mean score of 2.74 as compared to a value of 8.62 in malignant[table1] tumors ($P = 0.000$). There was also a marked difference in MN[Figuer2] score between the benign The mean score (0.75) and malignant the mean score(3.70) [table1].

An analysis of variance (anova) test showed a significant difference in MN and Ag NOR scores between benign and malignant tumor ($P < 0.000$). The Pearson's correlation coefficient showed positive correlations between MN and Ag NOR scoring in the benign and malignant.

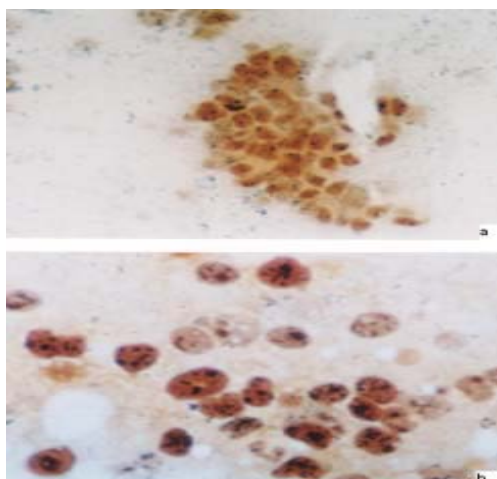


Figure 1: (a) Fine, discrete, and regular AgNOR dots in benign ductal epithelial cells (Silver stain, x100). (b) Coarse, dark, irregularly distributed AgNOR dots in malignant cells (Silver stain, x100).

Table(1): The mean score of AgNOR and MN

Type of tumor	Number of case	Mean age	Mean micro nuclei score	Mean AgNOR score
Benign	74	30,8	0,75	2,74
Malignant	26	58,4	3,70	8,62



Figure (2): Breast smear shows micronuclei. Pap stained. 100X.

DISCUSSION

Carcinoma of the breast is on a rise in Sudan. Like other cancers even breast carcinomas are known to have chromosomal instabilities that play an important role in cancer development and progression. These instabilities can involve various oncogenes and tumor suppressor genes. Chromosomal instabilities in breast cancer occur in the form of P53 mutations, BRCA1, BRCA2 mutations and CHEK 2 mutations. These occur both in familial and sporadic carcinomas. Thus screening for chromosomal instabilities is very important. This can be done by using micro nuclei scoring, and by looking into the aneuploidy status as they are very sensitive indicators of chromosomal instabilities.[21,22]

In breast carcinoma patient's occurrence of micro nuclei has been investigated in lymphocytes which is procured from peripheral blood.[25] Pranab Dey *et al.*[26] have reported significant increase of micronuclei in buccal smears of patients with breast carcinoma raising the possibility that genetic damage in these patients is generalized and micronuclei can act as bio monitoring of DNA detection in these cases. Only few studies have described the occurrence of spontaneous micronuclei in breast carcinoma on fine needle aspirates.[21,22,27].

We have applied also the AgNOR staining, to study the scores in breast tumors.

This consists of small discrete intra nuclear dots and large structures resembling nucleoli which may stain uniformly or be seen as aggregates of dots within them.

Smith and Crocker[28] in 1988 tried to enumerate the number of AgNOR clusters per nucleus, the mean number of AgNORs per clusters and the number of satellite AgNORs in breast lesions and derived total-AgNOR count.

There were Several older studies assessed mean Ag NOR counts in breast cytology material [10-13].

Ag NOR count in Malignant tumors were average (8.62) and p value is significant(<0.000).

CONCLUSIONS

MN and AgNOR scoring on routinely stained smears of Malignant was significantly higher than in Benign and was relatively easy, reliable and reproducible.

Micronuclei and AgNOR scoring can be used as biomarkers on fine needle aspiration cytology smears of breast carcinoma.

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