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Use of Silver Stained Nucleolar Organizer Regions (AgNORs) as a Diagnostic Tool in Fine Needle Aspiration Cytology Smears of Palpable Breast Lumps

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ABSTRACT

Different modalities are used for the diagnosis of breast lesions. Fine needle aspiration cytology (FNAC) is now the most popular, single modality for diagnosing different breast diseases. One hundred patients with breast lesions underwent fine needle aspiration. In this study the FNAC smears were stained with routine stains and colloidal silver method for "AgNORs" staining. The "AgNOR" dots were counted in each case and analyzed statistically. The difference of the mean "AgNOR" numbers per cell noted on FNAC smears between benign neoplastic diseases of breast (range 5.45 to 8.44; mean 6.87; SD 0.92) and malignant neoplastic diseases of breast (range 12.94 to 25.38; mean 18.51; SD 2.90) was very highly significant with no overlap between the ranges of mean "AgNOR" counts in these groups.

It is concluded from this study that colloidal silver staining technique for "AgNORs" has a valuable cytological diagnostic significance in breast tumours. It is suggested that the technique should be employed as a special stain on FNAC smears of breast lesions.

INTRODUCTION

The main task of surgical pathology is an accurate microscopic diagnosis of the large majority of specimens sent to the laboratory. However, it can not answer all the questions and is far from ideal. Consequently, the pathologists have always searched for additional techniques to overcome these diagnostic problems[1].

The traditional methods of prognostication in histopathology have included histological typing, grading of tumours and an assessment of their proliferative potential. The nucleolar organizer regions (NORs) are loops of DNA present in the nucleoli of cells and are involved in protein synthesis[2]. These regions can be identified by light microscopy as discrete black dots, known as "AgNORs"[3]. The application of silver stain for nucleolar organizer regions has provided an interesting and promising investigation for diagnostic as well as prognostic purpose because, it is related to the proliferative potential of different benign and malignant lesions[4].

Nucleolar organizer regions (NORs) are loops of DNA (γ-DNA) which occur in nucleoli and ultimately direct ribosome and protein formation. The NOR-DNA possesses ribosomal RNA (γ-RNA) genes which are transcribed by RNA polymerase - 1 for the production of 18 S and 28 S ribosomal RNA[2,5-7]. In human and chimpanzee NORs are situated on the short arm of five acrocentric chromosomes *i.e.*, 13, 14, 15, 21 and 22[5,8-10]. These NORs bearing regions appear as achromatic gaps on short arms when metaphase preparations are examined with banding technique[8]. Although silver binding to nucleolar organizer region is attributable to proteins associated with these sites and have been characterized as B 23, C 23 and subunits of RNA polymerase – 1[8,9] rather than NORs themselves and it is highly specific[8].

Nucleolar organizer regions have associated histone and nonhistone proteins. The AgNORs reaction specifically involves the non - histone associated protein moiety. Thus it has been shown that the AgNORs reaction will still occur after the histone extraction. The carboxyl groups on the non -

histone proteins are thought to reduce the silver solution, forming micronuclei of silver. They are then developed in to larger aggregates of silver which are deposited at disulphide and sulphydryl group of (cystein and cystine) sites. The larger deposits are then quite clearly visible by light microscopy[11].

The "AgNORs" have been observed to be significantly increased in malignant cells[12]. This observation attracted much attention among the pathologists for the reason that the technique might help to differentiate malignant from a benign lesion in difficult situation and in small biopsy specimens[13]. Subsequently in a large series, "AgNORs" technique was applied to a variety of benign, dysplastic and malignant lesions and was found successful in differentiating these problematic cases[14].

Silver stained nucleolar organizer regions counts are high in whole cells than in cell sections. Since individual (cell) nucleus has a diameter larger than 3 micrometer, the usual thickness at which sections are cut. At this thickness the nuclei will not display absolute number of AgNORs, particularly in malignant cells with large nuclei[15,16]. Silver stained nucleolar organizer regions counts are high in cells in cytological smears than in cells in the histological sections, since individual (cell) nucleus has a diameter larger than 3 micrometer (the usual thickness at which sections are cut). At this thickness the nuclei will not display all the "AgNORs" especially if the cell is malignant with large nucleus[15,16].

Limited number of workers applied the "AgNORs" technique on FNAC smears of breast lesions[17-19]. Further studies are required to check the significance or otherwise of this technique on FNAC smears and on paraffin sections of the breast lesions.

AIMS AND OBJECTIVES

To compare silver stained nucleolar organizing regions (AgNORs) on fine needle aspiration cytological smears sections of benign and malignant breast diseases.

To assess the usefulness of AgNORs in the routine work of laboratory.

PATIENTS AND METHODS

A total number of 100 patients with palpable nodules in the breast, admitted in different wards or those referred to the outpatient aspiration cytology clinic of Histopathology Department of Shaikh Zayed Hospital were chosen for this study.

The smears were air dried for Giemsa staining[20]. Those for Papanicolaou stain were placed in alcohol[21], while others for colloidal silver staining were placed in a mixture of glacial acetic acid and alcohol (already prepared in a ratio of 1:3)[22]. The FNAC smears were stained by the Papanicolaou, Giemsa and colloidal silver stain (for AgNORs).

Colloidal Silver Stain For AgNORs

Principle

Carboxyl groups of intranucleolar non-histone proteins are involved in the "AgNORs" reactions. These groups initially bind to silver ions, causing the reduction of the ion to the metal. Submicroscopic nuclei of metallic silver form which then act as foci for the further deposition of silver drawn from the less electronegative sulphydryl groups of these non-histone proteins. The sulphydryl groups are causing development of the micro deposits of silver into the characteristic black dots visible at low microscopic magnification.

Solutions

Silver stained nucleolar organizer regions staining solution was made by combining solution A and solution B.

Solution A

This was prepared by dissolving white gelatin powder at a concentration of two percent W/V in distilled deionized water to which pure formic acid was added to a final concentration of one percent. The mixture was then gently shaken during slight heating till the gelatin was dissolved and a clear solution was obtained. The pH was adjusted to 3.0.

Solution B

This was prepared by dissolving silver nitrate crystals at a concentration of 50 percent W/V in distilled deionized water. The silver nitrate solution was kept under safe light conditions before use and

always prepared freshly. Solutions A and B were then mixed in a ratio of 1:2 volumes in order to obtain final working solution[5,22].

Method

These slides were kept in a large plastic tray containing wax with depressions for the glass slides under moist condition and a freshly prepared colloidal silver solution (for AgNORs) was dropped on to the slides with the aid of a dropping bottle. The petridish was kept in the dark for 30 minutes (for the smears) and 36 minutes (for the tissue sections, which were stained in a Coplin jar). Next the slides were washed with distilled, deionized water (three changes). Untreated silver was removed with 5.0% sodium thiosulphate solution for 3 minutes Washed in distilled deionized water. The smears were dried on a hot plate[5,22].

RESULT

The AgNORs appeared as discrete dark brown to black dots within nuclei which stained pale yellow[23].

Malignant neoplastic diseases of breast

Total 63 patients were included in this group. The range of mean "AgNORs" counted in malignant cells of FNAC smears was from 12.94 to 25.38 dots per nuclei, and overall mean of 18.51 dots/nuclei (SD=2.90). The counted number of "AgNORs" ranged from 9 (minimum) to 49 (maximum) dots/nuclei (Figs. 1, 2).

Benign neoplastic disease of breast

A total of 16 female patients were included in this group. The range of mean "AgNORs" dots was from 5.45 to 8.44 per ductal epithelial cell in FNAC smears with overall mean of 6.87 per ductal epithelial cell (SD=0.92). The counted number of "AgNORs" per ductal epithelial cell ranged from 2 (minimum) to 15 (maximum) dot /cell (Fig. 3).

Non-inflammatory, non-neoplastic diseases of breast

Total 14 patients were included in this group. The range of mean "AgNORs" counts in FNAC smears was from 4.46 to 8.80 dots per ductal epithelial cells with an overall mean of 7.48 per cell (SD=1.32). The counted number of "AgNORs" per ductal epithelial cell in these cases ranged from 2 (minimum) to 15 (maximum) dots/cell.

Inflammatory diseases of breast

Six female patients were included in this group. The range of mean "AgNORs" counted in FNAC cases of inflammatory breast diseases was from 4.71 to 8.78 per ductal epithelial cell with an over all mean of 6.18 dots/cell (SD=1.41). The counted number of "AgNORs" per ductal epithelial cell in these cases ranged from 1 (minimum) to 12 (maximum) dots/cell (Fig. 4).

Normal breast tissue

Only one 30 years old female patient was included in this group. The mean "AgNORs" counted in the FNAC smears was 5.66 (SD=2.26) and the counted number of "AgNORs" per ductal epithelial cell in this case, ranged from 1 (minimum) to 12 (maximum) dots/cell.

Comparative analysis of various groups

The findings in different groups were analyzed and compared with each other to assess the significance or otherwise of these findings.

Benign neoplastic diseases of breast versus malignant neoplastic diseases of breast

The difference of "AgNORs" counts between benign neoplastic diseases of breast (mean 6.87, median 6.88, SD 0.92, SEM 0.087) and the malignant neoplastic diseases of breast (mean 18.51, median 18.61, SD 2.90, SEM 0.066) was also very highly significant with a 'P' value <0.0001.

There was no overlap between the ranges of mean number of "AgNORs" in these two groups (benign neoplastic diseases 5.45 to 8.44, malignant neoplastic diseases 13.08 to 25.38) (Table 1).

Non-inflammatory non-neoplastic diseases of breast versus benign neoplastic diseases of breast

The "AgNORs" counts in non-inflammatory non-neoplastic disease of breast (mean 7.48, median 8.11, SD 1.32, SEM 0.052) found insignificantly different from "AgNORs" counts of benign neoplastic diseases of breast (mean 6.87, median 6.88, SD 0.92, SEM 0.087) with a 'P' value >0.05.

Table 1: **Overall evaluation of silver stained nucleolar organizer region (AgNOR) counts in different breast diseases in this study – Histological comparison.**

Group	Diagnosis	Mean No. of AgNORs per cell	't' value	'P' value
2A	Benign neoplastic diseases of breast	6.41		
1A	Malignant neoplastic diseases of breast	17.68	4.930	>0.0001
3A	Non-inflammatory non-neoplastic diseases of breast	7.04		
2A	Benign neoplastic diseases of breast	6.41	1.507	<0.005
4A	Inflammatory breast diseases	5.56		
3A	Non-inflammatory non-neoplastic diseases of breast	7.04	2.711	>0.005
5A	Normal breast tissue	5.47		
4A	Inflammatory breast diseases	5.56	0.276	>0.025

There was an overlap of range of mean "AgNORs" between non-inflammatory non-neoplastic diseases and the range of mean "AgNORs" in benign neoplastic diseases of breast (non-neoplastic non-inflammatory disease of breast 4.46 to 8.80, benign neoplastic diseases of breast 5.45 to 8.44).

Inflammatory diseases of breast versus non-inflammatory non-neoplastic diseases of breast

The "AgNORs" counts in inflammatory disease of breast (mean 6.18, median 5.70, SD 1.41, SEM 0.151) were significantly different from the counts in non-inflammatory non-neoplastic diseases of breast (mean 7.48, median 8.11, SD 1.32, SEM 0.052) with a 'P' value >0.025.

There was an overlap of range of mean "AgNORs" of inflammatory breast diseases and range of mean "AgNORs" of non-inflammatory non-neoplastic diseases of breast (inflammatory diseases of breast 4.71 to 8.78, non-inflammatory non-neoplastic diseases of breast 4.46 to 8.80).

Normal breast tissue versus inflammatory diseases of breast

Silver stained nucleolar organizer regions count in normal breast tissue (mean 5.66, median 6.00, SD=2.26, SEM=0.085) were found insignificantly different from the counts in inflammatory diseases of breast (mean 6.18, median 5.70, SD 1.41, SEM 0.151) with the P value >0.1.There was an overlap of mean "AgNORs" in normal breast tissue and the range of mean "AgNORs" in inflammatory disease of breast (normal breast tissue 5.66, inflammatory disease of breast 4.71 to 8.78).

Qualitative analysis and intranuclear distribution of "AgNORs"

In addition to counting "AgNORs" dots in different groups of breast diseases, the size, variation in contour and dispersion of these dots was also evaluated. The following observations were made:

Malignant neoplastic diseases of breast

The "AgNORs" dots observed in the malignant cells of this group, were of variable sizes ranging from coarse to fine dots. The staining character was much more variable and irregularity in the contour of some of these dots was evident.

Non-inflammatory non-neoplastic diseases of breast and benign neoplastic diseases of breast

The "AgNORs" observed in ductal epithelial cells revealed mild variation in their size and contour and the dispersion of these dots was slightly more as compared to the "AgNORs" mentioned in following groups.

Fig. 1: **Photomicrograph of a fine needle aspirate of a carcinoma (ductal in type) showing numerous dispersed "AgNORs" in the nuclei of these malignant cells ("AgNORs" stain x700)**

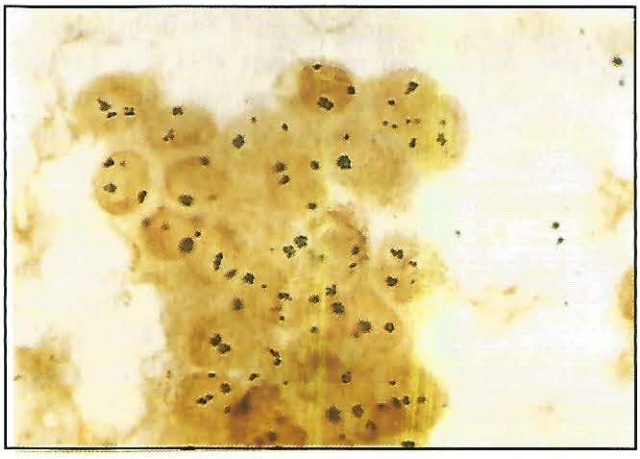

Fig. 2: **Photomicrograph of fine needle aspirate of an infiltrating lobular carcinoma. The nuclei of these malignant cells show AgNus and many dispersed "AgNORs" dots of irregular size ("AgNORs" stain x700)**

Inflammatory diseases of breast

The "AgNORs" noted in ductal epithelial cells of these groups appeared similar to those in normal breast tissue.

Normal breast tissue

The "AgNORs" appeared coarse and of same size and contour, the staining character remained uniform. The dispersion was not marked.

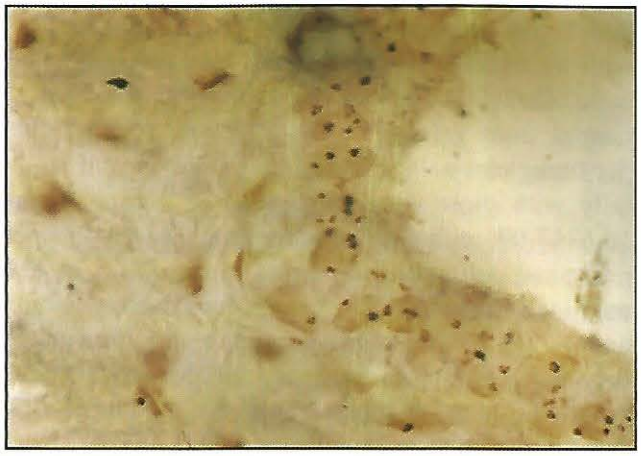

Fig. 3: **Photomicrograph of a section of fibroadenoma showing 3 to 6 "AgNORs" per epithelial cells ("AgNORs" stain x700)**

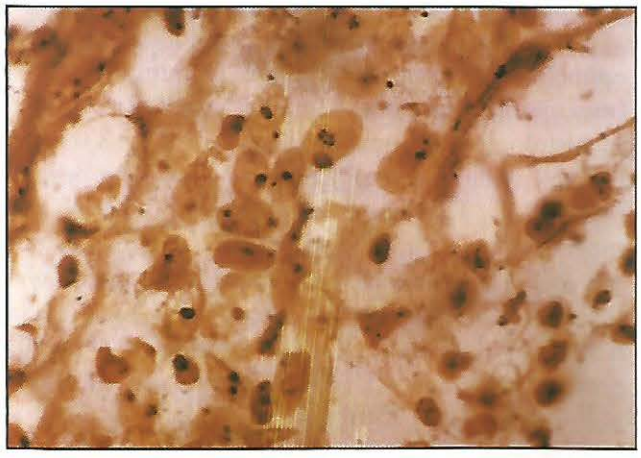

Fig. 4: **Photomicrograph of a section of chronic mastitis showing 3 to 8 "AgNORs" per epithelial cells ("AgNORs" stain x700)**

DISCUSSION

Malignant neoplastic diseases of breast

Total 63 patients were included in this group. The range of mean "AgNORs" counted in malignant epithelial cells of FNAC smears was from 12.94 to 25.38 with an overall mean of 18.51 per cell (SD=2.90). The counted number of "AgNORs" ranged from 9 (minimum) to 49 (maximum) dots per malignant cell. The range of mean "AgNORs" counted in the malignant epithelial cells in

histological sections was from 11.82 to 24.61 dots with an overall mean of 17.68 dots per malignant cell (SD=2.44). The counted number of "AgNORs" ranged from 9 (minimum) to 42 (maximum) dots per malignant cell. The results of the current study tally with those of Karmakar et al.[19] reported as 16.63±7.09, with a range between 6.9 to 40 dots per malignant cell and differ from those of Giri et al.[17] mentioned as 3.75±1.33 for non-invasive carcinoma and 4.22±1.18 for invasive carcinoma with a range between 1.5 to 6.0 dots per cell. Mourad et al.[18] reported a range of mean "AgNORs" counts as 1.96 to 3.4 in infiltrating ductal carcinomas.

Benign neoplastic diseases of breast

Total 16 patients were included in this group. The range of mean "AgNORs" dots was from 5.45 to 8.44 per ductal epithelial cells in FNAC smears with overall mean of 6.87 dots per cell (SD=0.92).

The counted number of "AgNORs" per ductal epithelial cell ranged from 2 (minimum) to 15 (maximum). The range of mean "AgNORs" counted in histological sections was from 5.0 to 8.05 dots per cell with an overall mean of 6.41 dots per cell (SD=1.04). The counted number of "AgNORs" ranged from 2 (minimum) to 12 (maximum) dots per cell. The result of the current study match with those of Karmakar et al.[19] accounted by them as 6.50±1.89 dots per cell with a range of 4 to 10.3 dots per cell, and differ from the results of Giri et al.[17], reported as 1.87±0.20.

Non-inflammatory non-neoplastic diseases of breast

Total 14 patients were included, out of which 12 female patients with diagnosis of fibrocystic disease and 2 male patients with diagnosis of gynaecomastia were reported.

The range of mean "AgNORs" counts in FNAC of this group was from 4.46 to 8.80 dots per ductal epithelial cell with an overall mean of 7.48 dots per cell (SD=1.32). The range of "AgNORs" count was from 2 (minimum) to 15 (maximum) dots per cell, which was similar to those in histological sections. The range of mean "AgNORs" counted in histological sections was from 4.28 to 8.23 dots per cell, with overall mean of 7.04 dots per cell (SD=1.29).

The overall mean "AgNORs" counted in cases of fibrocystic disease was 7.5 (SD=2.76). The result of the current study had matched with those of Karmakar et al.[19] reported as 6.35±1.99 ranging from 2.8 to 10.2 dots per cell and were approaching near to those mentioned by Dervan et al.[24] as 4.42±1.3, ranging from 1.48 to 6.69 dots per cell; but differ from other workers, Giri et al.[26] shown as 1.96±0.21; Giri et al.[17] as 1.96±0.24; Raymond et al.[25] as 1.7. All workers had noticed higher "AgNORs" counts in fibrocystic disease with epitheliosis than in fibrocystic disease without epitheliosis. Giri et al.[26] described "AgNORs" counts as 2.21±0.31; Giri et al[17] as 2.2±0.3 with a range of 1.56 to 2.5 dots per cell; Karmakar et al.[19] as 7.13±5.13 and ranged from 3.5 to 10.76 dots per cell in fibrocystic disease with epitheliosis.

The mean "AgNORs" counted in cases of gynaecomastia in the current study was 5.12 dots per cell in FNAC smears and 4.84 in histological sections and overall mean was 4.98 dots per cell (SD=0.214) these results agree with the results of Karmakar et al.[19] who reported as 5.43±1.87 with a range between 4.1 to 6.75 dots per cell.

Inflammatory diseases of breast

Six female patients were included in this group. The range of mean "AgNORs" counted in FNAC cases of this group was from 4.71 to 8.78 dots per ductal epithelial cell with an overall mean of 6.18 per cell (SD=1.41).

The range of mean "AgNORs" counted in histological sections of these 6 FNAC cases was from 4.56 to 7.12 dots per ductal epithelial cell with an overall mean of 5.56 per cell (SD=0.86).

The counted number of "AgNORs" in these cases ranged from 1 (minimum) to 12 (maximum) dots per ductal epithelial cell in both FNAC smears as well as in tissue sections. No worker had mentioned about the "AgNORs" counts in the inflammatory diseases of breast.

Normal breast tissue

The mean "AgNORs" counted in FNAC smears was 5.66 (SD=2.26) and the counted number of "AgNORs" per ductal epithelial cell in this case ranged from 1 (minimum) to 12 (maximum) dots.

The mean "AgNORs" studied in tissue

section of this case was 5.47 (SD=1.93) and the counted number of "AgNORs" per ductal epithelial cell ranged from 2 (minimum) to 10 (maximum) dots. This case was included as a normal control for "AgNORs" counts. The result of this case was higher than those reported by Raymond et al.[25] as 1.8 dots per cell in histological sections. This low "AgNORs" count by Raymond et al.[25] was probably because of not counting the dots in cluster which were counted separately in the current study.

CONCLUSION

1. This was achieved up to the very highly significant level between benign and malignant neoplastic diseases of breast (P value <0.0001) as compared to the correlation amongst various groups of benign breast diseases (P value vary from >0.25 to P>0.05) which were variables ranging from insignificant to significant levels.
2. The ranges and pooled means of "AgNORs" counts for ductal epithelial cells within benign and malignant neoplastic lesions were widely separated thus enabling the distinction to be readily made in FNAC.
3. By employing proper "AgNORs" staining method with appropriate timing and temperature, this technique can be utilized on small biopsies, FNAC smears, in helping to detect malignant breast disease. So that these patients could be cared off accordingly. Cases with higher "AgNORs" counts than control confirmed to be benign on biopsy must be followed up for malignancy in the future, bearing the prognostic value of "AgNORs" in mind.

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