Comparative Study of Silver Stained Nucleolar Organizer Regions (Agnors) between Fine Needle Aspiration Cytology (FNAC) and Histology 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 and later surgery and divided into 05 groups. The results were correlated. In this study silver stained nucleolar organizer regions (AgNORs) enumeration technique was applied to FNAC smears and paraffin sections of biopsies or mastectomy specimens of the same patient to judge usefulness in breast diseases. The "AgNOR" dots were counted in each case and analyzed statistically. The difference of the mean "AgNOR" numbers per cell noted on FNAC smears and histological sections between benign neoplastic diseases of breast and malignant neoplastic diseases of breast was very highly significant (`P' value <0.0001) with no overlap between the ranges of mean "AgNOR" counts in these groups. The technique was not fully able to differentiate between different benign diseases of breast on FNAC and histological sections with overlap between the mean "AgNOR" counts in these groups. It is concluded from this study that colloidal silver staining technique for "AgNORs" has a valuable histological and cytological diagnostic significance in breast tumours. It is suggested that the technique should be employed as a special stain on FNAC smears and paraffin sections of breast lesions.

Key words: Osteogenic sarcoma, osteosarcoma, high grade, intramedullary, magnetic resonance imaging.

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].

The "AgNORs" have been observed to be significantly increased in malignant cells[5] as compared to benign lesions in difficult situation and in small biopsy specimens[6]."AgNORs" technique was applied to a variety of benign, dysplastic and malignant lesions and was found successful in differentiating these problematic cases[7].

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) [8, 9].

Limited number of workers had applied the "AgNORs" technique to FNAC smears of breast lesions [12, 13, 14]. 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

1. To compare silver stained nucleolar organizing regions (AgNORs) on fine needle aspiration cytological smears and histological sections of benign and malignant breast diseases.
2. To assess the usefulness of AgNORs in routine laboratory work.

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

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were chosen for this study.

The smears were air dried for Giemsa staining [15]. Those for Papanicolaou stain were placed in alcohol[16], while others for colloidal silver staining were placed in a mixture of extra pure glacial acetic acid and absolute alcohol (already prepared in a ratio of 1:3)[17]. When blood/blood stained material was aspirated in larger quantity, it was processed as a clot in paraffin block, and the section was subsequently stained with heamatoxylin and eosin stain[16]. The FNAC smears were stained by the Papanicolaou[16], Giemsa[15] and colloidal silver stain (for AgNORs)[18].

Patients with breast nodules selected for fine needle aspiration cytology, when underwent surgery, and their lumpectomy/mastectomy or needle biopsy specimens were received by the Histopathology department, tissue blocks made from these specimens were processed routinely using automated tissue processor and sections made from the paraffin blocks were then subsequently stained with haematoxylin and eosin and with colloidal silver technique

COLLOIDAL SILVER STAIN FOR AgNORs

Principle: Carboxyl groups of intranucleolar non-histone proteins are involved in the "AgNOR" 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 convert the micro-deposits of silver into the characteristic black dots visible at low microscopic magnification[22.]

Method: Silver stained nucleolar organiser 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 (Crocker and Nar, 1987; McLemore and Lord, 1991).

.The FNAC slides were kept in a large plastic tray containing wax with depressions for the glass slides under moist conditions in dark with colloidal silver solution (for AgNORs) for 30 minutes (for the smears) and 36 minutes (for the tissue sections, which were stained in a Coplin jar)[17,18].

RESULTS

The AgNORs appeared as discrete dark brown to black dots within nuclei which stained pale yellow 18. Silver stained nucleolar organizer regions stained sections/smears were examined under the light microscope. AgNORs were counted under oil immersion (x100).for each case in 100 epithelial cells and a mean count per cell was calculated[9].

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/cell	't' value	'p' value
2A	Benign neoplastic diseases of breast	6.41 vs 17.68	4.930	>0.0001
1A	Malignant neoplastic diseases of breast			
3A	Non-inflammatory non-neoplastic disease of breast	7.04 vs 6.41	1.507	<0.005
2A	Benign neoplastic diseases of breast			
4A	Inflammatory breast diseases	5.56 vs 7.04	2.711	>0.005
3A	Non-inflammatory non-neoplastic diseases of breast			
5A	Normal breast tissue	5.47 vs 5.56	0.276	>0.025
4A	Inflammatory breast diseases			

Table 2: Overall evaluation of silver stained nucleolar organiser region (AgNOR) counts in different breast diseases in this study - Fine Needle Aspiration Cytology Comparison

Group	Diagnosis	Mean No. of AgNORs per cell	't' value	'p' value
2A	Benign neoplastic diseases of breast	6.87 vs 18.51	4.350	>0.0001
1A	Malignant neoplastic diseases of breast			
3A	Non-inflammatory non-neoplastic disease of breast	7.48 vs6.87	1.464	<0.05
2A	Benign neoplastic diseases of breast			
4A	Inflammatory breast diseases	6.18 vs7.48	2.053	>0.025
3A	Non-inflammatory non-neoplastic breast disese			
5A	Normal breast tissue	5.66 vs 6.186	0.982	>0.1
4A	Inflammatory breast diseases			

Table 3: Silver stained nucleolar organiser regions (AgNORs) comparison between normal breast tissue (group 5a) and inflammatory diseases of breast (Group 4A) - On Fine Needle Aspiration Cytology

Group	Diagnosis	No. of cases	AgNOR dots per ductal cell				
			Range	Mean	Median	SD	SEM
5A vs 4A	Normal breast tissue	1	1-12	5.66	6.00	2.26	0.085
	Inflammatory breast disease	6	1-12	6.18	5.70	1.41	0.151
			P value <0.1				
On histological Section							
5A vs 4A	Normal breast tissue	1	2-10	5.47	6.0	1.93	0.118
	Inflammatory breast disease	6	1-12	5.56	5.4	0.86	0.409
			P value <0.1				

Table 4: Silver stained nucleolar organiser regions (AgNORs) comparison between inflammatory disease of breast (Group 4A) and non-inflammatory non-neoplastic diseases of breast (Group 3A) - On Fine Needle Aspiration Cytology

Group	Diagnosis	No. of cases	AgNOR dots per ductal cell				
			Range	Mean	Median	SD	SEM
4A vs 3A	Normal breast tissue	6	4.71-8.78	6.18	5.70	1.41	0.151
	Non inflammatory non neoplastic disease	14	4.46 – 8.80	7.48	8.11	1.32	0.052
			P value <0.025				
On histological Section							
4A vs 3A	Normal breast tissue	6	4.56 – 7.12	5.56	5.40	0.86	0.409
	Non inflammatory non neoplastic disease	14	4.28 – 8.23	7.04	7.80	1.29	0.054
			P value <0.005				

Table 5: Silver stained nucleolar organiser regions (AgNORs) comparison between non-inflammatory non-neoplasticdiseases of breast (Group 3A) and benign neoplastic diseases of breast (Group 2A) - On Fine Needle Aspiration Cytology

Group	Diagnosis	No. of cases	AgNOR dots per ductal cell				
			Range	Mean	Median	SD	SEM
3A vs 2A	Non inflammatory non neoplastic diseases of breast	14	4.46-8.80	7.48	8.11	1.32	0.052
	Benign neoplastic diseases of breast	16	5.45-8.44	6.87	6.88	0.92	0.087
			P value <0.05				
On histological Section							
3A vs 2A	Non inflammatory non neoplastic diseases of breast	14	4.28-8.23	7.04	7.80	1.29	0.054
	Benign neoplastic diseases of breast	16	5.0- 8.05	6.41	6.32	1.04	0.068
			P value <0.005				

Table 6: Silver stained nucleolar organiser regions (AgNORs) comparison between benign neoplastic diseases of breast (Group 2A) and malignant neoplastic diseases of breast (Group 1A) - On Fine Needle Aspiration Cytology

Group	Diagnosis	No. of cases	AgNOR dots per ductal cell				
			Range	Mean	Median	SD	SEM
2A vs 1A	Benign neoplastic diseases of breast	16	5.45-8.44	6.87	6.88	0.92	0.087
	Malignant neoplastic diseases of breast	63	13.08-25.38	18.51	18.61	2.9	0.066
			P value <0.0001				
On histological Section							
2A vs 1A	Benign neoplastic diseases of breast	16	5.0-8.05	6.41	6.32	1.04	0.068
	Malignant neoplastic diseases of breast	63	12.60-24.61	17.68	18.08	2.44	0.093
			P value <0.0001				

DISCUSSION

In this study, mostly distilled deionized water was used. Nitric acid treated and distilled deionized water washed glassware was used. The pH of the developer solution (2% gelatin in 1% formic acid) was adjusted to 3.0. The smeared slides of FNAC were stained in a large plastic tray containing wax with depression for the glass slides. The "AgNORs" stain was dropped onto these slides, while the paraffin section mounted slides were stained in a vertical staining jar. The staining time for smears of cytology was reduced to 30 minutes and that of paraffin section was also reduced to 36 minutes as longer period of 60 minutes [17] or 45 minutes [18] were found to result in overstaining. The temperature was maintained upto 20°C in a cooled incubator (Gallen Kamp) and the slides were stained in the dark.

By employing this combination of staining technique together with specific timings and temperature, the "AgNOR's" were discernible as clear dark brown to black dots within the pale yellow stained nuclei.

Group 1A (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 (1995) reported by them 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 (1989) 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, Raymond et al (1989) had shown 4.3 to 6.2 with a mean "AgNORs" of 5.46±2.27 dots per malignant cell, Sivridis and Sims (1990) counted mean "AgNORs" of 2.81±0.61 in carcinomas with negative lymph nodes or less than 4 positive lymph nodes and a mean "AgNORs" of 8.57±2.63 in carcinomas with 4 or more positive lymph nodes. Mourad et al (1994) reported a range of mean "AgNOR" counts as 1.96 to 3.4 in infiltrating ductal carcinomas.

Group 2A (Benign neoplastic diseases of breast): Total 16 patients were included in this group. The range of mean "AgNOR" 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 (1995) 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 (1989), reported as 1.87±0.20; Raymond et al (1989) as 2.30; and Trere et al (1991) reported as 1.5±0.62 μm^2 area of "AgNOR" protein instead of "AgNORs" dots per cell.

Group 3A (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 "AgNOR" 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 (1995) 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 (1989) as 4.42±1.3, ranging from 1.48 to 6.69 dots per cell; but differ from other workers, Giri et al (1988) shown as 1.96±0.21; Giri et al (1989) as 1.96±0.24; Raymond et al (1989) as 1.7. All workers had noticed higher "AgNOR" counts in fibrocystic disease with epitheliosis than in fibrocystic disease without epitheliosis. Giri et al (1988) described "AgNOR" counts as 2.21±0.31; Giri et al (1989) as 2.2±0.3 with a range of 1.56 to 2.5 dots per cell; Raymond et al (1989) as 7.1 and Karmakar et al (1995) 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 (1995) who reported as 5.43±1.87 with a range between 4.1 to 6.75 dots per cell.

Group 4A (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 "AgNOR" counts in the inflammatory diseases of breast.

Group 5A (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 "AgNOR" counts. The result of this case was higher than those reported by Raymond et al (1989) as 1.8 dots per cell in histological sections. This low "AgNORs" count by Raymond et al (1989) was probably because of not counting the dots in cluster which were counted separately in the current study.

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

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