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Original Article

Significance of Silver Stained Nucleolar Organiser Region (AgNOR) in Fine Needle Aspiration Smears of Breast Lesions

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ABSTRACT

Background: *Breast cancer is the leading cause of cancer - related deaths in Asia. The number of intra-nuclear silver stained structures, termed AgNORs, is significantly higher in malignant cells than in normal, reactive or benign cells. The purpose of this study was to evaluate the AgNOR scores in FNA smears of breast lesions and their correlation with histopathology.* ***Aims & Objective:*** *To establish AgNOR staining as a diagnostic and prognostic tool in management of various breast lesions.* ***Methods:*** *This was a prospective study conducted over a period of 18 months may 2015 to November 2016. A total of 100 cases were included in the study. AgNOR stain was done in both cytology and scoring was done and analysed.* ***Results:*** *In FNAC aspirates, Mean AgNOR count ranged from 5 to 9.9 per high power field with a mean value of 2.888±2.553. The AgNOR dots morphology was homogenous, symmetric with regular contours in FNAC slides of benign breast lesion. In malignant breast lesions, the dots were asymmetric with irregular contours and were aggregated, smaller and more scattered.* ***Conclusions:*** *The present study showed that fine needle aspiration cytology is a useful modality for diagnosis of breast lesions. It has a high concordance with the histopathology . AgNOR count assessment provides a useful objective measure for segregation of different grades of tumor with 100% accuracy for detection of higher grade of lesions, as observed in present study. For differentiation of benign from malignant lesions too it has a high sensitivity as well as specificity. The usefulness of FNAC to evaluate nodal involvement also showed a 90% sensitivity and 95.6% specificity. On the basis of present study, it could be concluded that AgNOR count estimation using fine needle aspiration is a useful method to differentiate and diagnose breast lesions.*

Key words: *Fine needle aspiration, Nucleolar organizer regions, Argyrophilic Nuclear Organizer Region, Malignancy*

INTRODUCTION

Breast cancer is the leading cause of cancer-related deaths in Asia and in recent years is emerging as most common female malignancy in the developing Asian countries, overtaking cancer of the uterine cervix.

Fine needle aspiration (FNA) cytology of the breast has become an accepted diagnostic procedure throughout the world during the past few decades.[1,2] The technique has emerged as a patient friendly and out-patient procedure because of high percentage of true positive(s), nearly no false positive(s) and virtually no complications, and no requirement of anaesthesia. Considering the relevance of FNAC in the diagnosis of breast cancer, attempts have been made to improve its accuracy by adding new features.

Nucleolar organizer regions (NORs) are segments of DNA, that contain nucleoli which inhibit coding genes for ribosomal RNA that regulate the synthesis of cellular protein.[3] These genes have been located on the short arm of chromosomes 13, 14, 15, 21 and 22.There are nucleolar organiser region-associated proteins (NORPAS) that are associated with these regions.

NORs can be rapidly identified in light microscopic sections by a simple, one-step, colloidal silver technique by staining their associated proteins with colloidal silver and these silver stained reaction products represent the AgNORs and appear as black dots within nucleus.[4] An increased number of AgNORs is associated with increased

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tumor aggressiveness as the mean number of AgNORs per nucleus is higher in malignant than in benign tissues, higher in high grade malignancies and in tumours with a poor prognosis compared to those with good prognosis.[5]

Keeping in view these promising features of FNAC with AgNOR evaluation, the present study was planned with an aim to establish AgNOR staining as a diagnostic and prognostic tool in management of various breast lesions including precancerous lesions at tertiary care centre.

It is aimed to confirm the AgNOR diagnosis against Cytomorphology as a gold standard. Over the years, FNAC has proved to be a useful tool for preliminary diagnosis of breast cancer in combination with mammography and USG, addition of AgNOR in interpreting FNAC will help to establish the accuracy of FNAC as a single and confirmative test for differentiation of suspected, benign and malignant cases and will in turn reduce the burden of invasive procedures and histopathology as well as provide opportunity to initiate an early treatment in otherwise false negative cases.

Aims And Objectives

The present study was planned with an aim to assess the usefulness of AgNOR staining as a diagnostic and prognostic tool in management of various breast lesions. This aim was fulfilled with the help of following objectives:

1. To assess the efficacy of AgNOR staining tool on cytology smear in differentiating the benign and malignant breast diseases in terms of its sensitivity, specificity, positive and negative predictive values for malignant breast disease diagnosed on cytomorphology.
2. To find out a correlation between AgNOR findings on cytology smears, tumor stage and histopathological grade.

METHODS

Hundred breast aspirates from 100 patients (17 malignant, 29 benign, probably benign 41, probably malignant 11, fibrocystic disease 2) were selected at random from the cytology section in department of pathology, at tertiary care centre . In all cases a minimum of 2 smears were made which were both air dried and subsequently methanol fixed. One smear was stained routinely with May Grunewald Giemsa (MGG) and the other with AgNOR technique as follows: smears were hydrated in distilled deionized water before staining.

Procedure for Argyrophilic Nuclear Organizer Region (AgNOR)

Evaluation

This was done using Silver Nitrate Method as detailed below:

Silver nitrate method for AgNOR

Solution: A 50% Silver nitrate solution was prepared using the following composition:

Silver nitrate - 50g
Distilled water - 100ml

Gelatin Solution:

Gelatin - 2g
Formic Acid- 1ml
Distilled water -- 100 ml

Working solution

Silver nitrate solution – 2 part
Gelatin solution - 1 part

The above portions were mixed just before use.

For better use the staining procedure was done in very low light or in dark.

The volume of working solution used depended on the number of slides to be stained.

Slides were examined and representative areas with minimal cell overlap were demarcated for AgNOR counting. Counting was performed under oil immersion at X 1000 magnification. AgNORs were visualized as black dots within the nucleus against a yellow background. The dots were defined as black homogenous precipitates of varying size from tiny specks to well defined small rounded dots to larger angulated dots.

The dots were scattered in the nucleus as satellites or grouped together as clusters. A total of 100 cells were counted in each side for AgNOR dots and the average number of AgNOR per cell was determined. The AgNOR counts thus obtained was tabulated for each lesion.

RESULTS

The present study was carried out with an aim to assess the usefulness of AgNOR staining as a diagnostic and prognostic tool in management of various breast lesions. For this purpose, a total of 100 patients with suspected breast lesions were included in the assessment. Table 1 provides the age distribution of patients enrolled in the study.

In this study age group from 21 years to 64 years was included. Most of the patients were in 4th decade followed by 5th decade and only one case was more than 60 years of age group. Mean age of patients was 41.3±7.96 years (Table 1).

Table 1: Distribution of breast lump Age wise

S.No.	Age	No. of cases	%
1.	21-30 Yrs	6	6.0
2.	31-40 Yrs	49	49.0
3.	41-50 Yrs	31	31.0
4.	51-60 Yrs	13	13.0
5.	61-70 Yrs	1	1.0
	Mean Age±SD (Range) in years	41.3±7.96 (21-64)	

All the cases had unilateral involvement. Left side was slightly more commonly involved (52%) than the right side. In the context of Clinical Presentation heaviness in breast was the most common complaint (84%), complain of pain was in 7% of patients, nipple discharge was seen in 13% cases and retraction on nipple was in 21% cases. Breast lump of 70% patient were mobile and rest 30% was immobile on examination.

On clinical examination, a total of 41% cases were diagnosed as probably benign, 11% as probably malignant & fibrocystic disease in 2%, while 29% cases were concluded as benign and 17% as malignant (Table 2).

Table 2: Provisional Clinical Diagnosis (n=100)

SN	Diagnosis	Statistic (No. & %)
1.	Probably benign	41
2.	Probably malignant	11
3.	Fibrocystic disease	2
4.	Benign	29
5.	Malignant	17

In spectrum of cytomorphological diagnosis fibroadenoma was the most common finding and fibrocytic changes were seen in 4 cases. Intra ductal papilloma was suggested in 3 cases. 3 cases were with suppurative changes and concluded as breast abscess. In 4 cases the lesions were disappeared after aspiration and in conjunction to microscopy diagnosed as simple cyst. History of lactation was present in 2 cases and their cystic lesion were said to be galacotocele on cytological examination. Granuloma were found in 2 cases. In 2 cases lumps was of more than 7 cm in size with past history of increase in growth and concluded as phyllodes tumor. 30 cases were diagnosed ductal carcinoma NOS and further graded by Robinsons cytology method. 2 cases were of lobular carcinoma and only 1 case was inconclusive for diagnosis.(Table 3)

Among 32 malignant cases, 30 were ductal carcinoma and 2 lobular carcinomas respectively. Ductal carcinoma NOS were graded cytologically by Robinsons grading method and found to be Grade I = 12, Grade II = 08, Grade III= 10. Histopathological diagnosis could be done in only 40 cases. Of these 25 (62.5%) were benign cases and remaining 15 (37.5%) were malignant (Table 4).

In FNAC aspirates, Mean AgNOR count ranged from 5 to 9.9 per high power field with a mean value of 2.888±2.553 (Table 5).

Mean AgNOR count was observed to be significantly higher in malignant (5.67±2.83) as compared to benign (1.58±0.71) cases ($p<0.001$).With increasing grade, a significant increase in Mean AgNOR count was observed ($p<0.001$). Mean AgNOR count was 2.82±1.21, 5.58±0.75 and 9.01±0.81 for Grades I, II and III respectively (Table 6).

Mean AgNOR was higher for those having nodal involvement (8.00±1.93) as compared to those not having nodal involvement (4.61±2.54), thus showing a statistically significant association ($p=0.001$) (Table 6).

Table 3: Cytopathological Diagnosis

SN	Diagnosis	No.	%
1	Breast abscess	3	3.0
2	Chronic inflammatory (Non-Specific)	3	3.0
3.	Granulomatous lesion	2	2.0
4.	Simple cyst	4	4.0
5.	Galacotocele	2	2.0
6.	Intraductal papilloma	3	3.0
7.	Fibrocytic changes	4	4.0
8.	Fibroadenoma	44	44.0
9.	Phyllodes	2	2.0
10.	DC-NOS	30	30.0
11.	Lobular Carcinoma	2	2.0
12.	Inconclusive	1	1.0
	Total	100	100.0

DISCUSSION

Breast cancer is responsible for the maximum number of cancer related deaths in women. An early diagnosis and timely intervention helps to increase the survival of cancer women. Imaging techniques are less specific and histopathology is one of the last steps in the diagnostic work-up algorithm, early diagnosis is challenging. It is often seen that in many cases the histopathological outcome turns out to be a benign in which surgical intervention could have been either delayed or was not required at all. Thus, there is a need to promote such minimally invasive techniques that can provide results as good as histopathology.

Fine needle aspiration cytology (FNAC) has evolved as a method with reasonable diagnostic efficacy in distinguishing breast cancer from a normal healthy breast. Although FNAC has reduced the need for histopathology to a great extent, however it is important to stress that aspiration technique requires practice and skill and the interpretation of the results requires experience.[6]

Argyrophilic Nuclear Organizer Region (AgNOR) count has emerged as a new technique that helps to provide an objectivity to fine needle aspiration cytology and has shown to be promising in distinguishing benign from malignant and amongst different cytopathological grades. In present study, we made an attempt to evaluate the efficacy of AgNOR for evaluation of different suspicious breast masses. For this purpose, a total of 100 patients with suspected breast lesions were included in the assessment.

Table 4: Correlation between Cytological and Histopathological Diagnosis (n=40)

SN	Diagnosis	Cytology Dx	HPE Dx	Concordance of FNAC with HPE	HPE Diagnosis for Discordant Cases
1.	Breast abscess	3	-	-	-
2.	Chronic inflammatory (Non-Specific)	3	-	-	-
3.	Simple cyst	4	-	-	-
4.	Galacotocele	2	-	-	-
5.	Fibroadenoma	44	20	16/20 = 75%	2- fibrocystic Changes 1-Adenosis 1-Ductal Papilloma
6.	Fibrocytic changes	4	3	2/3=66.7%	1-Fibroadenoma
7.	DC-NOS	30	14	14/14=100%	-
8.	Intraductal papilloma	3	1	1/1=100	-
9.	Lobular Carcinoma	5	1	1/1=100%	-
10	Phyllodes	2	1	1/1=100%	-
11	Granulomatous lesion	2	-	-	-
12	Inconclusive	1	-	-	-

Table 5: AgNOR Count Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Mean AgNOR Count	100	0.6	9.9	2.888	2.55

Table 6: Association of Mean AgNOR count with different cytomorphological outcomes

SN	Outcome	Mean AgNOR	SD	Statistical Significance
1.	Malignancy Status			
	Benign (n=68)	1.58	0.71	't'=11.229;
	Malignant (n=32)	5.67	2.83	p<0.001
2.	Grade (n=30)			
	I (n=12)	2.82	1.21	F=108.31;
	II (n=8)	5.58	0.75	p<0.001
	III (n=10)	9.01	0.81	
3.	Nodal involvement			
	Yes (n=10)	8.00	1.93	't'=3.754;
	No (n=90)	4.61	2.54	p=0.001

The age of patients ranged from 21 years to 64 years was included. Majority of patients (55%) were ≤40 years of age. mean age of patients in present study corresponded with the mean age of patients with breast cancer as reported by Schwartz *et al.* (2012) who reported the mean age of breast cancer to be 49 years.

In present study, all the cases had unilateral involvement with left side slightly more commonly involved (52%) than the right side. Heaviness in breast was the most common complaint (84%), complaint of pain was noted in 7% of patients, nipple discharge was seen in 13% cases and retraction on nipple was in 21% cases. Breast lump of 70% patient were mobile and rest 30% was immobile on examination. All these clinical features place a patient under suspicious malignancy and do not provide a useful clue to final diagnosis.

Table 7: Mean AgNOR count in benign and malignant breast masses as observed in different contemporary case series

SN	Author (Year)	Sample size	Mean AgNOR count in benign cases	Mean AgNOR count in malignant cases	Remarks
1.	Dhakhwa *et al.* (2012).	110 (38 malignant, 72 benign)	2.63±1.36	6.26±1.19	Significant
2.	Darad *et al.* (2013)	79 52 Benign, 27 Malignant	3.45±1.19	9.54±2.54	Significant
3.	Dixit *et al.* (2013).	80 41 Benign 39 Malignant	2.63±1.36	8.42±2.53	Significant
4.	Nepal and Talwar (2014)	40	1.734	4.508	
5.	Basher *et al.* (2016)	100 76 Benign 24 Malignant	2.74	8.62	
6.	Mahajan *et al.* (2016)	50 38 Benign 12 Malignant	3.33±1.49	6.53±2.73	Significant
7.	Present study (2016)	100 67 Benign 32 Malignant 1 Inconclusive	1.58±0.71	5.67±2.83	Significant

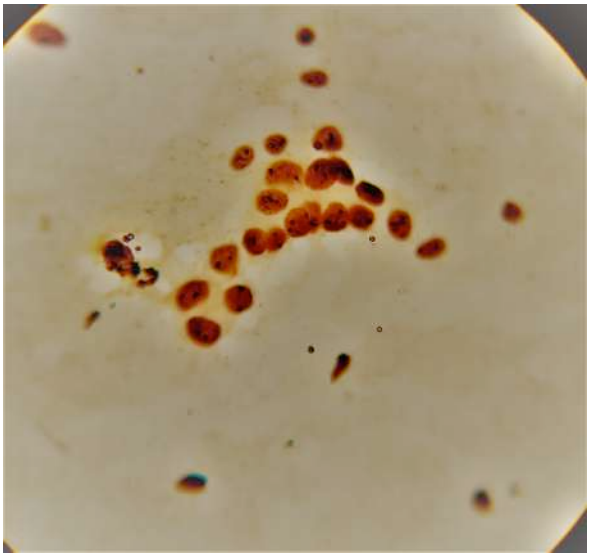

Fig 1: AgNOR Score about 1-3 per nuclei

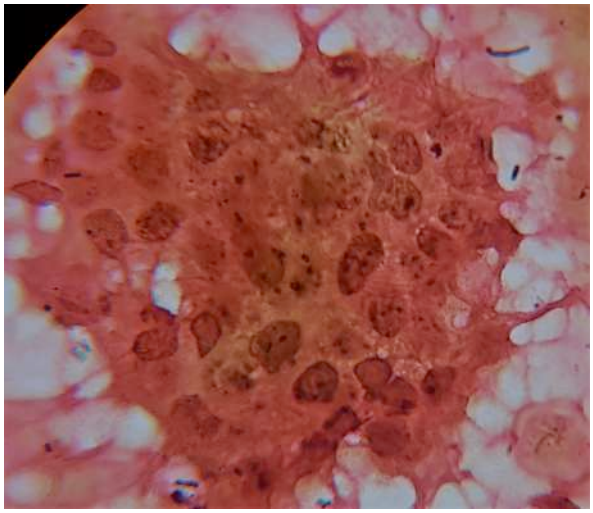

Fig 2: AgNOR score 3-5 per nuclei

On the basis of clinical diagnosis, a total of 41% cases were diagnosed as probably benign, 11% as probably malignant and fibrocystic disease in 2%, while 29% cases were concluded as benign and 17% as malignant. Thus the malignancy rate as per clinical diagnosis was 28% (17% malignant and 11% probably malignant).

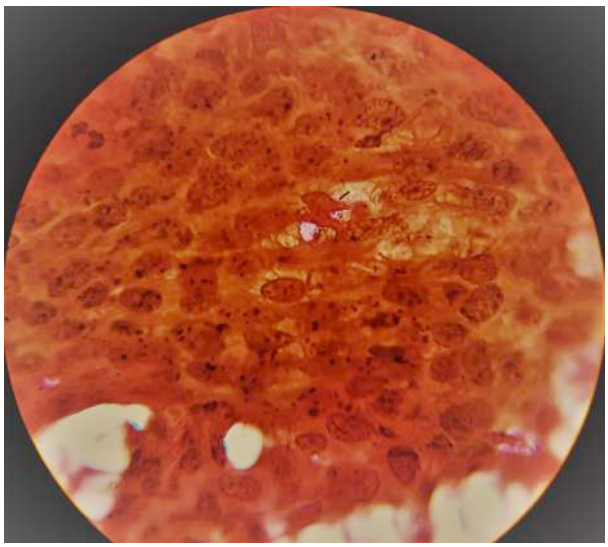

Fig 3: AgNOR Score about 6-7 per nuclei

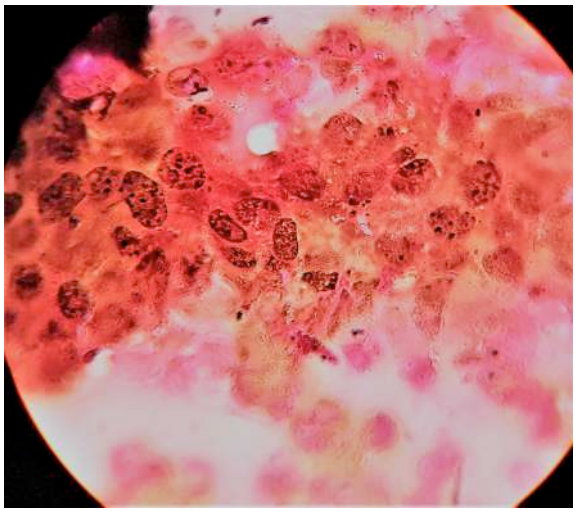

Fig: 4 AgNOR Score about 8-9 per nuclei

However, on mammography, a total of 36 (36%) cases were diagnosed as malignant, 57 (57%) as benign and 7

(7%) as inconclusive. Mammography is the primary imaging modality for breast cancer screening and diagnosis. Addition of mammography helped to identify 8 additional cases, thus indicating that breast masses need a thorough evaluation and highlighted the limitation of clinical diagnosis.

In present study, cytopathology was considered to be the final diagnosis. Cytopathologically, a total of 67 (67%) cases were diagnosed as benign including inflammatory lesions, 32 (32%) were diagnosed as malignant. Only one aspirate was paucicellular and haemodiluted to conclude definite diagnosis. Thus, the malignancy rate was 37% in present study.

In literature, variable malignancy rates in breast masses have been reported. Starvos *et al.* (1995)[9] reported rate of malignancy to be 17% in solid breast nodules. Drew *et al.* (1999) reported this rate in 53.5% of their suspected cases. Kaplan (2001) in clinically and mammographically negative women reported the malignancy rate to be only 0.3%.

In present study among malignant cases ductal carcinoma was the most common type this finding is agreement with various epidemiological studies that place ductal carcinoma to be the most common type comprising nearly 75-80% of breast carcinoma types Similar to our study, Wasif *et al.* (2009) also reported a higher rate of invasive carcinoma in their study which was dominated by ductal cancer (94%) and had only 6% of lobular carcinoma cases. In present study too, there were only 6.3% cases of lobular carcinoma.

In present study, ductal carcinoma NOS were graded cytologically by Robinsons grading method. Of 30 cases of ductal carcinoma, 12 (40%) were Grade I, 8 (26.7%) were Grade II and 10 (33.3%) were Grade III. Contrary to findings of present study, Sinha *et al.* (2014) in their study reported Grade III (n=32/59; 54.2%) to be the most dominating grade while Grade I comprised only 8.5% of their cases.

In present study mean AgNOR count ranged from 5 to 9.9 per high power field with a mean value of 2.888±2.553. The range of AgNOR count has been reported to vary in different studies. In a study by Hasnan *et al.* (1996) reported range of Mean AgNOR count to be 2.47 to 17.15. Mahajan *et al.*(2016) too in their study reported the range of mean AgNOR values to be 1.4 to 11.2 in fine needle aspirates obtained in their study. In another study, Bhatt *et al.* (2013) found the mean AgNOR count in various breast lesions to range from 0 to 6.99.

An overview of Table 7 above shows that in all the series in malignant cases, mean AgNOR count was higher as compared to benign cases.

In present study, this value was 1.58±0.71 which is close to the value obtained by Nepal and Talwar (2014). For malignant cases this range was 4.508 (Nepal and Talwar, 2014)[19] to 9.54±2.54 (Darad *et al*., 2013).

In present study, mean value for malignant cases was 5.67±2.83 which is close to the value 6.26±1.19 reported by Dhakhwa *et al*. (2012).

CONCLUSION

The present study showed that fine needle aspiration cytology is a useful modality for diagnosis of breast lesions. It has a high concordance with the histopathology (87.5%). AgNOR count assessment provides a useful objective measure for segregation of different grades of tumor with 100% accuracy for detection of higher grade (Grade III) of lesions, as observed in present study. For differentiation of benign from malignant lesions too it has a high sensitivity (87.5%) as well as specificity (91.2%). The usefulness of FNAC to evaluate nodal involvement also showed a 90% sensitivity and 95.6% specificity. Considering the fact that a large proportion of our study population was young at age, FNAC as a preliminary assessment tool is recommended to avoid the burden of histopathology in every suspicious case.

On the basis of present study, it could be concluded that AgNOR count estimation using fine needle aspiration is a useful method to differentiate and diagnose breast lesions. In present study, it was found to be highly efficient in differentiating high grade from lower grades and nodal involvement. A higher AgNOR count helped to differentiate between benign and malignant lesions. The findings in present study were promising. However, one of the limitations of study was sample size. Further studies on larger sample size are recommended.

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