

Qualitative and Quantitative Analysis of AgNORs in FNAC Smears of Palpable Breast Lumps

Tamanna Binta Hasan^{1*}
Shoibul Karim²
Mohammad Ismail Hossain³
Md Quamrul Hassan⁴
Sayeda Nasreen³
Pradip Bhattacharjee³
Md Zillur Rahman³

¹Department of Pathology
University of Science and Technology Chittagong (USTC)
Chittagong, Bangladesh.

²Department of Surgery
Ibrahim Iqbal Memorial Hospital &
Medical College (BGC Trust)
Chittagong, Bangladesh.

³Department of Pathology
Chittagong Medical College
Chittagong, Bangladesh.

⁴Department of Pathology
Cox'sBazar Medical College
Cox'sBazar, Bangladesh.

*Correspondence to:

Dr. Tamanna Binta Hasan
Assistant Professor
Department of Pathology
University of Science and Technology Chittagong (USTC)
Chittagong, Bangladesh.
Mobile: +880 01819615210
Email: tui8bd@yahoo.com.

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Abstract

Background: The study aimed to evaluate the efficacy of AgNORs on FNAC smears of breast lesion. **Methods:** FNAC were done in 200 female patients of breast lump consecutively, Papanicolau and AgNOR staining was done in all 200 cases of FNA smears. Among them biopsy and histopathology were done in 99 cases. The findings of FNAC and AgNOR analysis were compared to histopathological findings. **Results:** By FNAC, 53 (26.5%) cases were non-neoplastic benign, 89(44.5%) cases were benign neoplasm, 07 (3.5%) cases were atypical ductal hyperplasia and 51(25.5%) cases were malignant (Duct cell carcinoma). Among them histopathology was done in 99 cases. Out of 40 FNAC malignant cases, histopathologically all were proved malignant. Out of 42 benign cases, 1 was found malignant. 4 atypical ductal hyperplasia were also diagnosed as malignant histopathologically. True positive cases are 40, True negative cases are 36, false positive are nil (0), and false negative is 01. The sensitivity is 97.56%, specificity is 100%, positive predictive value is 100%, Negative predictive value is 97.3%, and accuracy is 98.7%. AgNOR impression were analysed in 99 histopathologically confirmed cases. The results showed benign impression in 50 cases and malignant impression in 48 cases. 01 histopathologically malignant case was impressed as benign by AgNOR and 01 as suspicious which may be included as malignant by AgNOR impression. 2 histopathologically benign cases showed higher proliferative activities and counted as malignant. True positive cases are 46, True negative 49, false positive is 02 and false negative is 01. The sensitivity is 97.87%, Specificity 96.07%, PPV 95.83%, NPV 98%, Overall accuracy is 96.93%. **Conclusion:** The efficacy of FNAC and AgNOR analysis in the diagnosis of breast lesion was found more or less similar and mild over lapping.

Key words: Papanicolau; AgNORs; FNAC; Histopathology.

INTRODUCTION

Breast cancer is the top most cancer in women both in the developed and the developing world. Breast cancer survival rates vary greatly worldwide, ranging from 80% or over in developed countries, around 60% in middle-income countries and below 40% in low income countries. The low survival rates in less developed countries can be explained mainly by the lack of early detection programmes, resulting in a high proportion of women presenting with late-stage disease, as well as by the lack of adequate diagnosis and overall management facilities¹.

The mitotic rate is an important characteristic of malignant tumors. A high mitotic rate is common in malignant neoplasm. In addition to the ability to separate benign from malignant tumors based on their mitotic activity, the number of mitosis within a given tumor provides a rough indication on the rate of tumor proliferation and its diagnosis^{2,3}.

The number of Nucleolar Organizer Regions (NORs) has been considered to reflect the proliferating activity of cells and to serve as an indicator of the malignancy level of tumour⁴. Their frequency within the nuclei is significantly higher in malignant cells than in normal, reactive or benign neoplastic cells^{5,6,7}.

NORs are loops of DNA in the human genome that have been associated with protein synthesis and ribosomal activity and are located in the short arm of the acrocentric chromosomes 13, 14, 15, 21 and 22^{8,9,10}.

These regions can be easily identified by light microscopy as discrete black dots after the application of silver stain, known as 'Argyrophilic Nucleolar Organizer Regions' (AgNORs)¹¹. Initially used as a parameter for the diagnosis of malignancy, the AgNOR parameter was found to be also useful for assessing the prognosis of cancer diseases¹².

AgNOR count is a reproducible simple, efficient and inexpensive method, which can be used as an adjunct to routine H & E staining. The staining technique is a relatively simple and rapid one^{12,13}.

Fine Needle Aspiration Cytology (FNAC) is now an integral part of the pre-operative investigation and therapeutic protocol of breast lesions⁷. FNAC has an advantage of being an immediate and excellent method for on-site examination and one-stop diagnosis at breast outpatient clinics. Since the majority of patients have benign disease, they benefit from rapid diagnosis and discharge from the clinic¹⁴. Mastectomies can be prevented by early diagnosis and open biopsies can be reduced¹⁵. However, from time to time, cytological picture may be equivocal or inconclusive⁸. The clinical use of FNAC has been questioned because of the variability in results reported. The use of Papanicolaou staining alone generates false-negative and false-positive results and the diagnostic accuracy is approximately 90%¹⁶. False negativity of FNAC was also reported by Mendoza et al¹⁷. Addition of AgNORs analysis on FNA smear will definitely strengthen the accuracy of FNAC of breast in differentiating reactive, benign and malignant tumours of the breast. Although, the number of AgNORs is indicator of proliferative index, the addition of the measurement of size and area of dispersion gives improved diagnostic and prognostic specificity¹⁸.

No AgNOR study of breast lesions has been done so far in our country. This study was done in a view of evaluating the role of AgNORs analysis in FNA smear of breast lesions.

MATERIALS AND METHODS

It is a cross sectional descriptive study carried out in the Department of Pathology, Chittagong Medical College (CMC), Chittagong, during the period of one year from July 2013 to June 2014. 200 Consecutive female patients, irrespective of their age, presenting with clinically palpable breast lumps attending in the Department of Pathology, CMC, referred from CMCH (Chittagong Medical College Hospital) and other hospitals and clinics or individual physicians of Chittagong were included in the study.

Informed written consent was taken from all the patients, included in this study, after full explanation of nature, purpose and potential risks of the study in the local language.

A brief Clinical history was taken from each patient and was recorded in a preformed questionnaire proforma. Each patient was examined thoroughly and informations were recorded in details in all cases.

The patients presenting with palpable breast lump were undergone FNA in the FNAC lab of the Department of Pathology, CMC, Chittagong. At least four smears were prepared in each case, out of them two were immediately fixed in 95% alcohol for Papanicolaou stain and two of them air dried followed by fixation in 95% alcohol for AgNOR stain. Two alcohol fixed smear were stained by Papanicolaou method. AgNOR staining were performed by using silver nitrate, gelatin and formic acid on air-dried, alcohol-fixed FNA smears¹⁹.

Excision biopsy was done from the cytologically diagnosed malignant cases and from cases, where excisions were indicated clinically and histopathological specimen was collected in these cases. The biopsy specimens, after processing & sections, blocks were prepared and slides were made and stained by Haematoxylin & Eosin (H&E).

Examinations of the stained smear and sections

- i) **Pap stained smear:** Smears stained by Papanicolaou were examined in light microscope. The cellularity and the morphological details were thoroughly studied to reach a diagnosis regarding the type of lesions.
- ii) **AgNOR stained smear:** AgNOR stained smear were examined under light microscope using oil immersion lens with magnification of 100 x and 100 randomly selected duct epithelial cells were studied for AgNOR count, size and dispersion.

AgNOR count (mAgNOR)

The NORs were seen as clear black dots in the nuclei. AgNOR dots present in cluster form in the nuclei were considered as one. The count was the mean number of AgNORs in 100 tumour nuclei (mAgNOR).

AgNOR size

The grading of size variation was performed according to method used by Hossain et al and Khan et al^{19,20}. Scores of distribution are given below:

- 0: More or less uniform in size
- 1+: Two different sizes
- 2+: More than two different sizes (But not those of 3+)
- 3+: Including all sizes & grade

Proliferative AgNOR (pAgNOR)

AgNOR proliferative index (pAgNOR) = percentage of cells with 5 or more AgNOR dots. pAgNOR more than 8% was considered to display high proliferative activity. Malignant lesions show high proliferative activity. We considered <8 for benign lesions, 8-11 for suspicious lesions and >11 for malignant lesions Hossain et al¹⁹.

Parameter	Benign	Suspicious	Malignant
mAgNOR	Variable, but usually <3	Variable, usually >3	Variable, usually >3
pAgNOR	<8%	8-11%	>11%
Size grade	0 - 1+	2+ - 3+, variable	2+ - 3+, variable

2 of the 3 parameter were taken to decide diagnosis and confirmed by histopathology.

iii) **H&E stained sections:** The stained sections were examined in the light microscope to get a definite diagnosis of the lesions and its type.

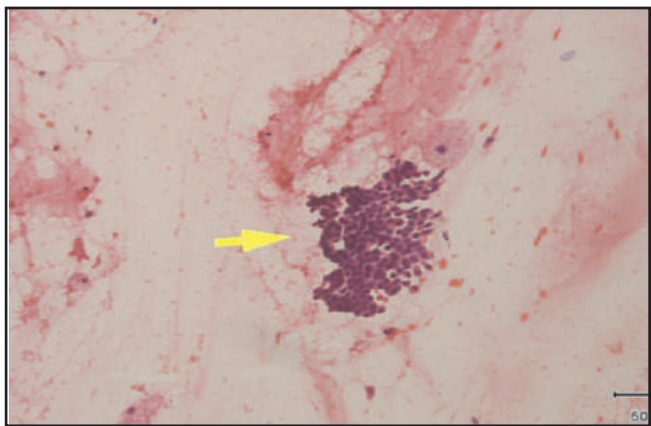


Figure 1 : Fibrocystic Change 100 X (Pap stain)FNA smear.

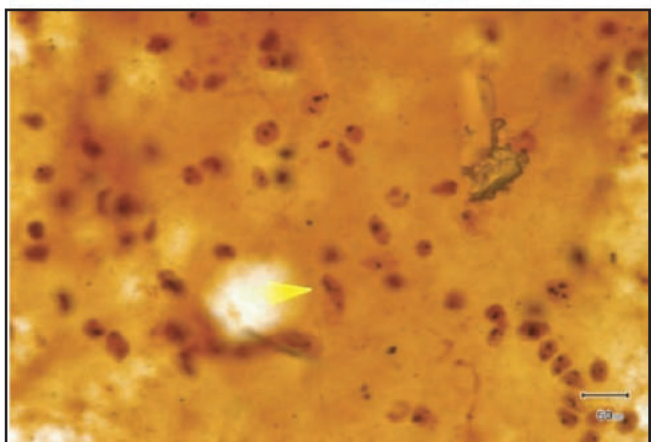


Figure 2 : Fibrocystic Change 1000 X (AgNOR stain).

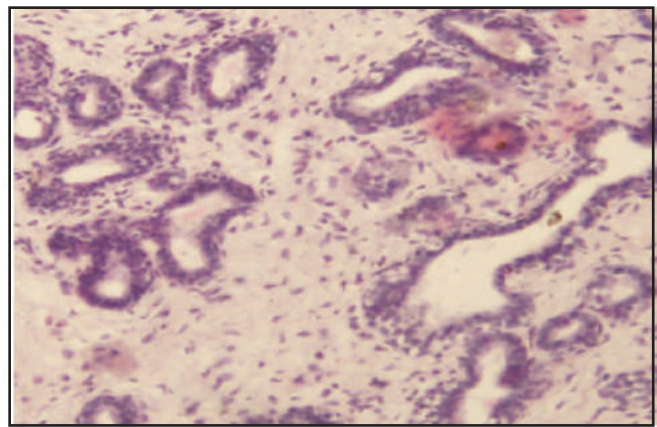


Figure 3 : Fibrocystic Change 400 X (H & E stain) Histopathology section.

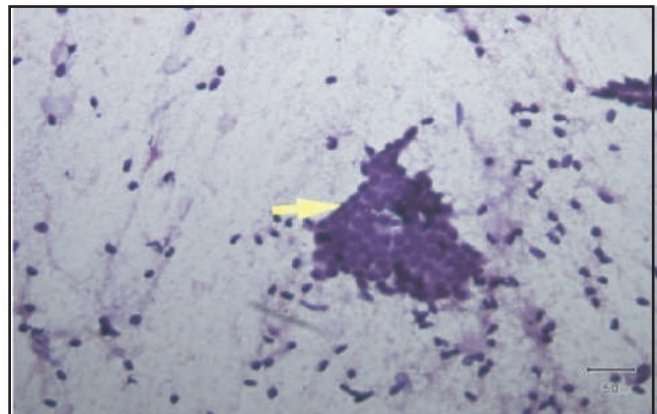


Figure 4 : Fibro adenoma 400 X (Pap stain) FNA smear.

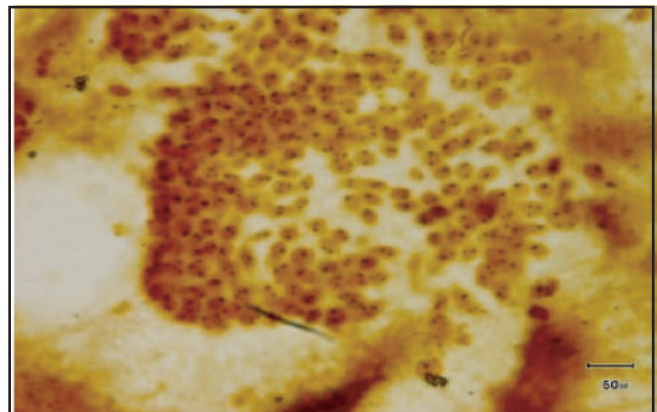


Figure 5 : Fibroadenoma 1000 X (AgNOR stain) FNA smear.

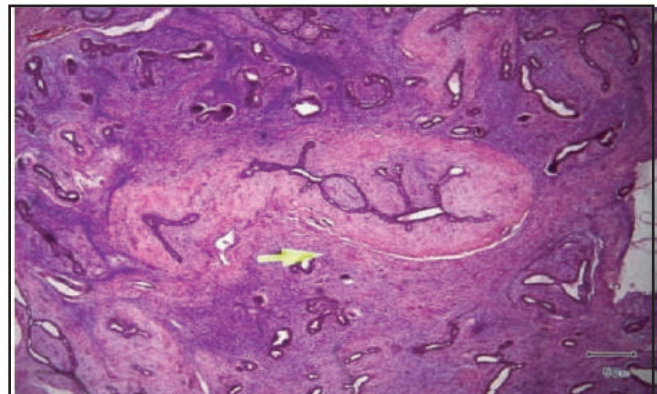


Figure 6 : Fibroadenoma 400 X (H & E stain) Histopathology section.

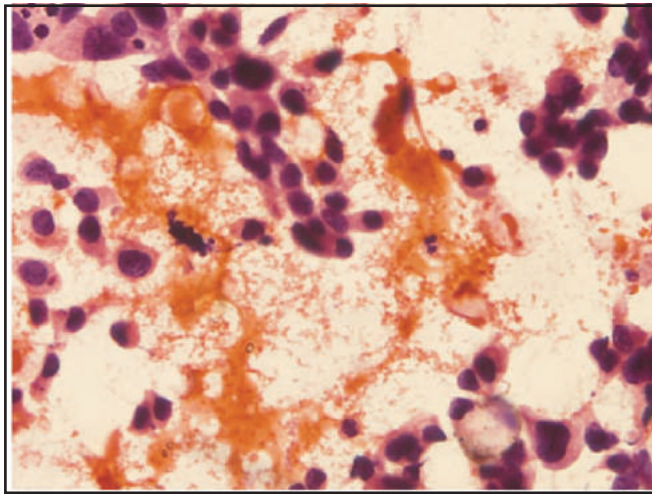


Figure 7 : Duct Cell Carcinoma 400 X (Pap stain) FNA smear.

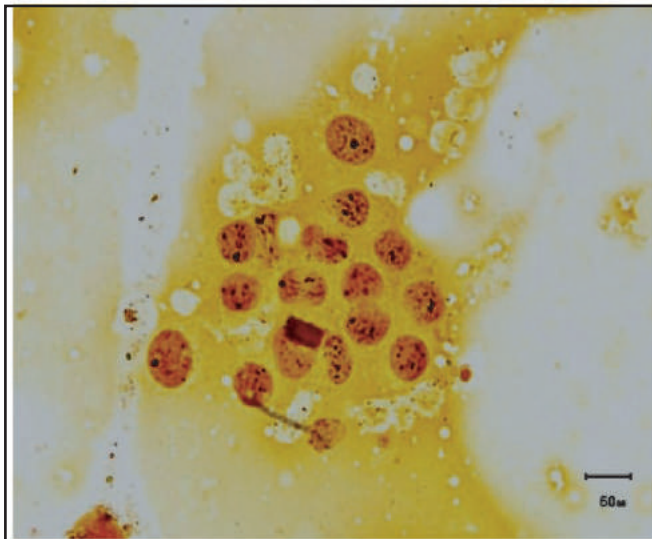


Figure 8 : Duct Cell Carcinoma 1000 X (AgNOR stain).

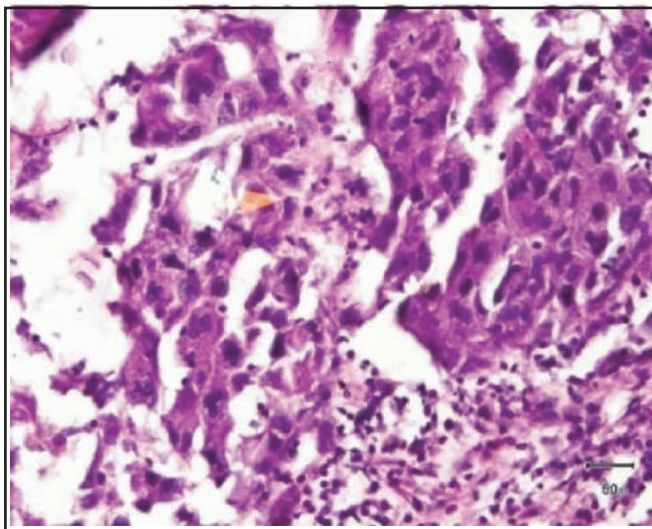


Figure 9 : Duct Cell Carcinoma 400 X (H & E stain) Histopathology.

RESULTS

The age range of the 200 patients was 15-70 years and mean age was 33.65 years.. The cases were divided into different age groups (1= upto 20 years, 2= 21-30 years, 3=31-40 years, 4=41-50 years, 5=51-60 years and 6= over 60 years.). The number & percentage of cases in different age groups The history of 200 patients, clinical findings, FNAC, AgNOR findings and histopathological findings were noted in a preformed data sheet. A total 51 malignant cases were found.Among the 200 patients by FNAC, 53 (26.5%) cases were non-neoplastic benign, 89(44.5%) cases were benign neoplasm, 07 (3.5%) cases were atypical ductal hyperplasia and 51 (25.5%) cases were malignant lesion. Out of 53 non-neoplastic benign cases, 07 cases were inflammatory lesion, 28 cases were fibrocystic changes, and 18 cases were non-specific or inconclusive. Out of 89 benign neoplasms all were fibroadenoma and out of 51 malignant cases, all were duct cell carcinoma (Table 1 & 2).

Table 1 : Distribution of breast diseases according to age group (n=200).

Age Range	FNAC Category			Total	
	Non-neoplastic benign	Benign neoplasm	Atypical ductal hyperplasia		
≤20 yrs	10	40	00	00 (00.00%)	50 (25%)
21-30 yrs	13	39	02	07 (13.72%)	61 (30.5%)
31-40 yrs	13	05	01	09 (17.65%)	28 (14%)
41-50 yrs	12	03	04	16 (31.37%)	35 (17.5%)
51-60 yrs	05	01	00	13 (25.49%)	19 (9.5%)
61-70 yrs	00	01	00	06 (11.77%)	07 (3.5%)
Total	53 (26.5%)	89 (44.5%)	07 (3.5%)	51 (25.50%)	200 (100%)

Table 2 : Frequency of breast diseases (n=200).

FNAC Impression	FNAC Diagnosis	Frequency & percent
Non-neoplastic benign	Inflammatory	07 (3.5%)
	Fibrocystic Changes	28 (14%)
	Others	18 (9%)
		53 (26.5%)
Benign neoplasm	Fibroadenoma	89 (44.5%)
Atypical ductal hyperplasia	Atypical ductal hyperplasia	7 (3.5%)
Malignant	Duct cell carcinoma	51 (25.5%)
Total		200 (100%)

AgNOR impression in FNA smears

AgNOR analyses were done in all 200 cases of FNA smears. Out of 200 cases, 53 cases were non-neoplastic benign lesions, 89 cases were benign neoplasm, 07 cases were atypical ductal hyperplasia and 51 cases were malignant lesions. Overall mean mAgNOR were 3.00 (SD±2.14), mean pAgNOR were 9.40 (SD±11.50), mean AgNOR size were 0.66 (SD±.87). Mean mAgNOR in case of non-neoplastic benign lesions were 1.52 (SD±1.40), in benign neoplasm were 2.18 (SD±.91) and in malignant lesions were 5.64 (SD±1.73). Mean pAgNOR in non-neoplastic benign lesions were 3.75 (SD±7.25), in benign neoplasm were 4.98(SD±3.68) and in malignant lesions were 21.47 (SD±13.99). Mean AgNOR size, in non-neoplastic benign

lesions were 0.38 (SD±0.90), in benign neoplasm were 0.47 (SD±0.62) and in malignant lesions were 1.12 (SD±0.86).

136 patients showed <3 range of mAgNOR and 63 patients showed >3 range of mAgNOR. 141(70.5%) patients showed <8% grading of pAgNOR and 59(29.5%) patient showed >11% pAgNOR. AgNOR size grading showed 1(0.5%) cases 0+, 173(86.5%) cases 1+, 4(02%) cases 2+ and 22(11%) cases showed 3+ with much over lapping between benign and malignant lesions.

All this findings are shown on table 3 (a, b and c).

Table 3 : Frequency of different parameter of AgNORs(n=200).

3(a)mAgNOR

m AgNOR range	FNAC			Malignant	Total
	Non-neoplastic benign	Benign neoplasm	Atypical ductal hyperplasia		
1 (<3)	50	86	0	0	136
2 (3)	0	0	1	0	1
3 (>3)	3	3	6	51	63
Total	53	89	7	51	200

3(b)pAgNOR

p AgNOR grading	FNAC			Malignant	Total
	Non-neoplastic benign	Benign neoplasm	Atypical ductal hyperplasia		
1 (<8%)	50	87	2	2	141
3 (>11%)	3	2	5	49	59
Total	53	89	7	51	200

3(c)AgNOR size grade

AgNOR size grading	FNAC			Malignant	Total
	Non-neoplastic benign	Benign neoplasm	Atypical ductal hyperplasia		
0+	0	0	0	1	1(0.5%)
1+	49	87	3	34	173(86.5)
2+	0	2	0	2	4(2%)
3+	4	0	4	14	22(11%)
Total	53	89	7	51	200(100%)

Among the 200 cases of breast lesions, biopsy and histopathology were done in 99 cases. Histopathological diagnoses showed 14 (14.14%) non-neoplastic benign (Fibrocystic changes), 37 (37.37 %) were benign neoplasm. (36 Fibroadenoma and 1 Phylodestumour, which were diagnosed as fibroadenoma by FNAC) and 48 (48.49 %) were malignant (Duct cell carcinoma). The results are shown in Table 4.

Table 4 : Distribution of histopathological findings (n=99).

Histopathologic typing	Frequency	Percent
FCC	14	14.14
FA	36	36.36
Phylodestumor	01	01.01
Duct cell carcinoma	48	48.49
Total	99	100.0

FNAC results of histopathologically diagnosed 99 cases showed 13 non-neoplastic benign case, 42 were benign neoplasm (Fibroadenoma), 4 atypical ductal hyperplasia, and 40 cases were duct cell carcinoma. Out of 40 FNAC malignant cases, histopathologically all were proved malignant. Out of 42 benign cases, 1 was found malignant. 4 atypical ductal hyperplasia were also diagnosed as malignant histopathologically. True positive cases are 40, True negative cases are 36, false positive are nil (0), and false negative is 01. (Non neoplastic benign and atypical ductal hyperplasia are excluded from the statistics). The sensitivity is 97.56%, specificity is 100%, positive predictive value is 100%, Negative predictive value is 97.3%, and accuracy is 98.7%. The association between FNAC and histopathological diagnoses are shown in the Table 5 (a and b).

Table 5(a): Association between FNAC and histopathological findings (n=99).

FNAC Category	Histopathology			Not done	Total
	Non-neoplastic Benign	benign Neoplasm	Malignant		
Non-neoplastic benign	9	1	3	40	53
Benign neoplasm	5	36	1	47	89
ADH	0	0	4	3	7
Malignant	0	0	40	11	51
Total	14	37	48	101	200

$\chi^2=161.83$, $p<0001$, Highly significant (Chi-Square test)

Table V(b): Statistical evaluation of FNAC in the diagnosis of Breast lesion (n=99).

Validity test (Chi-square test)	Result
Sensitivity	97.56%
Specificity	100%
Positive predictive value	100%
Negative predictive value	97.3%
Accuracy	98.7%

AgNOR impression was analysed in 99 histopathologically confirmed cases. The results showed benign impression in 50 cases and malignant impression in 48 cases. 01 histopathologically malignant case was impressed as benign by AgNOR and 01 as suspicious which was included as malignant

by AgNOR impression. 2 histopathologically benign cases showed higher proliferative activities and counted as malignant. True positive cases are 46, True negative 49, False positive is 02 and false negative is 01. The sensitivity is 97.87%, specificity 96.07%, PPV 95.83%, NPV 98%, Overall accuracy is 96.93%. The association between AgNOR and histopathological diagnoses are shown in the Table 6 (a).

Table 6 (a) : Association between AgNOR and histopathological diagnosis (n=99).

AgNOR Impression	histopathological Diagnosis			Total
	Non-neoplastic benign	Benign	Malignant	
Benign	14	35	1	50
Suspicious/ Atypical	0	0	1	01
Malignant	0	02	46	48
Total	14	37	48	99

$\chi^2 = 134.29$; $p < 0001$; Highly significant

Table 6 (b) : Statistical evaluation of AgNOR impression on FNA smears in the diagnosis of breast lesion (n=99).

Validity test (Chi-square test)	Result
Sensitivity	97.87%
Specificity	96.07%
Positive predictive value	95.83%
Negative predictive value	98%
Accuracy	96.93%

DISCUSSION

FNAC has gained popularity as a procedure for diagnosis of breast lump in all over the world including Bangladesh, because of the simplicity of the procedure, good tolerance by the patients, rapidity, cost effectiveness and safety. However, from time to time, cytological picture may be equivocal or inconclusive. Sometimes, the clinical use of FNAC has been questioned because of the variability in results reported. Addition of another simple procedure AgNORs analysis on FNA smears simultaneously will strengthen the accuracy of FNAC of breast in differentiating reactive, benign and malignant tumours of the breast.

Papanicolaou and AgNOR analyses done on all FNA smears of 200 breast lesions. Among them, biopsy and histopathology were done in 99 cases and the findings were compared to respective FNA and AgNOR findings separately and statistically evaluated.

Out of 40 FNAC malignant cases, histopathologically all were proved malignant. Out of 42 benign cases, 1 was found malignant. 4 atypical ductal hyperplasia were also diagnosed as malignant histopathologically. 01 fibroadenoma diagnosed by FNAC was found Phyllodes tumour by histopathology. True positive cases are 40, True negative cases are 36, false positive are nil (0), and false negative is 01. (Non neoplastic benign and atypical ductal hyperplasia are excluded from the statistics). The sensitivity is 97.56%, specificity is 100%, positive predictive value is 100%, Negative predictive value is 97.3%, and accuracy is 98.7%.

There are limited studies regarding AgNOR analysis of breast lesions, most of which are based on mAgNOR count (Monoparametric) only. Ansari et al observed mean mAgNOR, 2.96 (SD±0.72) and pAgNOR 14.1 in benign neoplasm and mean mAgNOR 4.0 (SD±1.42), pAgNOR 36.6% in malignant neoplasm, which are similar to the findings of present study¹³. Ahmed et al showed mean mAgNOR in benign neoplasm was 1.26 and in malignant neoplasm was 2.57. Chughtai et al in their study observed mean mAgNOR in benign neoplasm 6.87(SD=0.92) and in malignant cases was 18.51(SD=2.90)¹¹. The findings of these two studies also support the findings of present study.

Histopathological diagnoses showed 14 (14.14 %) non-neoplastic benign case, 37(37.38 %) was benign neoplasm and 48 (48.48 %) cases were malignant. All the non-neoplastic benign cases were fibro-cystic diseases. Out of 37 benign neoplasms were 36 were fibro-adenoma and 01 was phylodestumour (Which was diagnosed as fibroadenoma by FNAC) and malignant neoplasm were invasive Duct Cell Carcinoma (NOS).

The results of AgNOR analysis showed benign impression in 51 cases and malignant impression in 46 cases. 01 histopathologically malignant case was impressed as benign by AgNOR and 01 as suspicious. 2 histopathologically benign cases were impressed as malignant by AgNOR analysis. True positive cases are 46, True negative are 49, false positive are 02 and false negative are 02. The sensitivity is 97.87%, specificity 96.07%, PPV 95.83%, NPV 98% and overall accuracy is 96.93%.

CONCLUSION

Qualitative and quantitative analysis of AgNORs on FNA smear can strengthen the efficacy of FNAC of palpable breast lump in differentiating reactive, benign and malignant tumours of the breast.

DISCLOSURE

All the authors declared no competing interest.

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