Evaluation of AgNORs on FNA smears and tissue sections in benign and malignant breast diseases

Deepti Mahajan[1,*], Kuldeep Singh[2], Yudh Vir Gupta[3]

[1]Assistant Professor, [2]Professor, [3]Professor & Head (Retd.), Dept. of Pathology, Govt. Medical College, Jammu

***Corresponding Author:**
Email: deeptim1974@yahoo.com

Abstract

Background and Objectives: AgNOR scoring on breast tissue has shown a significantly higher count in malignant breast lesions in comparison to benign lesions and AgNOR staining on breast aspiration smears has been found superior to staining of histological sections. The present study aims at a comparative evaluation of mean AgNOR (mAgNOR) counts on the cytologic smears and the corresponding histologic sections in the various benign and malignant lesions of the breast.

Methods: A one step silver colloidal staining procedure was employed for demonstration of AgNORs on aspiration smears and the corresponding histologic sections in fifty patients with breast lumps, 38 of these had a benign breast disease while 12 harboured a malignant lesion. AgNOR counts were expressed as mean AgNORs per nucleus (mAgNOR score).

Results: The AgNORs in benign lesions were fewer, small, uniform, and mostly centrally placed where as those in malignant lesions were irregular, generally large and scattered. The mean mAgNOR count in benign breast lesions was 3.3; S.D 1.49 on FNA smears and 2.3; S.D 1.22 on histologic sections ($p<0.001$). In malignant diseases of the breast the mean mAgNOR score was 6.53; S.D 2.73 on FNA smears and 4.98; S.D 1.53 on histologic sections ($p>0.1$). The mAgNOR scores for the malignant breast lesions were significantly higher than those for benign lesions on both cytologic smears and histologic sections ($p<0.001$).

Interpretation and Conclusion: Though a broad overlap between benign and malignant lesions limits its use as a sole diagnostic criterion, however, AgNOR scoring can prove to be a simple and yet a very useful tool in predicting tumour progression and patient survival.

Key words: AgNOR, mAgNOR score, carcinoma breast.

Access this article online

Quick Response Code:

Website: www.innovativepublication.com

DOI: 10.5958/2394-6792.2016.00034.X

Introduction

Diseases of the breast are widely prevalent and are of concern to the patient and clinician alike. Although most breast lesions are benign, carcinoma of the breast is the most common non-skin malignancy in adult women world-wide and attention to it often overshadows that given to the other breast lesions.[1] In India cancer of the breast is the second most common malignancy in females, next only to the cancer of uterine cervix.[2] Accurate prediction of tumour progression and patient survival is of definite significance in the management of breast cancer, thus mandating a continuous search for additional prognostic predictors.[1] Silver staining of nucleolar organizer regions (AgNOR technique) is a quick and simple method which provides information about cellular proliferative activity in hyperplastic and neoplastic conditions. Nucleolar organizer regions (NORs) are ribosomal DNA loops located on the short arms of acrocentric chromosomes which encode for the ribosomal RNA (rRNA) and are thus responsible for the development of the RNA containing nucleolus. The frequency of NORs per nucleus may reflect cell ploidy with an increased expression of NOR sites in actively proliferating cells.[3] Studies of interphase nuclei by morphological, cytogenetic and cell kinetic methods suggest a close relationship between the number of interphase NORs and state of cellular proliferation.[4] NORs can be visualized as black dots by staining with silver nitrate and the structures thus demonstrated are termed "Argyrophilic Nucleolar Organizer Regions" or "AgNORs". Quantification of AgNORs can, therefore, aid in diagnosis and prognostication as their frequency is claimed to be significantly higher in malignant cells than in normal, reactive or benign neoplastic cells.[5]

Initially described as a sequential three step procedure, the AgNOR technique was later abbreviated to a simple one step sequence, rendering the results more reproducible.[6,7] When applied to routinely processed paraffin embedded breast tissue, the total AgNORs in malignant breast lesions have been observed to significantly exceed those of normal breast and benign lesions.[8] In malignant lesions of the breast AgNOR counts have been observed to correlate with tumour size, mitoses and desmoplasia with ER, PR negative tumours showing higher NOR counts, thus serving as a useful prognostic marker.[9] AgNOR staining on breast aspiration smears also gives very good discrimination between benign and malignant lesions and is found superior to staining of histologic

sections due to lack of background staining, better dispersion of NORs and easy counting of the better discernible AgNOR dots on FNA smears.[10,11,12]

Materials and Methods

The study was conducted on fifty patients (49 females and one male) who reported to the cytology section of the Department of Pathology, Government Medical College Jammu for fine needle aspiration of palpable breast lumps. Adequate sampling was ensured and a minimum of five smears were made in each case, two of these were immediately fixed in 95% ethanol for Papanicolaou (Pap) staining[13] and the remaining were kept air-dried for May Grunwald Giemsa (MGG)[13] and AgNOR staining. Histopathologic correlation with post-operative lumpectomy or mastectomy was done in each case except one. Formalin fixed and routinely processed tissue sections were stained with the Haematoxylin and eosin stain[14] and at least one tissue section in each case were subjected to AgNOR staining. A condition was labelled as benign or malignant on the basis of the definitive histological diagnosis. Only one case that was reported as squamous cell carcinoma on cytology was included in the study without the availability of the respective tissue sections, since the patient expired a few days after FNAC was done. However, this case had sufficiently distinctive cytological features and AgNOR pattern to merit inclusion in the study.

AgNOR staining and counting procedure:[8] Air dried, alcohol fixed smears and formalin fixed paraffin embedded tissue sections were stained by one step silver colloid staining method as follows:

2g gelatin powder was dissolved in 1g/dl aqueous formic acid at room temperature to give a 2% solution of gelatin in 1% aqueous formic acid (Solution-A). 50g silver nitrate and 100ml distilled water were thoroughly mixed in a pre-cleaned glass beaker under dark room conditions to give a 50% aqueous silver nitrate solution (Solution-B). One volume of Solution-A and two volumes of Solution-B were mixed in dark room to form the silver colloid solution for staining of nucleolar organizer regions. The smears and the dewaxed tissue sections were rehydrated to deionized water and incubated with a freshly prepared silver colloid solution for 40 minutes, at room temperature, in a dark room. The silver colloid was washed off with distilled, deionized water and the smears/sections were dehydrated, cleared in xylene and mounted in DPX.

AgNORs were seen as dark brown or black dots, usually > 1micron, within the nucleus against a golden brown background. AgNOR counting was done under an oil immersion objective at a magnification of 1000X in 100 randomly chosen epithelial cells. The number of individually discernible and separable black dots in each nucleus was recorded and the dots present in clusters or inseparable aggregates were counted as one. The dots overlying the nuclear membrane were not counted to prevent errors due to variation in section thickness. Finally the mean AgNOR (mAgNOR) score per nucleus was calculated for each case.

Results

In the present study mAgNOR scoring was done on 38 benign and 12 malignant lesions of the breast. The AgNOR dots were fewer and smaller in benign diseases. They were discrete, regular, well rounded and central in position with a few clustered marginal dots (Fig. 2 and 3). Similar morphology of AgNOR dots in benign breast lesions was observed on both FNA smears and tissue sections, though they were better defined in cytological preparations. The mean mAgNOR score (±SD) in benign lesions of the breast was 3.3(±1.49) on FNA smears and 2.53(±1.22) on histologic sections (Table 1, Fig. 1). Proliferative benign lesions like the cellular fibroadenomas, fibrocystic disease or fibroadenomatosis wth significant adenosis and epitheliosis and Phyllodes tumour showed higher counts (Table 1, Fig. 4). Applying the Student's paired t-test, significantly higher mAgNOR counts were observed on FNA smears than the corresponding tissue sections with a t-stat = 9.28($p<0.001$).

The AgNORs in malignant breast lesions were irregular, angulated and showed variations in shape and size (Fig. 5, 6, 7). They were generally large, clustered or dispersed throughout the nucleus (Fig. 5). The mean mAgNOR score (±SD) in malignant lesions of the breast was 6.53(±2.73) on FNA smears and 4.98(±1.53) on tissue sections (Table 2, Fig. 1). The case of squamous cell carcinoma showed the highest score, followed by infiltrating ductal carcinoma breast (2 cases) and a case of Non Hodgkin lymphoma breast. Though, the mAgNOR counts in tissue sections were slightly lower than those observed in the corresponding cytologic smears, the difference was not statistically significant on applying the Student's paired t-test (t-stat = 1.61 i.e. $p>0.01$).

For the comparison of mAgNOR scores between benign and malignant breast lesions (Fig. 1), unpaired or the pooled t-test was used. On FNA smears the t-stat value was -5.23 ($p<0.001$) and on tissue sections t-stat was -5.57 ($p<0.001$). Hence, the difference between mAgNOR scores in the benign and malignant breast diseases on both aspiration smears as well as tissue sections was statistically highly significant. However, a broad range of overlap (4.3 to 8.0 on smears and 2.0 to 7.2 on sections) in mAgNOR scores of benign and malignant lesions was observed.

Table 1

mAgNOR scores in benign breast lesions			
S. No	**Diagnosis**	**mAgNOR score**	
		FNA smears	**Tissue Sections**
Case 1	Fibroadenoma	2.2	2.1
Case 2		2.1	1.4
Case 3		1.9	1.1
Case 4		2.7	1.2
Case 5		3.3	2.1
Case 6		3.4	2.0
Case 7		2.8	2.6
Case 8		2.4	2.1
Case 9		3.2	2.5
Case 10		2.4	1.9
Case 11		4.5	3.2
Case 12		3.8	2.9
Case 13		4.4	3.1
Case 14		3.5	2.9
Case 15		4.1	3.2
Case 16		3.5	2.8
Case 17	Fibroadenomatosis	2.4	1.5
Case 18		8.0	7.2
Case 19		3.0	1.2
Case 20		5.0	4.2
Case 21		6.7	4.1
Case 22		5.1	4.2
Case 23		4.1	2.7
Case 24	Fibrocystic disease	1.4	1.0
Case 25		2.5	1.6
Case 26		1.9	2.2
Case 27		3.6	3.1
Case 28		2.4	1.6
Case 29		4.1	2.2
Case 30	Intraductal papilloma	4.0	3.5
Case 31		1.0	1.1
Case 32		2.2	1.3
Case 33	Tuberculosis	1.5	-
Case 34	Gynaecomastia	2.6	1.8
Case 35	Lactational adenoma	4.1	3.0
Case 36	Benign Phyllodes tumour	6.5	4.9
Case 37	Breast abscess	1.4	1.1
Case 38	Sclerosing adenosis	2.8	2.2

Mean mAgNOR score: 3.33, S.D 1.49 (FNA smears); 2.53, S.D 1.22 (Tissue sections).
t-stat = 9.28 ($p<0.001$) [paired t test].

Table 2

mAgNOR scores in malignant breast lesions			
S. No	**Diagnosis**	**mAgNOR score**	
		FNA smears	**Tissue Sections**
Case 1	Infiltrating ductal carcinoma	4.9	2.0
Case 2		10.0	5.4
Case 3		4.3	5.5
Case 4		6.4	2.9
Case 5		6.2	4.6
Case 6		5.8	5.5
Case 7		6.5	4.8

Case 8		9.3	7.4
Case 9		1.1	5.1
Case 10	Intraductal carcinoma	5.2	4.8
Case 11	Non Hodgkin lymphoma	7.5	6.8
Case 12	Squamous cell carcinoma	11.2	-

Mean mAgNOR score: 6.53, S.D 2.73 (FNA smears); 4.98, S.D 1.53 (Tissue sections).
t-stat = 1.61 (p>0.1) [paired t test].
Benign Vs malignant lesions [unpaired/pooled t test]:
FNA smears t-stat = -5.23 (p<0.001); tissue sections t-stat = -5.57 (p<0.001).

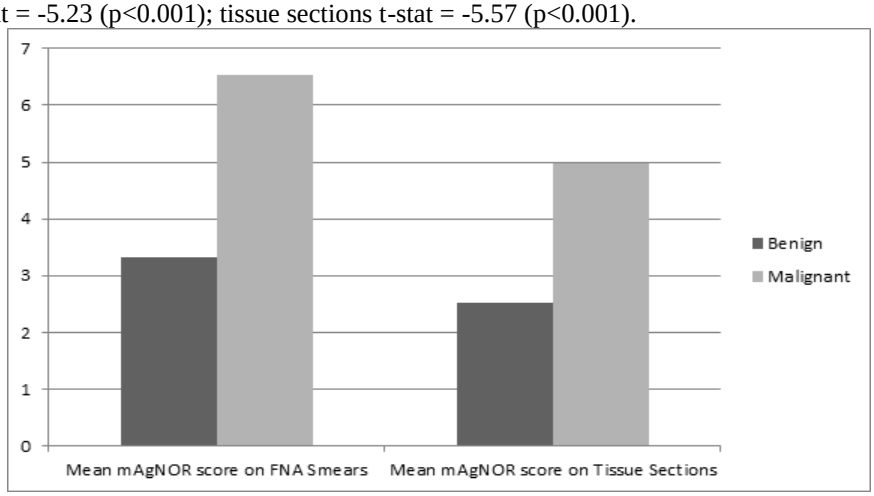

Fig. 1: Bar chart showing comparison of the mean mAgNOR scores between benign and malignant breast diseases

Table 3

Comparison of AgNOR scores as reported in various studies				
Study	**Mean mAgNOR(±S.D)**			
	FNA Smears		**Tissue Sections**	
	Benign	**Malignant**	**Benign**	**Malignant**
Smith and Crocker (1988)[8]			5.60±1.50	13.30±9.10
Giri et al (1989)[3]	2.80±1.50	4.40±1.20	1.87±0.20 (fibroadenoma), 2.21±0.30 (epitheliosis)	4.22±1.18 (infiltrating ductal carcinoma), 3.75±1.33 (intra ductal carcinoma)
Meehan et al (1994)[16]	4.44±2.0	9.52±2.20		
Agarwal et al (1995)[17]			3.40±1.02	9.10±1.40
Simha et al (1996)[9]			1.80 (excluding Phyllodes tumour)	3.5
Srivastava et al (1996)[10]	3.27±2.15	9.94±2.91		
Basu et al (1997)[11]	2.02±0.30 (fibroadenoma), 1.33±0.15 (fibroadenosis)	1.97±0.14	2.12±0.96 (fibroadenoma), 1.42±0.41 (fibroadenosis)	2.29±0.91
Dasgupta et al (1997)[18]	2	12.08	1.59 (fibroadenoma), 1.61 (fibrocystic disease)	12.10
Mehrotra et al (1998)[12]	3.08±0.75	7.10±1.54	3.20±1.03	8.50±1.11
Present study	3.33±1.49	6.53±2.73	2.53±1.22	4.98±1.53

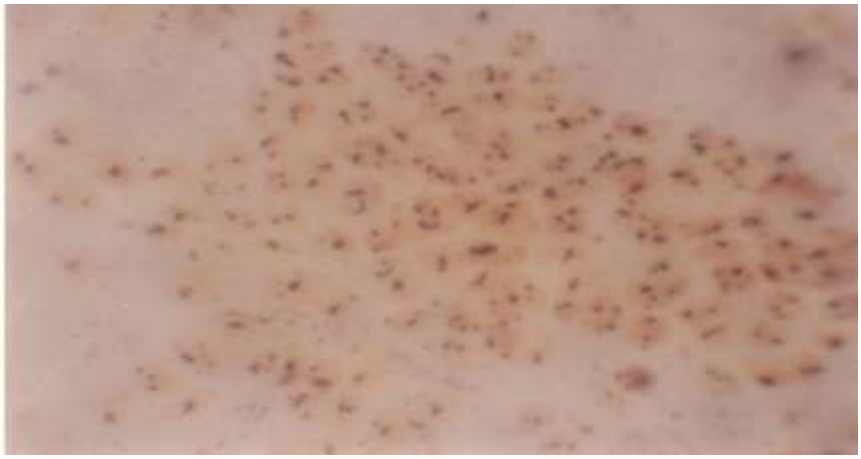

Fig. 2: Photomicrograph showing AgNORs on FNA smears in a case of fibroadenoma breast. Well rounded 2 to 5 dots per nuclear profile are seen (X400)

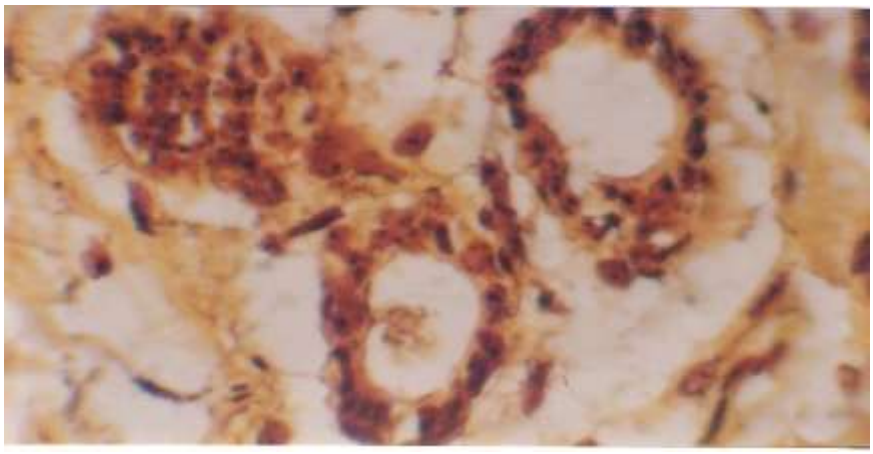

Fig. 3: Photomicrograph showing AgNORs on tissue section in fibroadenoma (X400)

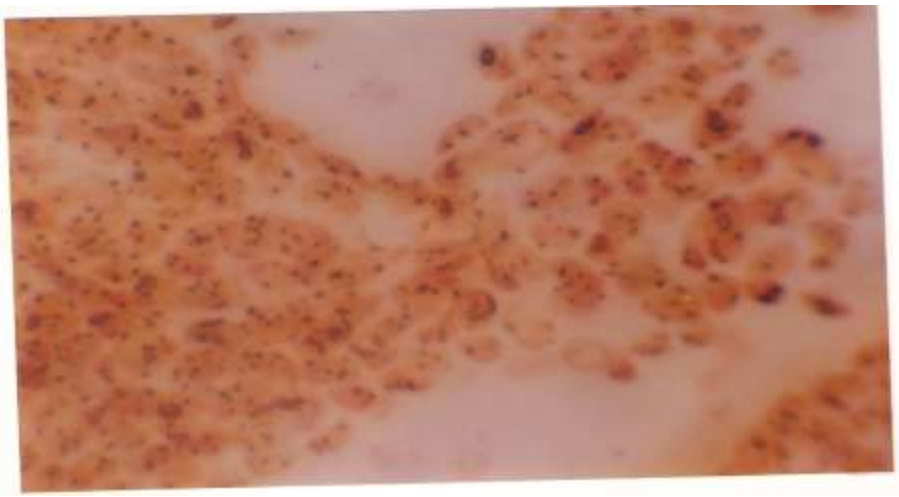

Fig. 4: Photomicrograph showing AgNORs on FNA smears in fibroadenosis. Well rounded, discrete 3 to 6 dots per nucleus are seen (X400)

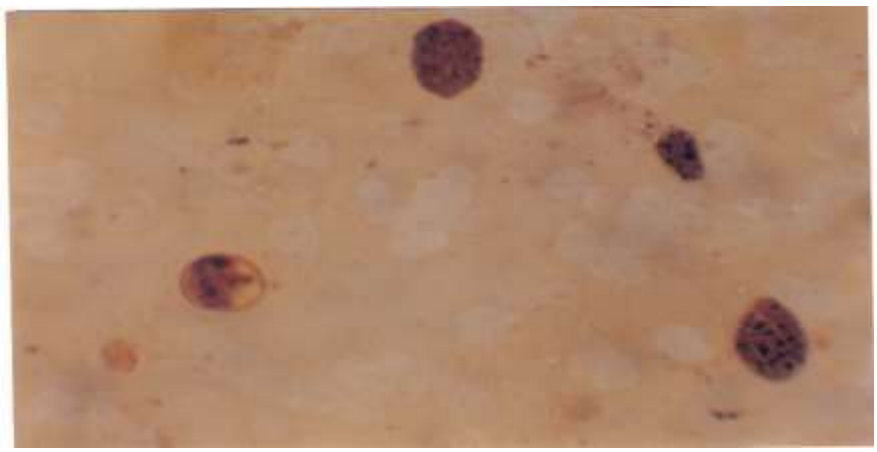

Fig. 5: Photomicrograph showing AgNORs on aspirates of carcinoma breast. Up to 10 to 12 irregularly dispersed dots are present per nucleus(X400)

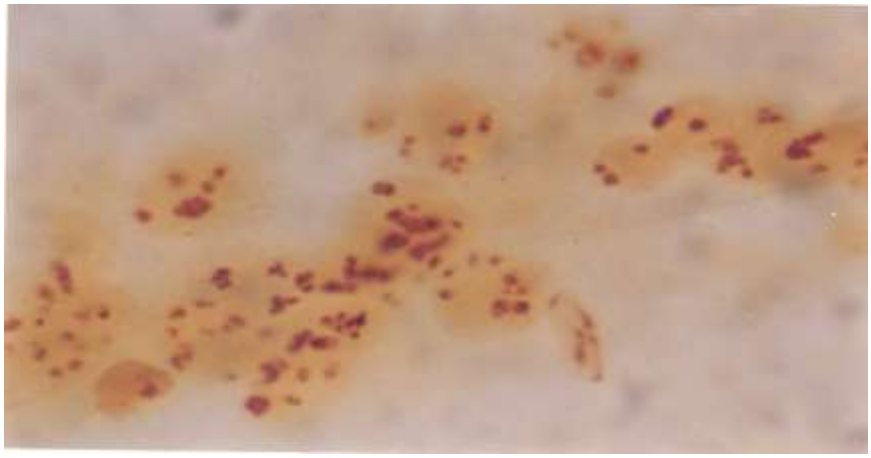

Fig. 6: Photomicrograph showing AgNORs on FNAC from a case of non-Hodgkin lymphoma breast. 3 to 8 dots are present per nucleus (X1000)

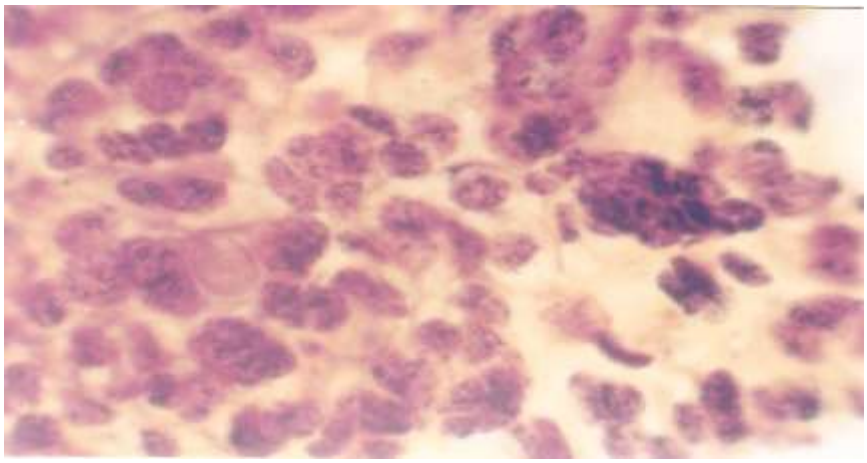

Fig. 7: Photomicrograph showing AgNORs on tissue section in a case of carcinoma breast. AgNORs are seen as large irregular clumps and blotches

Discussion

A quantifiable increase in the mean AgNOR count of cell population in tissue sections or FNA smears can result if:

- cell proliferation is so active that nucleolar dissociation is present in many cells,
- there is a defect of nucleolar association resulting in AgNOR dispersion,

- cell-ploidy increases resulting in an absolute increase in the number of NOR bearing chromosomes or
- transcriptional activity increases resulting in prominence of the otherwise inconspicuous NORs.[15]

In malignant lesions, the number of proliferating cells, transcriptional activity and in some cases cell-ploidy increase resulting in an increase in AgNOR counts compared to benign lesions.[3]

In the present study mAgNOR scoring was done on 38 benign and 12 malignant lesions of the breast. For benign lesions, significantly higher mAgNOR counts were observed on FNA smears than the corresponding tissue sections ($p<0.001$) which may be attributed to the fact that the cells on smears are in monolayers and air-drying displays the structure of the nucleus better as the cells are flattened. So the dots can be easily recognized and counted.[12] Though, the mAgNOR counts in tissue sections were slightly lower than those observed in the corresponding cytologic smears in malignant lesions too, this difference was not statistically significant ($p>0.01$).

The difference in mAgNOR scores between the benign and malignant breast diseases on aspiration smears as well as tissue sections was statistically highly significant ($p<0.001$). A broad range of overlap (4.3 to 8.0 on smears and 2.0 to 7.2 on sections) in mAgNOR scores of benign and malignant lesions was observed, thus precluding the use of AgNOR scoring as an independent, sole diagnostic criterion. But it can be a useful tool in certain defined conditions, for example, in the present study the mAgNOR score of 2.2 observed in sclerosing adenosis is much lower than the average mAgNOR count of 4.98 observed on tissue sections in malignant lesions of the breast and can be used to differentiate between sclerosing adenosis and carcinoma breast where the histological distinction is sometimes difficult.

Studies on AgNORs published in the literature show a wide variation in AgNOR scores on both cytological smears and histological sections (Table 3). This variation can be accounted for by the choice of the fixative used, length of incubation in silver nitrate solution, subjective impression of dot distinction and subjective variation in counting technique.[16] Heterogeneity of tumours with regard to cell proliferation and difficulty in control and standardization of section thickness can further enhance the disagreement in AgNOR counts.[17] However, majority of the studies are in agreement on the observation that AgNOR counts are significantly higher in malignant lesions of the breast in comparison to the benign lesions (Table 3). In carcinoma breast, AgNORs have been shown to correlate with tumour size, axillary lymph node status, tumour grade, S-phase fraction, mitoses, desmoplasia , ER, PR status and numerous other prognostic indicators.[9,17,19] Though, the enthusiasum for this technique has somewhat waned in recent years, judging from the marked drop in articles on the subject[20], the present study reiterates the utility of AgNORs as a simple and inexpensive method for analysis of cell proliferation in place of the more expensive and complicated procedures like flow cytometry, immunohistochemistry and thymidine labelling index. Therefore, one can safely conclude that the morphology and distribution of AgNORs in the nucleus when applied in conjunction with the mAgNOR scores can be a useful adjunct to diagnostic histopathology in predicting tumour progression and patient survival especially in a centre like ours where more sophisticated techniques like immunohistochemistry or flowcytometry are not yet fully established and are not performed routinely.

References

1. Lester SC. The Breast. In Kumar V, Abbas AK, Fausto N, Aster JC. Robbins and Cotran Pathologic Basis of Disease, 8th edition, Elsevier, 2010:1065-1096.
2. Murthy NS, Juneja A, Sehgal A, Prabhakar AK, Luthra UK. Cancer projection by the turn of the century – Indian Science. Ind J Cancer, 1990;27:74-82.
3. Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JCE. Silver binding nucleolar organizer regions (AgNORs) in benign and malignant breast lesions: Correlations with ploidy and growth phase by DNA flow cytometry. J Pathol, 1989;157:307-313.
4. Jan-Mohamed RM, Armstrong SJ, Crocker J, Leyland MJ, Hulten MA. The relationship between the number of interphase NORs and NOR bearing chromosomes in non-Hodgkin's lymphoma. J Pathol, 1989;158:3-7.
5. Crocker J, Skilbeck N. Nucleolar organizer region associated proteins in cutaneous melanotic lesions: A quantitative study. J Clin Pathol, 1987;40:885-889.
6. Goodpasture C, Bloom SE. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma, 1975;53:37-50.
7. Howell WM, Black DA. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: A one step method. Experientia, 1980;36:14-15.
8. Smith R, Crocker J. Evaluation of nucleolar organizer region associated proteins in breast malignancy. Histopathology, 1988;12:113-125.
9. Simha M, Menon M, Doctor V. Prognostic value of argyrophilic nucleolar organizer regions (AgNORs) in breast lesions. Ind J Cancer, 1996;33:76-85.
10. Srivastava A, Goel M, Gupta M, Agarwal PK, Tandon S, Misra RP. Comparison of AgNOR counts in fine needle aspirates and tissue sections of breast neoplasms. J Cytol, 1996;13:11-13.
11. Basu A, Sanyal S, Bhattacharyya A, Bhattacharyya S, Dasgupta S. A comparative study of silver binding nucleolar organizer regions (AgNORs) of breast lesions in histological sections and fine needle aspiration smears. J Indian Med Assoc, 1997;95:443-447.
12. Mehrotra A, Chandra T. Statistical significance of AgNOR counts in FNAC smears and corresponding histopathological sections. Ind J Exp Biol, 1998;36:162-166.
13. Bales CE. Laboratory Techniques. In Koss LG, Melamed MR eds. Koss' diagnostic cytology and its

histopathologic bases, 5th edition, Lippincott, Williams and Wilkins, 2006:1569-1634.
14. Williams I, Gamble M. The Haematoxylins and eosin. In Bancroft JD, Gamble M eds. Theory and practice of histological techniques, 5th edition, Churchill Livingstone, 2002:125-138.
15. Underwood JCE, Giri DD. Editorial: Nucleolar organizer regions as diagnostic discriminants for malignancy. J Pathol, 1988;155:95-96.
16. Meehan SM, Magee H, Carney DN, Dervan PA. The diagnostic value of silver nucleolar organizer region assessment in breast cytology. Am J Clin Pathol, 1994;101:689-693.
17. Agarwal PK, Mehrotra A, Chandra T. Diagnostic relevance of silver stained nucleolar organizer regions in benign and malignant breast lesions. Ind J Expt Biol, 1995;33:715-721.
18. Dasgupta A, Ghosh RN, Sarkar R, Laha RN, Ghosh TK, Mukherjee C. Argyrophilic nucleolar organizer regions (AgNORs) in breast lesions. J Indian Med Assoc, 1997;95:492-494.
19. Mourad WA, Erkman-Balis B, Livingston S, Shoukri M, Cox CE, Nicosia SV, Rowlands Jr DT. Argyrophilic nucleolar organizer regions in breast carcinoma. Correlation with DNA flow cytometry, histopathology and lymph node status. Cancer, 1992;69:1739-1744.
20. Pich A, Margaria E, ChiusaL. Significance of AgNORs in tumor pathology. Pathologica, 2002;94:2-9.