

Nucleolar organizer regions distribution in fine needle aspiration cytological smears from breast lesions

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Abstract

Fine needle aspiration (FNA) cytology is now an integral part of the pre-operative investigation of breast lesions and the therapeutic protocol is today often planned on the basis of cytodiagnosis. However, from time to time the cytological picture may be equivocal or inconclusive. In recent years, nucleolar organizer region (NOR) scores have been explored for potential value in the diagnosis of malignancy as the scores in malignant nuclei are seen to be higher than in benign or reactive nuclei.

With a view to applying NOR scoring in the evaluation of cytologically equivocal cases, we adopted the argyrophil technique for staining NORs (AgNOR) in FNA cytological smears of 56 breast lesions, comprising 31 benign and 25 malignant lesions. Histological correlation was possible in 26 of these cases (17 malignant and 9 benign) and AgNOR scoring was done on paraffin sections of these as well. There was a significant difference between mean AgNOR scores in benign and malignant lesions in the cytological smears ($P < 0.001$). The AgNOR scores ranged from 2.5 to 5.0 per cell in benign lesions and 5.8 to 17.2 per cell in malignant lesions. None of the cases fell into the gray zone of overlap. One malignant lesion that was cytologically equivocal showed a mean AgNOR score of 6.08. The AgNOR scores on histological sections also showed a statistically significant difference ($P < 0.001$) between benign and malignant lesions with mean scores ranging from 1.34 to 2.58 dots per cell in benign lesions and scores of 2.42 to 5.28 dots per cell in malignant lesions. However, the scores overlapped in four cases and therefore it was considered unsuitable for routine diagnostic work.

From this preliminary study, we conclude that an FNA AgNOR score of 5.0 and less strongly favours a benign lesion whereas a score above 5.0 would be in favour of a malignant lesion. A larger study would be needed to verify our impression that AgNOR scoring can be useful in cytologically equivocal cases.

Key words: Breast carcinoma, AgNORs, FNA cytology, histopathology.

INTRODUCTION

Fine needle aspiration (FNA) cytology of the breast has become an accepted diagnostic procedure throughout the world during the past few decades.¹⁻⁸ The procedure proved to be useful in the clinical management of patients including the institution of more effective and humane preoperative counselling.⁹ Although it carries a high degree of sensitivity (89%) and specificity (97%) in experienced hands,¹⁰ from time to time the cytologic picture may be equivocal, necessitating further work up before definitive therapy. Ever since cytogenetic studies showed ectopic nucleolar organizer regions (NOR) or unusual NOR patterns in certain malignancies^{11,12} pathologists have been excitedly exploring the potential of NOR estimation in the diagnosis of malignancy and in the differentiation of malignant from borderline lesions.¹³⁻²¹

NORs are loops of DNA projecting into the nucleoli of the interphase nuclei and they are thought to encode for ribosomal RNA. In the human karyotype, NORs are located on each of the short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22.²² It has been shown that the number of NORs within nuclei is significantly higher in malignant cells than in normal, reactive or benign neoplastic cells. A recently described one-step silver-staining technique, localizing proteins associated with NORs (AgNORs), made it possible for NORs to be demonstrated relatively easily in routinely processed histological sections and cytological smears.²³

There have been varied reports about the utility of AgNOR enumeration.²⁴⁻²⁶ Prognostic studies have indicated that counting and AgNOR morphology may provide information with regard to nodal metastases, supplementary to that obtained by established methodology.^{27,28} There

are, however, only scanty reports on AgNOR enumeration in FNA smears of breast lesions.^{29,30}

With the view of establishing the range of NOR in benign and malignant lesions and evaluating the role of AgNOR scoring in equivocal cases, we applied the AgNOR technique to breast aspirate smears.

MATERIALS AND METHODS

Fifty-six breast aspirates from 56 patients (31 benign and 25 malignant) were selected at random from the Breast clinic operating at the University Hospital, Kuala Lumpur. Of these, material for histological study was available in 9 benign and 17 malignant cases. 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 Grunwald Giemsa (MGG) and the other with AgNOR technique as follows : smears were hydrated in distilled deionized water before staining. The silver colloid staining solution was obtained by dissolving 2 gm/dl gelatin in 1% aqueous formic acid (1gm/dl). This was mixed with twice the volume of 50 gm/dl aqueous silver nitrate solution. Both the solutions could be made into stock solutions separately but the aqueous silver nitrate solution needed to be protected from any light source during storage. The stock solutions could last for at least one month duration. At each staining session, the solutions were mixed and poured into a Coplin jar that contained six to eight selected slides. The Coplin jar was immediately covered with a rolled black paper, to avoid exposure to sunlight and kept in a light-proof cupboard, at room temperature for 30-35 minutes which is the optimum time at which the dots were best visualized. The slides were then washed with distilled deionized water, taken through alcohol to xylene and mounted in DPX medium. No counterstaining was performed.

The AgNOR dots were counted at a magnification of X100 (objective lens) oil

immersion and 200 randomly selected epithelial cells were counted. Care was taken to avoid recounting the same field. A strict counting protocol as recommended was followed.³¹ In all smears, cells in well spread areas, where the morphology of the cells was best defined, were counted. The AgNOR count was taken as a total of the dots dispersed in the nucleoli and in the nucleoplasm. The clusters where resolution into smaller dots was not clear were taken as a single unit.

Material for histological study was processed routinely and paraffin sections were hydrated through ethanol to distilled deionized water and stained similarly for AgNOR. The difference between AgNOR scores of malignant and benign cells on aspiration smears and 3-µm thick sections was analysed using the student's t-test.

RESULTS

In all the specimens, well-defined black silver stained dots were observed in the nuclei on cytological smears. Overlapping of cells was seen in many areas, but errors were avoided by counting AgNORs at peripheries of clumps or in well-spread areas.

Range of breast lesions

Out of 31 benign breast lesions, 18 cases were diagnosed on cytology as fibroadenoma, 7 as fibrocystic condition, 4 cases as benign proliferative lesions and 2 as benign phyllodes tumour. Smears from fibroadenoma (FA) showed good cellularity with cohesive clusters of monomorphic epithelial cells, many bipolar nuclei (Fig. 1) and stromal fragments. Apocrine cells with abundant cytoplasm dominated the cytological picture in fibrocystic condition (FCC - Fig. 2). Proliferative lesions that lacked characteristic features of FA such as stromal fragments and numerous bipolar nuclei and features of FCC such as apocrine cells were

TABLE 1: Cytohistological correlation in 9 benign breast lesions

Histodiagnosis	No. of cases	Cytodiagnosis		
		Fibroadenoma	Phyllodes tumour	Fibrocystic change
Fibroadenoma	7	6	1	
Phyllodes tumour	1	—	1	—
Fibrocystic change	1	—		1

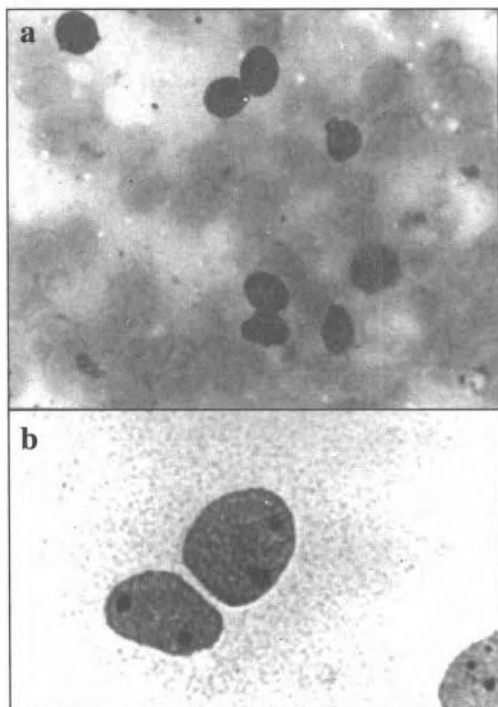


FIG. 1a: Smears from fibroadenoma showing bipolar nuclei (MGG \times 400).

1b: AgNOR staining of bipolar nuclei in fibroadenoma (AgNOR \times 1000)

labelled as benign proliferative lesion (BPL). The cytohistological correlation is given for 9 cases in which histological material was available (Table 1).

In 31 cases, a cytological diagnosis of carcinoma breast (No special type - NST) were made. Smears showed dissociated and clustered highly pleomorphic epithelial cells showing focal ductal pattern (Fig. 3). In 17 of these cases, histological material was available and showed infiltrating duct carcinoma (NST -Fig. 3). In one case where the cytological picture was equivocal, histological study showed infiltrating duct carcinoma (NST) with extensive intraductal carcinoma.

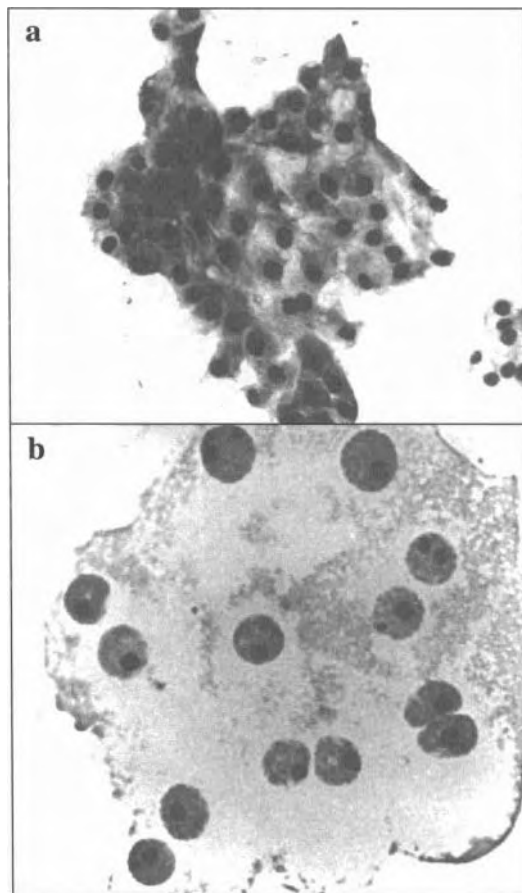


FIG. 2a: Cluster of apocrine cells in fibrocystic condition (MGG \times 300).

2b: AgNOR staining of apocrine cells showing 1 to 2 dots per nucleolus (AgNOR \times 1000)

AgNOR scores

The mean AgNOR scores in cytological smears of benign lesions ranged from 2.47 to 5.03 dots per cell with a mean of 3.82 and a standard deviation of \pm 0.58 (Fig. 1 and 3). The mean AgNOR score for malignant lesions ranged from 5.78 to 17.15 dots per cell with a mean of 9.42 (Fig. 4) and a standard deviation of \pm 3.16 (Table 2). The difference in AgNOR scores of

TABLE 2: Range, mean and standard deviation of AgNOR counts in cytological smears of benign and malignant breast lesions.

	Benign	Malignant
Range (dots per cell)	2.47 to 5.0	5.78 to 17.15
Mean	3.82	9.42
Standard deviation	0.58	3.16

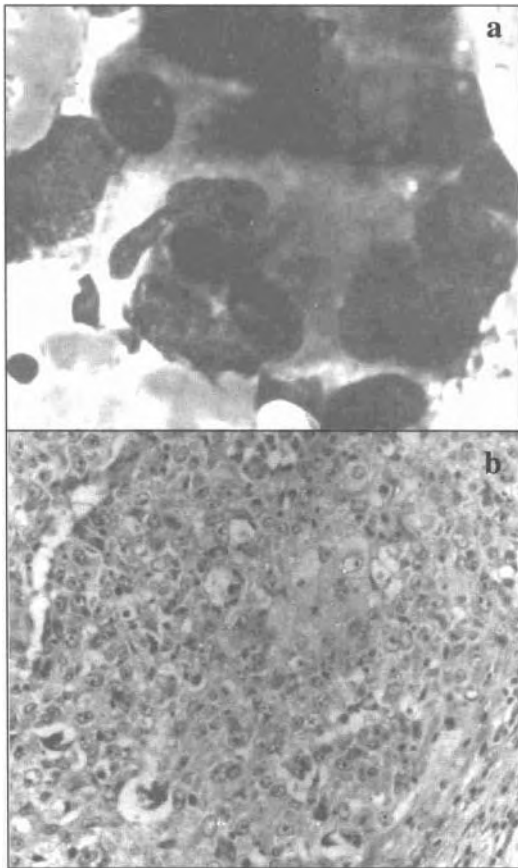


FIG. 3a: Clusters of pleomorphic malignant cells in FNA smear from infiltrating duct carcinoma of the breast (MGG \times 1000).

3b: Histological section from infiltrating duct carcinoma of the breast (H & E \times 400).

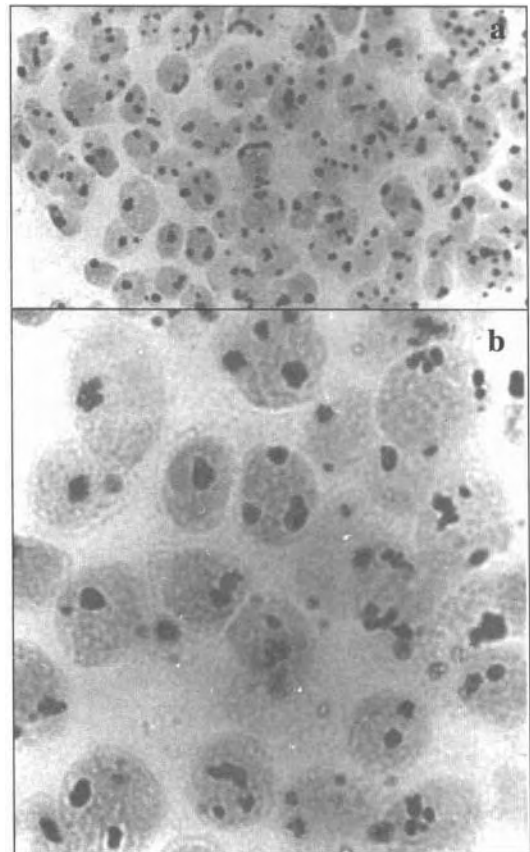


FIG. 4a: AgNOR staining in smear from a case of infiltrating ductal carcinoma of the breast (AgNOR \times 400)

4b: AgNOR staining in smear from a case of infiltrating ductal carcinoma of the breast (AgNOR \times 800).

benign and malignant breast lesions on cytological smears was statistically significant ($P < 0.001$).

The number of dots in histological sections from breast lesions was significantly lower than in cytological smears (Table 3). The dispersion within nucleoli could not be as well discerned on histology as in cytology. The benign lesions in histological sections had mean AgNOR scores ranging from 1.34 to 2.58 dots per cell (Fig. 5 and 6) whereas the malignant counterparts

scored from 2.42 to 5.28 dots per cell (Fig. 7). This difference was statistically significant ($P < 0.001$).

On the whole, the mean AgNOR score on histological sections was lower compared to that of cytological smears. The average score for benign and malignant breast lesions on histological sections was 2.12 and 4.12 respectively compared to the average score of 3.82 and 9.42 respectively on cytological smears. While AgNOR scores in

TABLE 3: Range, mean and standard deviation of AgNOR counts in histological sections of benign and malignant breast lesions

	Benign	Malignant
Range (dots per cell)	1.34 to 2.8	2.42 to 5.28
Mean	2.12	4.12
Standard deviation	0.52	0.82

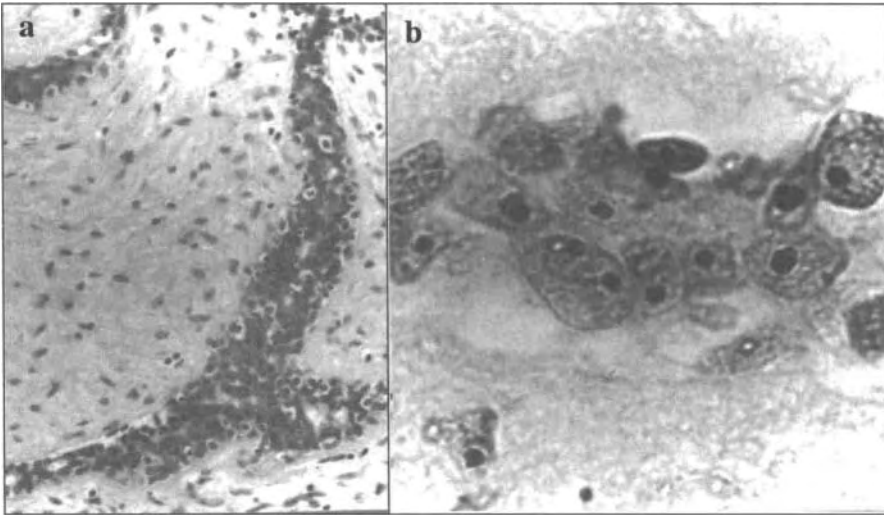


FIG. 5a: Section from fibroadenoma (H & E \times 400)

5b: AgNOR staining of a histological section from a fibroadenoma (AgNOR \times 1000)

cytological smears did not show any overlap between benign and malignant lesions, the scores in histological sections overlapped in 4 cases

DISCUSSION

In recent years, NORs have been claimed to be of use in differentiating malignant from benign conditions^{24,32} and predicting the grades of malignancy in some cases.³³ In the present study, it was found that the range of AgNOR scores in cytological smears of benign and malignant breast lesions showed a statistically significant difference (i.e. $p < 0.001$) with consistently lower scoring in benign lesions compared to their

malignant counterparts. Also, there was no overlap in that cases with AgNOR scores below 5.0 were benign while those with scores of 5.0 and above were malignant.

Misra and Kumar²⁹ found one case out of 45 that fell into the gray zone of overlap. That single case was noted to be a benign phyllodes tumour. In all other cases in that study, a cut-off score of 5 AgNOR dots on cytology smears was concluded as a value that differentiated benign from malignant breast lesions. Giri *et al.*,³⁰ enumerating AgNOR in FNA smears from 25 breast lesions, found overlapping values in one malignant and three benign cases. Although they felt that counts

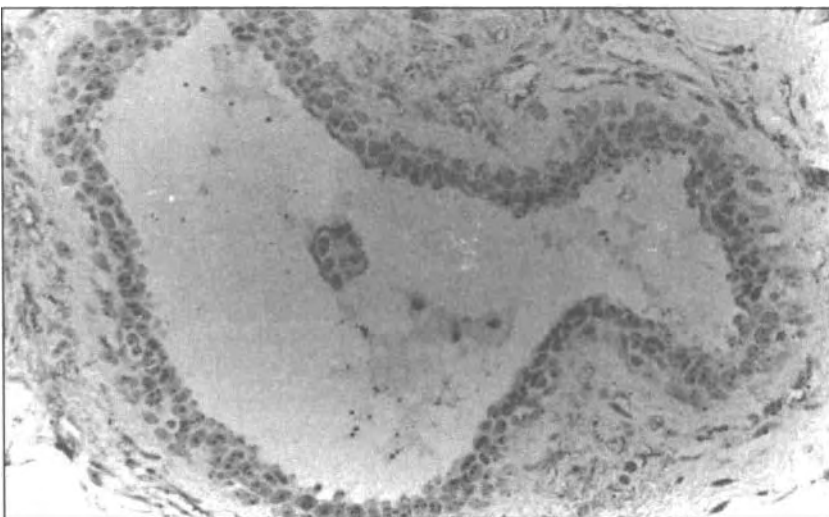


FIG. 6: AgNOR staining in section from a breast lesion showing fibrocystic change (AgNOR \times 400).

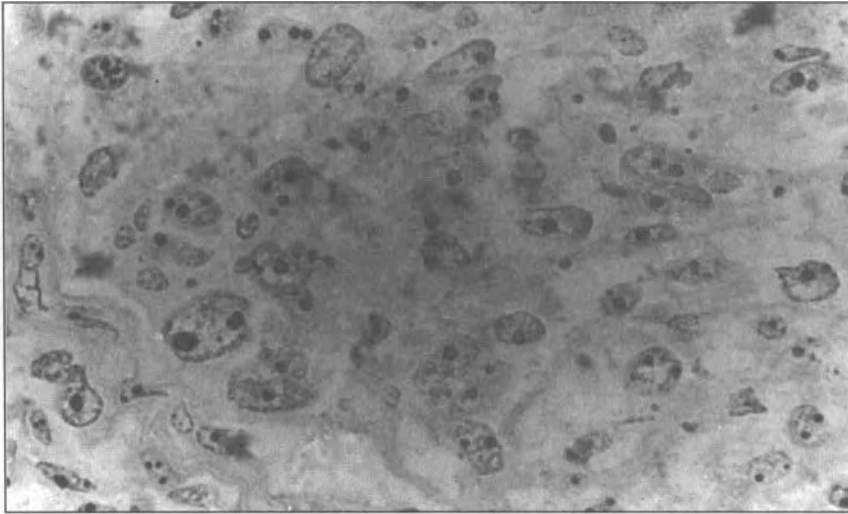


FIG. 7: AgNOR staining in histological section from a case of infiltrating duct carcinoma of the breast (AgNOR \times 800).

over 4.0 dots per cell were highly suggestive of malignancy, they concluded that the discrimination of benign from malignant breast lesions could not be done reliably using AgNOR technique and as such cannot be recommended for routine diagnostic work. In one of our cases showing equivocal cytologic features such as the presence of cell clusters with some lobular and papillary patterns with cells -showing mild pleomorphism and vacuolated cytoplasm, the AgNOR score was 6.08 and histopathology proved it to be an infiltrating duct carcinoma with extensive in-situ component. This case illustrates the potential value of AgNOR counting in equivocal cases.

AgNOR counts on histological sections were consistently lower as compared to those in cytological smears. This overall low count could possibly be due to an inability in distinguishing individual dots discretely in nucleoli of histological sections, resulting in many of them being counted as one in a single nucleolus.

Even though the mean AgNOR scores in benign and malignant breast lesions in histological sections showed a statistically significant difference ($P < 0.001$), 1 malignant and 3 benign cases fell into the gray zone of overlap; thus AgNOR counting of the breast lesions in histological sections probably cannot be advocated for routine work.

Errors in the enumeration of AgNOR can occur and the intra- and inter-observer error (i.e. percentage difference between mean counts) lies, in published series, between 2 and 7 per cent.³¹

This is acceptable for most tissues or tumours and may, in part, be the result of heterogeneity of cell populations. Counting of cells such as histiocytes (which have low AgNOR counts) or lymphoid cells (which not uncommonly infiltrate malignant tumours and which usually have two AgNOR dots in the resting state) can adversely affect the results. The second factor of importance in this context is that of section thickness. While thick sections may contain all NOR profiles their scoring may be difficult owing to the section thickness. On the other hand, in thin sections where NORs can readily be separated, many are lost. A section thickness of 3- μ m has been suggested as a compromise, although the loss of AgNORs in cells with multiple sites from 3- μ m sections may 'compress' data between high- and low-count specimens.³¹

Counting of discrete AgNORs within a nucleolus as one by some observers and as multiple by others further contributes to discrepancy. Although by differential focussing many of the discrete AgNORs can be discerned, this is much easier in cytological smears. The simplicity and superiority of staining of cytological smears and its potential role in cytologically equivocal cases makes AgNOR staining of aspiration smears a valuable asset in the evaluation of breast lesions.

In conclusion, the present study indicates that in FNA cytologic smears of breast lesions, an AgNOR score of 5.0 and less strongly favours a benign lesion whereas a score of above 5.0 would be in favour of a malignant lesion. However a study with a larger number of cases would be

desirable to confirm our preliminary findings

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