VALUE OF AgNOR PARAMETERS IN EVALUATION OF EPITHELIAL BREAST LESIONS ON CYTOLOGY

Pathology

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ABSTRACT

Introduction: Fine needle aspiration cytology (FNAC) is traditionally been used as a component of triple assessment tool for evaluation of breast lesions. FNAC being done as a simple outpatient procedure offers a rapid and safe diagnostic approach for breast lesions. The aim of the present study is to assess the value of AgNOR parameters in cytology smears for differentiating benign proliferative and non-proliferative lesions from malignant lesions of breast. And also to correlate the value of AgNOR parameters with Robinson's cytological scoring system for malignant breast lesions.

Materials and Methods: The present prospective study was conducted at the Cytology Laboratory of the Department of Pathology, Tirunelveli Medical College for 1 year duration. 100 cases with breast lump were randomly chosen and aspirated smears were stained with Hematoxylin & Eosin and AgNOR stains. The AgNOR parameters were then evaluated.

Results: All the AgNOR parameters showed statistically significant increase in values progressively from non-proliferative benign breast lesions to proliferative breast lesions and then to malignant breast lesions.

Conclusion: AgNOR parameters are of great help in differentiating benign from malignant neoplasm and also in differentiating proliferative vs non-proliferative lesions of breast. Mean AgNOR score and SAPA score were found to be more superior in comparison to other AgNOR parameters.

KEYWORDS

AgNOR, Fine needle aspiration cytology, Benign and Malignant breast lesions.

INTRODUCTION

Breast carcinoma is one of the most common non-skin malignancies and is the leading cause of death in women, representing approximately 25% of all cancers with 1.67 million new breast cancer cases diagnosed yearly and with a mortality rate of 12.7%¹. However, non-cancerous conditions of the breast are far more common than carcinomas. The incidence of female breast cancer is rising rapidly between the age group of 35 to 50 years worldwide. Invasive ductal carcinoma comprises the largest group of malignancy constituting about 40% - 75% of all breast carcinomas².

The advent of the medical science has conquered many of the infectious diseases that formerly destroyed large populations of the mankind, but cancer still remains as an unconquerable disease of the non-epidemic kind. It's ironic and tragic that lesions arising from the breast, readily accessible to self-examination and clinical diagnosis, continue to take a heavy toll on the female population. Clinical examination, mammographic imaging studies and fine needle aspiration cytology (FNAC) have traditionally been used as a triple assessment tool for evaluation of breast lesions³. Among these the application of FNAC undoubtedly has a major influence on the diagnosis of breast lesions.

FNAC offers a rapid and safe diagnostic approach for breast lesions. Even then the lesions of the breast pose various diagnostic difficulties in distinguishing benign from malignant lesions. Since most of the malignant neoplasm show increased proliferative activity, research had been directed towards finding a reliable proliferative marker. Nucleolar Organizer Regions (NOR's) forms one of the earliest proliferation marker discovered and utilised by Ploton et al⁴ to distinguish benign from malignant neoplasm, the latter showing more abundant NORs. The AgNOR staining is quiet simple and easily affordable compared to other expensive and complex procedures like enumeration of S phase fraction (SPF), Proliferating Cell Nuclear Antigen (PCNA), Ki - 67 index and MIB - 1 index⁵.

"Earlier the diagnosis of the lesion better is the prognosis". Having this concept in our mind, our present study has been conducted to assess the accuracy of AgNOR as a proliferation marker in differentiating benign and malignant epithelial breast lesions on FNAC.

MATERIALS AND METHODS

This prospective study was conducted at the Cytology Laboratory of the Department of Pathology, Tirunelveli Medical College spanning a period of about 12 months. Out of the total 423 breast cytology cases 100 cases were randomly chosen taking into account 40 malignant and 60 benign cases encompassing both proliferative (31 cases) and non-

proliferative lesions (29 cases).

FNAC was performed on all patients who presented with palpable breast lesions. The material aspirated was expressed on to glass slides, smeared and fixed immediately in 95% ethanol. The slides were stained subsequently with Hematoxylin and Eosin (H&E) and AgNOR stain

Cytological grading of breast carcinomas was done according to the Robinson et al⁶. grading system. These 100 smears stained with AgNOR stain were examined meticulously for number and morphology of AgNOR dots within the nucleus. AgNORs are visualised as blackish or brown dots in a pale yellow background, both in the nucleolus and within the nucleoplasm.

There enumeration and analysis of AgNOR parameters based on their count, morphology and distribution were performed on all the smears and the observations were recorded. The AgNOR parameters examined include

- Mean AgNOR count
- AgNOR proliferative index
- AgNOR size variation grading
- AgNOR distribution in the nuclei
- Subjective AgNOR Pattern Assessment (SAPA).

Mean AgNOR Count:

The number of AgNORs within the nuclei of 100 neoplastic cells is counted using a 100X objective. The mean numbers of NORs per nucleus were then calculated and results were expressed as a mean count $\pm$ Standard Deviation.

Proliferative AgNOR Index (pAgNOR):

pAgNOR is the percentage of neoplastic cells showing more than five AgNOR dots within the nucleus of neoplastic cells.

Distribution And Size Variation Of AgNORs⁷:

The size variation and the distribution of the AgNORs within the nucleus are evaluated and given a score of 0, 1+, 2+ & 3+ according to the criteria given in Table 1 and 2.

Table 1. AgNORs Size Variation Grading

AgNOR Size Variation	Score
More or less uniform	0
Two different sizes	1+
More than two different sizes (but not those of 3+)	2+
All grades and sizes including too minute to be counted	3+

Table 2. AgNOR Distribution In The Nuclei

AgNOR distribution - nuclei	Score
Limited to nucleoli	0
Occasional dispersion outside nucleoli	1+
Moderate dispersion outside nucleoli	2+
Widely dispersed throughout the nucleus	3+

Subjective AgNOR Pattern Assessment (SAPA)*:

The SAPA scoring of the AgNORs are done based upon the criteria given in Table 3.

Table 3. Subjective AgNOR Pattern Assessment

Parameters	Score
Estimated number per cell	
Few (<5)	1
Several (5-10)	2
Many (>10)	3
Variation satellite shape and size (Score each)	
Uniform	1
Moderate variation	2
Marked variation	3
Variation cluster shape and size (Score each)	
Uniform	1
Moderate variation	2
Marked variation	3

Correlation study between the Robinson's grading system and the AgNOR scoring system for grading malignant neoplasm of breast was conducted. Descriptive analysis was carried out by mean and standard deviation for quantitative variables, frequency and proportion for categorical variables, P-values are generated using SPSS software version.

OBSERVATION AND RESULTS

The H&E staining along with AgNOR staining and enumeration was performed on 100 randomly selected cases which revealed mean AgNOR score of 3.55 in benign lesions (Fig. 1, 2), while mean AgNOR was higher in malignant lesions with a score of 7.35 (Fig. 3-5). All the AgNOR parameters showed statistically significant higher values in malignant lesions compared to the benign ones (P value < 0.05 – significant, *0.001 - Highly significant value). These findings are tabulated in table 4.

Figure: 1 A) Aspirate of fibrocystic disease showing honeycombing of uniform ductal epithelial cells (H&E, 100X). B) Fibrocystic change of breast showing uniform duct epithelial cell clusters (H&E, 400X). C&D) Uniform duct epithelial cell clusters in honeycomb arrangement showing only a few AgNOR dots (AgNOR, 400X)

Figure: 2 A) Large sheets of mildly atypical duct epithelial cells with holes indicating a cribriform pattern (H&E, 100X) B) Finger like papillary structures seen in intraductal papilloma of breast (H&E, 400X). C&D) Atypical hyperplastic duct epithelial cell cluster of breast showing 4-5 AgNOR dots per nucleus (AgNOR, 400X)

Table 4. AgNOR Score Of Benign Vs Malignant Breast Lesions

AgNOR parameters	Benign lesions			Malignant lesions			P-value
	Range	Mean	S.D.	Range	Mean	S.D.	
Mean AgNOR	1.67-6.21	3.55	+/-1.32	5.8-9.5	7.35	+/-0.82	<0.001*
p AgNOR	3 - 51	23.6	+/-13.06	40 - 71	50.88	+/-6.60	<0.001*
AgNOR size variation	0 - 2	0.7	+/-0.646	2 - 3	2.25	+/-0.44	<0.001*
AgNOR Distribution	0 - 2	0.72	+/-0.613	2 - 3	2.25	+/-0.44	<0.001*
SAPA score	3 - 5	3.72	+/-0.783	5 - 8	6.7	+/-0.88	<0.001*

Figure: 3 A&B) Cellular smear showing dyscohesive clusters of epithelial cells with mild cellular pleomorphism and nuclear atypia - Grade I (H&E, 400X). C&D) Dyscohesive clusters of pleomorphic duct epithelial cells showing increased number of AgNORs (AgNOR, 400X)

Out of 60 randomly chosen benign lesions 29 were non-proliferative and 31 were proliferative lesions. The proliferative lesions encompassed epithelial hyperplasia, intra ductal papilloma and atypical ductal hyperplasia. The non-proliferative benign lesions encompassed fibrocystic disease / fibroadenosis. These AgNOR values showed a statistically significant correlation with regard to proliferative and non-proliferative lesions of the breast (table 5).

Figure: 4 A&B) Dyscohesive clusters of epithelial cells with moderate nuclear enlargement & atypia - Grade II (H&E, 400X). C&D) Clusters of duct epithelial cells with moderate nuclear pleomorphism & atypia showing higher number of AgNORs per nucleus (AgNOR, 400X)

Table 5. AgNOR Score Of Non-proliferative Vs Proliferative Breast Lesions

AgNOR parameters	Non-proliferative			Proliferative lesions			P-value
	Range	Mean	S.D.	Range	Mean	S.D.	
Mean AgNOR	1.67-3.35	2.38	+/-0.55	2.78-6.21	4.65	+/- 0.77	<0.001*
p AgNOR	3 - 25	12.41	+/-6.42	18 - 51	30.07	+/- 7.93	<0.001*
AgNOR size variation	0 - 1	0.17	+/-0.38	1 - 2	1.19	+/- 0.40	<0.001*
AgNOR Distribution	0 - 1	0.24	+/-0.44	1 - 2	1.16	+/- 0.37	<0.001*
SAPA score	3 - 4	3.03	+/-0.19	3 - 5	4.36	+/- 0.55	<0.001*

Table 7. Robinson's Score Vs AgNOR Parameters

No of cases	Robinson Grade	Robinson's Score			Mean AgNOR			P-value
		Range	Mean	S.D.	Range	Mean	S.D.	
8	I	7-10	8.88	+/-1.13	5.8-7.4	6.57	+/-0.65	<0.001*
26	II	12-14	12.96	+/-0.77	6-8.7	7.37	+/-0.66	<0.001*
6	III	15-17	16.17	+/-0.75	7.9-9.8	8.32	+/-0.65	<0.001*
No of cases	Robinson Grade	Robinson's Score			pAgNOR			P-value
		Range	Mean	S.D.	Range	Mean	S.D.	
8	I	7 - 10	8.88	+/-1.13	40 - 52	44.5	+/-4.47	<0.001*
26	II	12-14	12.96	+/-0.77	41 - 62	51.1	+/-4.89	0.141
6	III	15-17	16.17	+/-0.75	49 - 71	58.3	+/-7.76	<0.001*
No of cases	Robinson Grade	Robinson's Score			SAPA score			P-value
		Range	Mean	S.D.	Range	Mean	S.D.	
8	I	7 - 10	8.88	+/-1.13	5 - 7	5.75	+/-0.71	<0.001*
26	II	12-14	12.96	+/-0.77	6 - 8	6.73	+/-0.37	<0.001*
6	III	15-17	16.17	+/-0.75	7 - 8	7.83	+/- 0.38	<0.001*

Figure: 5 A&B) Highly dyscohesive clusters of duct epithelial cells showing obvious nuclear enlargement & overlapping - Grade III (H&E, 400X). C&D) Highly dyscohesive duct epithelial cell clusters showing numerous AgNORs per nucleus (AgNOR, 400X).

Out of the 31 benign proliferative epithelial breast lesions, there was a gradual and steady increase in AgNOR values from epithelial hyperplasia (18 cases) to atypical ductal hyperplasia (11 cases) and then to intra ductal papilloma (2 cases). But the differences were very subtle and there was overlap between AgNOR counts with regard to individual values of these lesions. Only the mAgNOR and pAgNOR values were found to be statistically significant in differentiating various subtypes of benign proliferative epithelial breast lesions (Table 6).

Table 6. AgNOR Score Of Proliferative Epithelial Breast Lesions

AgNOR parameters	Epithelial Hyperplasia		Atypical Ductal Hyperplasia		Intra Ductal Papilloma		P-value
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Mean AgNOR	4.42	+/-0.84	4.90	+/-0.52	5.35	+/-0.69	<0.001*
p AgNOR	31.72	+/-8.50	36.73	+/-6.28	40.5	+/-2.12	<0.001*
AgNOR size variation	1.16	+/-0.38	1.18	+/-0.41	1.5	+/-0.71	<0.05
AgNOR Distribution	1.17	+/-0.39	1.19	+/-0.40	1	+/-0.21	0.052
SAPA score	4.33	+/-0.49	4.36	+/-0.67	4.5	+/-0.71	0.542

The mean AgNOR and SAPA score showed a gradual increase in correlation with the Robinson's grade for malignant neoplasms of breast and were statistically significant. Although proliferative AgNOR index score showed proportional increase with increasing grades of the breast carcinomas, these were significant statistically only for grade I and grade III tumours and not for grade II tumours. Likewise there was an increased variation in the size and distribution of the AgNOR dots as the Robinson's cytological grade of the malignant breast neoplasm increased. Correlation between the Robinson's score and AgNOR parameters in 40 cases of malignant breast neoplasm are tabulated in table 7.

DISCUSSION

In our study the frequency, number and dispersion of AgNOR dots are consistently higher in malignant neoplasm compared to the benign neoplasm of breast. In this regard AgNORs have attracted much attention as a proliferative marker and as an indicator for aggressiveness of the lesion and has a potential value in diagnostic cytology.

Benign Breast Lesions:

Simha M et al⁹, Dasgupta A et al¹⁰, Kumar A et al¹¹ reported mean AgNOR count of 1.8, 1.61 and 1.88 respectively for benign breast lesions. In our study mAgNOR count for benign neoplasm was 3.55, with values ranging from 1.67 to 6.21 and a SAPA score of 3.72. Among the 60 cases of benign lesions 29 cases of non-proliferative breast lesions showed a mAgNOR count of 2.38 with values ranging from 1.67 to 3.35 and a SAPA score of 3.03, the remaining 31 cases of proliferative breast lesions showed a higher mAgNOR score of 4.65 with values ranging from 2.78 to 6.21 and a SAPA score of 4.35. All the AgNOR parameters showed a higher AgNOR values in proliferative lesions compared to non-proliferative lesions of breast.

Among the proliferative breast lesions epithelial hyperplasia showed a mAgNOR value of 4.42 and a SAPA score of 4.33, atypical ductal hyperplasia showed mAgNOR value of 4.90 and a SAPA score of 4.36 and intraductal papilloma showed mAgNOR value of 5.35 with a SAPA score of 4.5. The variations in AgNOR values between the different subtypes of proliferative breast lesions are very subtle. The mAgNOR and pAgNOR values were only found to be of significance in differentiating subtypes of benign proliferative epithelial breast lesions on cytology, while AgNOR size variation, pattern of AgNOR distribution within nucleus and SAPA score are not found to be of much significance.

Malignant Breast Lesions:

Kim I S et al¹², Kumar A et al¹¹ and Dasgupta A et al¹⁰ reported a mAgNOR count of 5.09, 6.57 and 12.10 respectively for malignant breast lesions. In our study malignant breast neoplasms showed a mAgNOR count of 7.35 with individual values ranging from 5.8 to 9.54 and a SAPA score of 6.7. Among 40 malignant lesions grade I tumours showed mAgNOR value of 6.57 with SAPA score of 5.75, grade II tumours showed mAgNOR value of 7.37 with SAPA score of 6.73 and grade III tumours showed mAgNOR value of 8.32 with a SAPA score of 7.83. There was a steady increase in mAgNOR values and SAPA score in concordance with Robinson's cytological grade of the lesions. All the other AgNOR parameters also showed a gradual, steady and significant increase in the values as the Robinson's cytological grade of the tumour progressed higher.

Malignant vs Benign Lesions:

Simha M et al⁹, came out with the finding that AgNOR values are much higher in malignant neoplasm compared to the benign ones. Dasgupta A et al¹⁰ and Hasnan J et al¹³ had similar findings with higher AgNOR counts for malignant neoplasm of breast compared to benign neoplasm, they also observed that there was no overlap between the AgNOR values of benign and malignant lesions of breast. Ansari H A et al¹⁴ observed a mAgNOR & pAgNOR values of 2.9 & 14% for benign breast lesions and 4.0 & 36.6% for malignant breast lesions respectively.

Madhavi, B. V et al¹⁵ on assessing 70 breast lesions found a mAgNOR count of 2.45 for benign and mAgNOR count of 4.76 for malignant breast lesions. Khanna AK et al⁸ assessed 27 benign and 46 malignant breast lesions by using two parameters both Subjective AgNOR Pattern Assessment (SAPA) and mAgNOR count. They found that mAgNOR count and SAPA were quite similar in differentiating benign from malignant lesions.

Our present study showed a higher mAgNOR, pAgNOR and SAPA scores for malignant neoplasm compared to lower mAgNOR, pAgNOR and SAPA scores for benign neoplasm of breast. Our study also showed a considerable variation in the AgNOR size and distribution within nucleus for malignant neoplasm compared to a meagre variation in the AgNOR size and distribution for the benign neoplasm of breast.

In a study by Dhakhwa R et al¹⁶ on 110 breast lumps they observed that if the cut off score for AgNOR count is taken as 6 then the specificity is 88.9% and sensitivity is 89.5%, if the cut off value for SAPA score is taken as 8 then the specificity is 83.3% and sensitivity is 89.5% for

differentiating benign from malignant neoplasms. From our study we observed that a mAgNOR cut off value of 6 and SAPA score cut off value of 5 can be effectively utilised in differentiating benign from malignant neoplasm of the breast with a sensitivity of 92.5% and specificity of 90%.

There was a considerable variation in the absolute value of mAgNOR counts by different studies. These can be attributed to the fact that different authors' count AgNOR dots differently, some authors count clustered dots as a single dot when individual NORs could not be easily discerned, while others leave off such cells where NORs could not be easily discerned. These variation could have also stemmed up from the fact that there was a lack of standardisation in the counting of AgNOR dots and also due to inter observer variability. This explains for the variation in these values. Crocker et al¹⁷ recommended the counting of 100 cells as a standardised approach to AgNOR dot enumeration and this was followed in our study.

CONCLUSION

FNAC as a preoperative diagnosis is primarily aimed at distinguishing benign from malignant neoplasm. But pathologists are sometimes fraught with diagnostic dilemmas in cytology. In such difficult situations, AgNOR as a proliferative marker is of great help in differentiating benign from malignant neoplasm and also in differentiating proliferative vs non-proliferative lesions of breast. Mean AgNOR score and SAPA score were found to be more superior compared to other AgNOR parameters and provide additional information in cases that provide diagnostic difficulty in routine FNAC. SAPA score was more reproducible compared to mAgNOR count. AgNOR staining being a simple one step silver staining technique can be reliably and effectively utilised to differentiate the above lesions of breast. As a cell proliferative marker AgNOR count and SAPA score represent proliferating cellular activity and hence predict the biological behaviour of the breast neoplasm.

REFERENCES

1. Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., ... & Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer*, 136(5), E359-E386.
2. Makki, J. (2015). Diversity of breast carcinoma: histological subtypes and clinical relevance. *Clinical Medicine Insights: Pathology*, 8, CPath-S31563.
3. Ogbuanyia, A. U. O., Anyanwu, S. N., Iyare, E. F., & Nwigwe, C. G. (2020). The role of fine needle aspiration cytology in triple assessment of patients with malignant breast lumps. *Nigerian Journal of Surgery: Official Publication of the Nigerian Surgical Research Society*, 26(1), 35.
4. Ploton, D., Menager, M., Jeannesson, P., Himber, G., Pigeon, F., & Adnet, J. J. (1986). Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. *The Histochemical Journal*, 18(1), 5-14.
5. Van Diest, P. J., Brugal, G., & Baak, J. P. (1998). Proliferation markers in tumours: interpretation and clinical value. *Journal of clinical pathology*, 51(10), 716.
6. Robinson, I. A., McKee, G., Nicholson, A., Jackson, P. A., Cook, M. G., D'Arcy, J., & Kissin, M. W. (1994). Prognostic value of cytological grading of fine-needle aspirates from breast carcinomas. *The Lancet*, 343(8903), 947-949.
7. Parveen, S., Mehboob, R., Khan, A. A., Bukhari, M. H., & Ahmad, F. J. (2013). Novel strategy to diagnose and grade hepatocellular carcinoma.
8. Khanna, A. K., Ansari, M. A., Kumar, M., & Khanna, A. (2001). Correlation between AgNOR count and subjective AgNOR pattern assessment score in cytology and histology of breast lumps. *Analytical and quantitative cytology and histology*, 23(6), 388-394.
9. Simha, M., Menon, M., & Doctor, V. (1996). Prognostic value of argyrophilic nucleolar organiser regions (AgNORs) in breast lesions. *Indian Journal of Cancer*, 33(2), 76-85.
10. Dasgupta, A., Ghosh, R. N., Sarkar, R., Laha, R. N., Ghosh, T. K., & Mukherjee, C. (1997). Argyrophilic nucleolar organiser regions (AgNORs) in breast lesions. *Journal of the Indian Medical Association*, 95(9), 492-494.
11. Kumar, A., Kushwaha, A. K., Kumar, M., & Gupta, S. (1997). Argyrophilic nucleolar organiser regions: their value and correlation with clinical prognostic factors in breast carcinoma. *Journal of surgical oncology*, 65(3), 201-204.
12. Kim, I. S., Kim, Y. S., & Kim, A. (1994). Quantitation of AgNORs in Breast Lesions Using Image Analysis. *Journal of the Korean Cancer Association*, 26(5), 756-764.
13. Hasnan, J., & Jayaram, G. (1996). Nucleolar organizer regions distribution in fine needle aspiration cytological smears from breast lesions. *Malaysian Journal of Pathology*, 18, 35-42.
14. Ansari, H. A., Mehdi, G., Maheshwari, V., & Siddiqui, S. A. (2008). Evaluation of AgNOR scores in aspiration cytology smears of breast tumors. *Journal of Cytology*, 25(3), 100.
15. Madhavi, B. V., & Ravishankar, B. (2016). Study of AgNOR counts in cytological smears of breast lesions. *Indian Journal of Basic and Applied Medical Research*, 5(4), 380-386.
16. Dhakhwa, R., Jha, R., Sayami, G., & Shrestha, H. G. (2012). Study of AgNOR count and SAPA score in fine needle aspirates of breast lumps. *Journal of Kathmandu Medical College*, 1(1), pp.3-9.
17. Crocker, J., Boldy, D. A., & Egan, M. J. (1989). How should we count AgNORs? Proposals for a standardized approach. *The Journal of pathology*, 158(3), 185-188.