

A STUDY OF SILVER STAINING NUCLEOLAR ORGANISING REGIONS AGNOR SCORE AS PROGNOSTIC MARKER IN BREAST LESIONS IN A TERTIARY CARE HOSPITAL IN HYDERABAD

Bhavani Shankar Nithyananda¹, Bheeshma Byram², Fatima Anjum³, Swathi Cheruku⁴

¹Associate Professor, Department of Pathology, Osmania Medical College, Hyderabad.

²Professor, Department of Pathology, Modern Government Maternity Hospital, Hyderabad.

³Senior Resident, Department of Pathology, Modern Government Maternity Hospital, Hyderabad.

⁴Senior Resident, Department of Pathology, Modern Government Maternity Hospital, Hyderabad.

ABSTRACT

BACKGROUND

Nucleolar Organising Regions (NORS) are segments of DNA closely associated with nucleolus, which code for ribosomal DNA consisting of non-histone proteins, which are argyrophilic. They are demonstrated as black, brown spots on staining with silver colloidal staining technique. Hence, they are called AgNORs. The assessment of AgNORs correlates with rate of proliferation, cell cycle time, since shorter the cell cycle, greater the ribosomal activity and protein synthesis higher AgNOR count.¹ AgNORs have been studied on NHL, GIT neoplasms, skin, gynaecological neoplasms, pulmonary neoplasms, prostate, bladder and breast cancer. In breast cancer, AgNOR can be used for early detection, grading and staging of disease.

In this study, we have tried to correlate AgNOR character to morphological prognostic markers.

MATERIALS AND METHODS

A total of 159 cases presenting with lump in the breast were included in the study. Specimens were subjected to histopathological examination. Sections cut three micron thickness were stained for AgNOR with silver colloidal AgNOR staining technique. AgNOR count was done on both benign and malignant lesions.

RESULTS

Benign breast lesions were more common compared to malignant lesions. The age of the patients ranged from 15-79 yrs. The mean age for benign lesions was 31 yrs. and for malignant lesions 43 yrs. The most common benign lesion was fibroadenoma and most common malignant lesion was infiltrating ductal carcinoma. AgNOR spots were seen as black/brown spots. AgNOR score was higher in malignant lesions when compared to benign lesions. Among malignant lesions, AgNOR score was found to be higher in high grade, metastatic tumours.

CONCLUSION

AgNOR count is a useful technique, which can supplement other prognostic markers like Ki-67, mitotic index. A long follow up study is required to confirm the prognostic utility of AgNOR count done in this study.

KEYWORDS

Argyrophilic, AgNOR, Breast, Benign, Malignant.

HOW TO CITE THIS ARTICLE: Nithyananda BS, Byram B, Anjum F, et al. A study of silver staining nucleolar organising regions AgNOR score as prognostic marker in breast lesions in a tertiary care hospital in Hyderabad. J. Evid. Based Med. Healthc. 2017; 4(83), 4890-4893. DOI: 10.18410/jebmh/2017/975

BACKGROUND

Nucleolar Organising Regions (NORs) are loops of ribosomal DNA that are transcribed to ribosomal RNA by RNA polymerase and then translated by the ribosomes into proteins. In humans, NORs are located on the short arms of the acrocentric chromosome 13, 14, 15, 21 and 22. The NOR

associated non-histone proteins such as RNA polymerase-1, C-23 (nucleolin) - a 100 KD molecule and B-23 (Neuratin)- a 78 KD phosphoprotein are argyrophilic. This property has been used to stain the NORs by the method described as the AgNOR-staining technique.^{2,3}

The AgNOR staining technique has been used to stain NORs in metaphase and interphase nucleoli in cell cultures and in preparation for electron microscopy. The amount of staining reflects activity of rRNA gene and can be considered a marker for protein synthesis, which in turn depicts proliferation rate of a given cell. The number of AgNORs thus reflect nuclear and cellular activity.^{4,5} Ultrastructurally, the silver is localised to fibrillar structures and sometimes to the dense fibrillar component of nucleolus.

Nucleolus is the morphological expression of synthesis and maturation of ribosomal RNA from amplified DNA

Financial or Other, Competing Interest: None.

Submission 12-08-2017, Peer Review 21-08-2017,

Acceptance 29-08-2017, Published 13-10-2017.

Corresponding Author:

Dr. Bheeshma Byram,

House No. 4-1-46, Surya Residency,

Flat No. 501, Road No. 3.

Snehapuri Colony PO, Nacharam, TS 500076.

E-mail: bheeshma60@gmail.com

DOI: 10.18410/jebmh/2017/975



(rDNA). Pluton and Menager described NORs as functional subunits of nucleolus in which actively transcribed rDNA is surrounded by numerous regulatory proteins some of which are Argyrophilic Non-Histone proteins (AgNOR).³ By using a cytochemical reaction based on argyrophilic, active NORs, maybe stained by precipitation of metallic silver granules whose quantity is directly related to the nucleolar activity.

Counts of NOR reflect proliferative activity as well as ploidy and are useful diagnostic markers of hyperplasia and neoplasia. Pluton and Menager³ concluded that the number of NORs can be related to grading of neoplasia. A quantitative increase in the number of NORs in malignancy has been postulated due to active cell proliferation such that AgNORs are dispersed throughout the nucleus or due to defect in nucleolar association resulting in AgNOR dispersion or an absolute increase in AgNOR bearing chromosomes due to increase in cell ploidy. In tumours, increased AgNOR counts in interphase nuclei are more likely to be due to cell proliferation than to abnormal ploidy because diploid cells in the proliferative cells are transiently tetraploid in G2 phase resulting in temporary doubling of the number of NOR bearing eccentric chromosomes.

It must however be noted that the number of dots revealed in interphase nuclei by AgNOR technique does not necessarily correspond to the actual number of NORs in the karyotype. As the AgNORs are often small, coalescent or overlapping, their apparent number is invariably less than the actual number present. A marked discrepancy is seen in AgNOR counts if slides are prepared from chromosomal spreads as compared to histology sections because it is difficult to perceive AgNORs when they are present within a relatively small nucleolus. In malignancy, they are scattered and are easily countable. Thus, the AgNOR counts denote more than the numerical index of dispersion than the absolute number.⁶

Immediately, before and after mitotic division, the NORs disperse and then re-aggregate thus leading to an increase in countable AgNORs in nuclear profiles.

This staining of chromosomes has shown that only active NORs can be stained and each individual has characteristic model number of AgNORs.⁷ This raises the prospect of determining the familial transmission of individual acrocentric chromosomes.

Several studies have shown correlation between AgNOR counts and cell proliferation indices like S. phase and Ki-67. Ki-67 is an established proliferative index number. AgNOR counts and Ki-67 scores have been found to correlate very well in non-Hodgkin's lymphoma (Hall et al)⁶ and breast lesion. One disadvantage of Ki-67 is that frozen section for fresh cells are required. AgNOR counts can be used to study cell kinetics where frozen sections or fresh cells are not available.

Smith R, Crocker et al¹ studied NORs in breast lesions and demonstrated high count in malignant lesions when compared with benign lesions. They studied 46 patients of breast malignancy and found that the enumeration of AgNOR maybe an adjunct to accurate diagnosis.

Aims and Objectives

This study is a prospective study, which was carried out to demonstrate AgNOR staining characteristics in benign and malignant breast lesions and to establish the utility of AgNOR staining characteristics in prognostication of breast malignancies.

MATERIALS AND METHODS

This study was conducted in Department of Pathology, Gandhi Medical College, for a period of 2 years and included 159 cases presenting with lump in the breast. These cases were examined and were initially subjected to fine needle aspiration cytology. These cases were subsequently operated and surgical specimens were subjected to histopathological examination. Size of the tumour and lymph node status was specifically noted. Paraffin sections were stained with H and E and histological diagnosis was made. Sections were also stained with silver colloidal technique and AgNOR counts were done.

Malignant lesions were graded based on modified Bloom and Richardson's score⁸ and AgNOR counts were analysed in each of these grades of malignant lesions. Prognostic index based on the formula of Nottingham Tenovus study was calculated and the count was compared and analysed.

Staining Procedure (Chromie et al)⁹

The slides were cut at 3 μ thickness and immersed in a Coplin jar containing saturated glycerine solution for 15 mins.

The sections were hydrated and placed on butter paper in a row over a hot plate with the temperature regulated to between 40°-50°C. 3 mL of colloidal developer solution and 6 mL of 50% aqueous silver nitrate solution were mixed and warmed gently till a light brown colour developed. The sections were then covered with the mixture and left over the hot plate for 30 minutes and then washed in distilled water. The slides were immersed in 10% nitric acid solution for 30 seconds and washed in distilled water. The slides were dehydrated and mounted.

Counting Procedure- The argyrophilic protein associated with nucleolar organiser regions are revealed as dense round black dots, both within the nucleolus and elsewhere in the nucleoplasm. They were observed using a 100X. 100 nuclei were studied in each case and the mean number of AgNORs per 100 nucleus calculated. The cells in the basal layer were taken into account and the counting done. All silver-stained structures, both intra and extra nuclear were counted. Undispersed AgNORs, which appeared as closed clusters and which cannot be resolved were counted as one structure.

RESULTS

A total of 159 cases were analysed, out of which, 106 were benign and 53 were malignant. The age of the patients ranged from 15-79 yrs. Benign lesions predominantly occurred in the age group of 20-39 yrs. with mean age being 31 yrs. Malignant lesions predominantly occurred in the age group of 30-49 yrs. with mean age being 43 yrs. The most common benign lesion was fibroadenoma and most common malignant lesion was infiltrating ductal carcinoma. Only 2

cases of lobular carcinoma and one each case of carcinosarcoma and lymphoma were noted.

Size of benign lesion ranged from 2-6 cms with an average of 3.5 cms. Size of malignant lesions ranged from 2 -7 cms. Based on size lesions are divided into 3 groups (<2 cms, 2-5 cms and above 5 cms). Majority of cases were in 2-5 cms group followed by more than 5 cms.

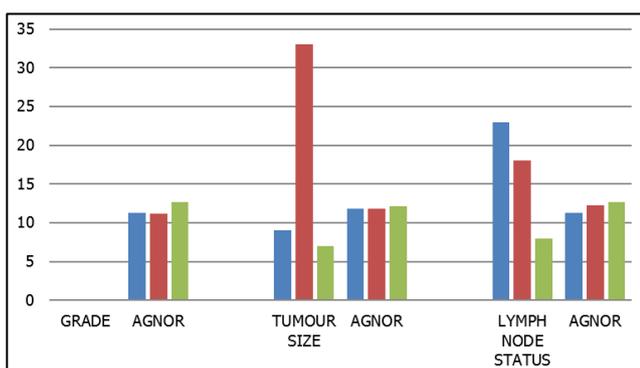
Lymph nodal involvement was divided into 3 groups- node negative, 1-3 nodes, >3 nodes. Most of the cases were node negative (26) followed by 1-3 nodes (19). 49 cases of invasive duct cell carcinoma were histological graded based on modified Bloom and Richardson's score. Majority of cases are grade II (25), followed by grade III (22).

AgNOR staining was done on all 159 cases.

Type of Lesion	No. of Cases	No. of Cells	AgNOR Range	Avg. AgNOR Count/Cell
Fibrocystic disease	19	1900	2-7	3.5
Fibroadenoma	77	1700	2-8	4.1
Phyllodes	4	400	2-7	3.9
Gynaecomastia	6	600	2-4	3.2
Invasive ductal carcinoma	49	4900	7-16	11.88
Invasive lobular carcinoma	2	200	8-14	10.97
Carcinosarcoma	1	100	8-15	11.9
Lymphoma	1	100	8-14	11.01
Total	159			

Table 1. AgNOR Score in Various Benign and Malignant Lesions (N=159)

AgNOR counts in benign lesions with an average of 3.67 are distinctively less than the count in malignant lesions of 11.47. AgNOR counts in invasive ductal carcinoma cases with average score 11.88 is higher than in invasive lobular carcinoma with an average score 10.97.



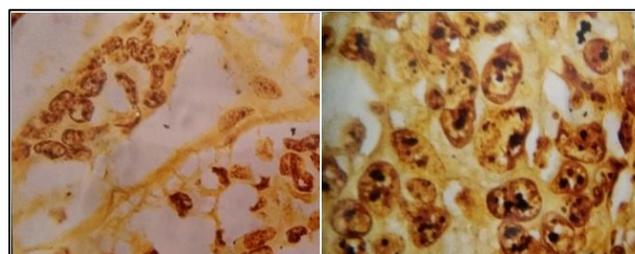
Graph 1. AgNOR Score According to Size, Grade and Lymph Node Status of Invasive Ductal Carcinoma

Prognostic Index	No. of Cases	Mean Average of AgNOR Cases
<3.4	1	10.75
3.4 to 5.4	29	11.35
>5.4	19	12.76

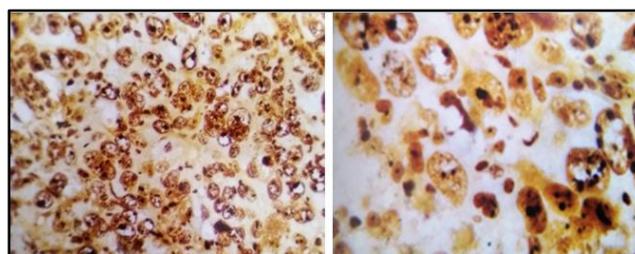
Table 2. AgNOR Score and Composite Prognostic Index of Cases of Invasive Duct Cell Carcinoma (N=49)



Photomicrograph 1. Showing Low Agnor Count in Fibroadenoma and Fibrocystic Disease (AgNOR 1000X)



Photomicrograph 2. Showing High AgNOR Count in IDC Grade 1 and Grade 2 (AgNOR 1000X)



Photomicrograph 3. Showing High AgNOR Count in IDC Grade 3 and Lobular Carcinoma (AgNOR 1000X)

DISCUSSION

Prognostic markers are objective parameters from which the likely course of disease and relative sensitivity to particular treatment can be predicted.

In the present study, the utility of AgNOR count as prognostic marker is analysed by correlating with the histomorphological parameters of prognosis. AgNOR counts were analysed in histopathology slides.

Our study correlated with study done by Dasgupta et al¹⁰ and Rajeevan et al¹¹ who observed mean AgNOR score in benign lesions to be 5.3 and 6.20 and in malignant lesions to be 11.2 and 12.08, respectively.

Mean AgNOR count in benign lesions in present study is 4.01 and for malignant lesions is 11.60. The quantification of AgNORs is dependent on degree of dispersion and ideally 100 or more well-dispersed cells should be selected in giving the correct average count.

Rajeevan et al¹¹ showed higher AgNOR score in invasive ductal carcinoma (11.58) when compared to invasive lobular carcinoma.^{11,12} In present study, invasive duct cell carcinoma (11.88), which is aggressive than invasive lobular (10.97) showed higher count. Carcinosarcoma, which is also aggressive showed higher count.

A study done by Pratibha D, Kuruvilla S et al showed that higher counts were associated with high histological grade stating that histological grade reflects the biological

aggressiveness of tumour.¹² The AgNOR count supplements the information obtained from other prognostic indices like DNA ploidy and Ki-67. In our study, AgNOR count in Grade I (11.37) and Grade II (11.17) showed significant marked difference from that in Grade III (12.72) and was much higher when compared with Pratibha et al where a count of 4.83 was noted in Grade III when compared to 3.42 and 4.56 in Grade I and Grade II. The relatively lower count observed by Pratibha et al is due to counting of groups and clusters as one.

In the present study, there was overlap between in average counting Grade I and Grade II. This is because of small sample size of Grade I (2 cases only). The nuclear characters are very significant in Grade III showing vesicular nuclei and prominent irregular dots are seen.

Increased AgNOR counts with increasing tumour size in our study (<5 cm-11.8 and >5 cm-12.10) correlated with Rajeevan et al (<5 cm-10 and >5 cm-13.1) who observed the same in his study.

Rajeevan K, Aravindan KP et al studied AgNOR score in breast malignancies with different lymph node status. He observed that the AgNOR score is seen to discriminate significantly the group with three or less lymph node and the group with more than three.¹¹ Difference in score is not distinctive within node negative group and 1-3 lymph node group.

In our present study, the AgNOR score was distinctive in all the three groups with relation to lymph node status, which was similar to a study done by Rajeevan et al.¹¹ Size and lymph node status has been time old morphological parameters of biological behaviour and aggressiveness of any tumour and AgNOR counts seem to be correlating with these parameters.

Our study showed significant correlation between the mean AgNOR count and the prognostic index where average AgNOR counts increased with increased tumour grade and the prognostic index. Higher prognostic index (>5.4) were associated with high AgNOR count (12.76), whereas prognostic index <3.4 were associated with AgNOR score of 10.75.

CONCLUSION

This study shows that AgNOR count delineates benign from malignant lesions. AgNOR staining is characteristic in malignant lesions and also correlates with histological grade of invasive ductal carcinoma. AgNOR count was comparatively higher in known aggressive tumours like invasive ductal carcinoma and carcinosarcoma unlike in less aggressive tumours like invasive lobular carcinoma. AgNOR

counts is a useful technique, which supplements other prognostic markers like Ki-67, mitotic index, PCNA, flow cytometry. AgNOR counts also correlated with prognostic index calculated for individual cases thereby suggesting that it has definite prognostic significance.

REFERENCES

- [1] Smith R, Crocker J. Evaluation of nucleolar organizer region associated proteins in breast malignancy. *Histopathology* 1988;12(2):113-125.
- [2] Goodpasture C, Bloom SE. Visualisation of NORs in mammalian chromosomes using silver staining. *J Chromosoma* 1975;53(1):37-50.
- [3] Ploton D, Menager M, Jeannesson P, et al. Improvement in the staining and the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. *Histochem J* 1986;18(1):5-14.
- [4] Hall PA, Crocker J, Watts A, et al. A comparison of nucleolar organizer region staining and Ki-67 immunostaining in non-Hodgkin's lymphoma. *Histopathology* 1988;12(4):373-81.
- [5] Eskelinen MJ, Lipponen PK, Collan Y, et al. The role of nucleolar organiser regions as prognostic factor in breast cancer. *Eur J Cancer* 1991;27(8):989-992.
- [6] Hall PA, Crocker J. A comparison of NOR staining and Ki-67 immunostaining in non-Hodgkin's lymphoma. *J Histopathology* 1988;12(4):373-381.
- [7] Buys CH, Osinga J. Selective staining of the same set of nucleolar phosphoproteins by silver and Giemsa. A combined biochemical and cytochemical study on staining of NORs. *Chromosoma* 1984;89(5):387-396.
- [8] Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 yrs. *Br J Cancer* 1957;11(3):359-377.
- [9] Cromie CJ, Benbo EW, Stoddarts RW. Pre-incubation with glycine solutions aids the demonstration of NORs associated proteins. *Histochemical Journal* 1988;20:722-724.
- [10] Dasgupta A, Ghosh RN, Sarkar R, et al. Argyrophilic nucleolar organiser regions (AgNORs) in breast lesions. *Indian J Pathol Microbiol* 1997;95(9):492-494.
- [11] Rajeevan K, Aravindan KP, Kumari BC. Value of AgNORs in fine-needle aspiration cytology of breast lesions. *Indian J Pathol Microbiol* 1995;38(1):17-24.
- [12] Pratibha D, Kuruvilla S. Value of AgNORs in premalignant and malignant lesions of the cervix. *Indian J Pathol Microbiol* 1995;38(1):11-16.