

Original article:

Study of AgNOR counts in cytological smears of breast lesions

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Abstract:

Introduction: Fine needle aspiration cytology is a routine outpatient procedure. The advantage of FNAC over biopsy is its simple technique and less time consuming. There has been a growing interest in the state of nucleus, DNA and proliferation markers. Nucleolar organizer regions are loops of DNA projecting into the nucleoli of interphase nuclei. Some of the NOR associated proteins are argyrophilic and can be demonstrated as black dots (AgNOR's) by silver staining technique. The aim of this study is to determine the efficiency of AgNOR technique in differentiating benign and malignant lesions in breast cytology, to find change in AgNOR count depending on the size of malignant lesion.

Methods: The present study is conducted in the department of pathology, Andhra Medical College, Visakhapatnam over a period of 1 year. Total 70 female patients with breast lump are aspirated and smears stained with H&E and with AgNOR staining. Only 11 surgical specimens are available for histopathology.

Observation and Results: The mean AgNOR count in benign breast lesions is 2.45 ± 0.05 and 4.76 ± 0.17 in malignant lesions. The mean AgNOR count in malignant breast lesions measuring 5cm or less is 4.52 ± 0.38 and 5.16 ± 0.93 in those measuring more than 5cm.

Conclusion: The mean AgNOR count in breast carcinoma is significantly raised in comparison with that of benign breast lesion. There is also significant difference in mean AgNOR count between malignant breast lesions measuring more than 5cm and those measuring 5cm or less.

Keywords: Fine needle aspiration cytology, Benign and malignant breast lesions, AgNOR counts

Introduction:

FNAC is a well established procedure for diagnosis of lesions of breast, thyroid, lymphnode etc. Breast lesions are a source of anxiety to the patients as well as to the treating doctor as carcinoma breast is fairly common.⁽¹⁾ It is the second most common cancer among females in India. (Jussavella and Gangadharan, 1974). The advantage of fine needle aspiration over biopsy methods is the simplicity of the technique. Fine needle aspiration is readily accepted by patients, the equipment is simple and inexpensive, and the whole procedure including

fixation and staining is so quick that a report can be issued quickly. There has been a growing interest in the state of nucleus, DNA and proliferation markers. Nucleolar organizer region (NORs) are loops of DNA projecting into the nucleoli of interphase nuclei, they are thought to encode for ribosomal RNA. NORs are normally restricted to the fine acrocentric chromosomes in the human karyotype. Some of the NOR associated proteins (NOR APs) are argyrophilic⁽³⁾ and can be demonstrated as black dots by a silver staining technique. The structures thus demonstrated are known as AgNORs⁽²⁾. The

principal advantages of the AgNOR technique is the relative simplicity of the staining method. Counts of AgNORs in the interphase nuclei may assist in the distinction between benign and malignant neoplasms and in the grading of the later. The number of countable AgNORs in interphase nuclei is probably related more to their dispersion through the nucleoplasm than to the actual number present. The AgNOR count may, therefore be an index of AgNOR dispersion rather than the actual number present in the karyotype. Dispersion in itself may reflect the proliferative state: before mitosis the division of the nucleoli and the NORs within the disperse, after mitosis the NORs reaggregate and nucleoli reform. Thus the AgNOR count rises before and after mitosis. Comparison with Ki67 immunostaining index shows a highly significant correlation in breast carcinoma (Dervan et al 1989) ⁽²⁾. The greatest advance in the methodology of AgNOR staining came from the groups of workers in Rheims, WHO described a one stage method run at room temperature (Plonton, 1986)⁽³⁾.

AgNOR technique has been applied in lymphomas (Crocker T and Nar, 1987)⁽⁴⁾ (Rama Gopalan and M Madhavan, 1995)⁽⁵⁾ cutaneous melanocytic lesions (Crocker J and Skilback, 1987)⁽⁶⁾, Non-Hodgkins lymphoma (Crocker J and Egan M, 1988) ⁽⁷⁾, Neuroblastoma (Derenzini M, 1989) ⁽⁸⁾.

Aims & objectives:

The aim of this study is to determine the efficiency of AgNOR technique in differentiating benign and malignant lesions in fine needle aspirates of breast lesions, to find change in AgNOR count depending on the size of malignant lesion.

Materials and Methods:

The present study entitled "Study of AgNOR counts in cytological smears of breast lesions" is conducted

in the department of pathology, Andhra Medical College, Visakhapatnam over a period of one year. The study included all the female patients with a breast lump referred to the department. All the cases undergoing FNAC were advised for histopathological study of the resected mass for cyto-histopathological correlation.

Prior to fine needle aspiration a detailed clinical history and a thorough clinical examination were carried out in all cases. FNAC was done for breast lumps and slides were fixed in 90% isopropyl alcohol for Haematoxylin and Eosin staining. Air dried smears were fixed in 95% ethanol for AgNOR stain. AgNOR staining requires freshly prepared working solution formed by mixing two parts of solution A and one part of solution B. Solution A is prepared by dissolving silver nitrate (50gm) in deionised water (100ml) in darkroom. Solution B composed of gelatin solution. 2 gm of gelatin is dissolved in 100ml of deionised water to which 1ml of formic acid is added. The test tube is agitated mechanically or placed in a water bath at 60°C till the gelatin mixes well. Freshly prepared working solution was poured over the slides and kept for 30minutes in darkroom at room temperature. Slides were washed in deionised distilled water and air dried. Dehydration in 95% and absolute alcohol. Later two changes of xylene and slides are mounted with DPX. Slides were examined and representative areas with minimal cell overlap were demarcated for AgNOR counting. Counting was performed under oil immersion at X 1000 magnification. AgNORs were visualized as black dots within the nucleus against a yellow background. The dots were defined as black homogenous precipitates of varying size from tiny specks to well defined small rounded dots to larger angulated dots. The dots were scattered in the nucleus as satellites or

grouped together as clusters. A total of 100 cells were counted in each side for AgNOR dots and the average number of AgNOR per cell was determined. The AgNOR counts thus obtained was tabulated for each lesion.

Observation& Results:

The present study is to evaluate the efficacy of AgNOR count in cytological aspirates from breast lump, to differentiate between benign and malignant lesions. The age distribution of the patients with breast lesions is shown in Table-1. In general, most of the patients with breast lesions are between 21 to 40 years. The incidence of benign lesions are seen at lower age when compared to malignant lesions which are seen commonly in later age groups.

In our observation, fibroadenoma is the commonest breast lesion. It constituted 65.71%. Next common lesion is carcinoma constituted 22.85%. Out of 70 patients studied cytologically, only in 11 cases surgical specimens are available for histopathological observation. The cytohistological correlation is 100% for carcinoma where as 5 cases out of 10 fibroadenoma are correlated.

AgNOR counts in different breast lesions are presented in Table-2. It is seen that the mean AgNOR count in breast carcinoma is highest (4.76 ± 0.17) followed by benign Phyllodes tumor (2.8 ± 0.75), fibrocystic disease (2.47 ± 0.20). The mean AgNOR counts in fibroadenoma are 2.45 ± 0.05 .

The mean AgNOR count in breast carcinoma is significantly raised in comparison with that of benign breast lesions. The mean AgNOR count in benign lesions is 2.45 ± 0.05 and mean AgNOR count in breast carcinoma is 4.76 ± 0.17 . This observation is presented in Table – 3

Mean AgNOR counts in malignant breast lesion of 16 patients with respect to the tumor size

are presented in Table – 4. The mean AgNOR count is 4.52 ± 0.38 in tumors measuring 5cm or less. The mean AgNOR count in tumors measuring more than 5cms is 5.16 ± 0.93 . There is significant difference in mean AgNOR count between malignant breast lesions measuring more than 5cm, and those measuring 5cm or less.

Conclusion & Discussion:

The histopathological technique is time consuming due to tissue processing, section cutting, preparation of slides and staining. The advantage of fine needle aspiration over biopsy technique is the simplicity of the technique. Fine needle aspiration is readily accepted by the patients, as it is less traumatic, inexpensive and does not require hospitalization. The whole procedure including fixation and staining is quick. The main draw backs of procedure are the need for the experienced cytopathologist to interpret smears and the risk of false negative results.

Microscopically the subjective impressions by which degree of malignancy is assessed that is nuclear pleomorphism, nuclear cytoplasmic ratio, loss of polarity and mitotic frequency are often unsatisfactory. Accordingly newer methods are being developed for describing malignancy more objectively and there has been growing interest in subcellular organellar level specifically in the state of the nucleus. One of the newer methods in DNA flow cytometry; the other approach is staining with monoclonal antibodies like ki 67.

One step staining technique in localizing proteins associated with nucleolar organizer regions (NORs) in the interphase nuclei has aroused considerable interest among pathologists. These NOR associated proteins are argyrophillic and can be demonstrated as black dots by silver staining technique. The structures thus demonstrated are

known as AgNORs. These can be discriminated between benign and malignant neoplasm and in the grading of the malignant lesions. AgNOR technique has been applied in lymphomas (Crocker J And Nar, 1987)⁽⁴⁾ (Rama Gopalan and M Madhavan, 1995)⁽⁵⁾, cutaneous melanocytic lesions (Crocker J and Skibeck, 1987)⁽⁶⁾, Non Hodgkins lymphoma (Crocker J And Egan M, 1988)⁽⁷⁾, Neuroblastoma (Derenzini M, 1989)⁽⁸⁾, Gall bladder lesions (Vatsala Misra, 1995)⁽⁹⁾.

Problems with silver staining technique in paraffin embedded tissue sections are apparent. First, staining intensity varies considerably with slight variation in staining time. This either obscures the individually clustered AgNORs within nucleoli if overstained or renders them very faint and therefore not assessable, if understained. Second, minor variations in section thickness affect the apparent number of AgNORs, thus necessitating sections of a uniform 3 micrometer thickness (Giri et al, 1989)⁽¹⁰⁾.

Since consistently obtaining 3 micrometer sections on routinely processed paraffin embedded blocks is potentially problematic, the technique of fine needle aspiration cytology smears is applied. More AgNORs are found in cytological preparation than in tissue sections as the whole nuclei in cytologic preparation have greater amount of available sites for silver precipitation than in 3 micrometer tissue section. (Boldy and colleagues, 1989)⁽¹¹⁾.

The present study aims at the evaluation of argyrophilic nucleolar organizer regions in breast cytological aspirations. The study included 70 cases and it is seen that most of the patients are in the age range of 21-40 years. In the present study, benign lesions comprised of 74.28%, malignant lesions

22.85% and miscellaneous 2.85%. Fibroadenoma was the commonest lesion. It constituted 65.71% followed by carcinoma (22.85%).

In the benign category, the majority are diagnosed as fibroadenoma (65.71%). The rest of the benign lesions are fibrocystic disease (5.71%) and benign phyllodes tumor (2.85%).

Out of 70 patients studied cytologically only in 11 cases surgical specimens are available for histopathological observation. The cytohistological correlation is 100% for carcinoma whereas 5 cases out of 10 fibroadenoma are correlated.

The mean AgNOR count in benign lesions of present study is 2.45 ± 0.05 . Similar observations were made by Rajeevan K, 1995 study⁽¹²⁾ In his study the mean AgNOR counts in benign lesions was 2.8. in Mangala, Simha's study (1996)⁽¹³⁾ the mean AgNOR count in benign lesions was 1.8. Kumar A (1997)⁽¹⁴⁾ observed, the mean AgNOR counts in benign lesion as 1.88 ± 0.19 .

The range of mean AgNOR count in fibroadenoma of present study 1.2 – 3 and the mean AgNOR count is 2.45 ± 0.05 . Similar observation was made by Dube MK⁽¹⁵⁾, 1995, in which the range of mean AgNOR count was 1.6 – 2.7. Basu et al (1997)⁽¹⁶⁾ observed a mean AgNOR count of 2.02 ± 0.30 in fibroadenoma. Rath Wolfson (1995)⁽¹⁷⁾ observed mean AgNOR count of 1.84 ± 0.46 in fibroadenoma.

The mean AgNOR count in fibrocystic disease of the present study is 2.47 ± 0.20 . LeaRath. Wolfson (1995) found mean AgNOR count of 1.76 ± 0.6 in fibrocystic disease. Pande et al found a mean AgNOR count 1.81 ± 0.11 in fibrocystic disease. Dube MK (1995)⁽¹⁵⁾ observed mean AgNOR count of 1.6 in fibrocystic disease.

The mean AgNOR count in fibroadenoma is 2.45 ± 0.05 and fibrocystic disease is 2.47 ± 0.20 . There is no statistically significant difference between these two groups. Similar observation was made by Lea Rath – Wolfson (1995)⁽¹⁷⁾. He observed no statistically significant difference between the two benign groups, fibrocystic change and fibroadenoma, the mean AgNOR count was 1.73 ± 0.6 and 1.84 ± 0.46 respectively.

The present study shows a significantly high mean AgNOR count in malignant lesions of breast. The mean AgNOR count is 4.76 ± 0.17 and the range of mean AgNOR count is 4-6.5. Dube M K (1995)⁽¹⁵⁾ observed mean AgNOR counts in the range of 2.7 – 9.9 and the mean AgNOR was 3.89. Rajeevan K (1995)⁽¹²⁾ study showed a mean AgNOR count of 5.4 and the range was 5 – 5.9 in malignant lesions. Kumar A (1997)⁽¹⁴⁾ study showed a mean AgNOR count of 6.61 ± 1.75 in breast carcinoma. Rzymocowska(1997) observed a mean AgNOR count of 4.83 ± 1.20 in intraductal carcinoma in his study. Gupta (1997)⁽¹⁹⁾ reported a mean AgNOR count of 6.26 in carcinoma breast. Mangala Simha et al (1996)⁽¹³⁾ study showed mean AgNOR count of 3.5 (2.4 – 5.5) in malignant breast lesions.

The present study shows a significant difference between benign and malignant lesions of breast. The mean AgNOR count in benign lesions is 2.45 ± 0.05 and in malignant lesions is 4.76 ± 0.17 . The mean count in malignant lesions is significantly increase in comparison with that of benign lesions. ($P < 0.001$). Similar observations were made by Rajeevan K, 1995⁽¹²⁾, in which the mean AgNOR count was significantly higher in malignant lesions ranging 5 – 5.9 and the mean AgNOR was 5.4. In benign lesions, the range of mean AgNOR was 2.7 – 3 and the mean AgNOR was 2.8. For Giri et al

(1989)⁽¹⁰⁾ the mean AgNOR counts of malignant lesions was 4.4 ± 1.2 and the mean AgNOR count of benign lesions 2.8 ± 0.4 . Similar observations were made by Kumar A (1997)⁽¹⁴⁾ where the AgNOR count of malignant lesions was significantly higher (6.61 ± 1.75) than in benign lesions (1.88 ± 0.19). In Dube MK (1995)⁽¹⁵⁾ study, the range of mean AgNOR count of malignant lesions was significantly higher (2.7 – 9.9) than the benign lesions (1.5 – 2.8). Mangala Simha et al (1996)⁽¹³⁾ recorded the mean AgNOR in benign lesions as 1.8 and 3.5 in malignant lesions.

In the present study, we found that there is correlation between the tumor size and the mean AgNOR count in malignant lesions. In the present study, the mean AgNOR count of malignant lesions measuring 5cm or less is 4.52 ± 0.17 as compared to 5.16 ± 0.10 in malignant breast lesions measuring more than 5cm in size. Similar observation was made by Rajeevan et al (1995) who found a high AgNOR count of 6.1 in malignant lesions measuring more than 5cm as compared to a mean AgNOR count of 5 in malignant lesions measuring less than 5cm. Megala Simha et al (1996)⁽¹³⁾ showed a mean AgNOR count of 3.1 in lesions less than 2.5 cm and mean AgNOR count of 3.5 in lesions measuring more than 2.5 cm. Simha et al (1996)⁽²⁰⁾, Gimenez Mas JA (1996)⁽²¹⁾, Kumar A (1997)⁽¹⁴⁾ and Bellotti M (1997)⁽²²⁾ observed a distinct rise in mean AgNOR counts in direct proportion to the size of the malignant lesion.

Besides the count of AgNOR dots, the size, shape and distribution are found to be different in various types of breast lesions studied. In benign lesions, a mixture of uniform looking small regular dots with occasional large cluster are found. In malignant lesions, the dots are often large, irregular

and mixed with some fine irregular dots. Basu A, (1997)⁽¹⁶⁾ study differentiated the appearance of the dots in different breast lesions. Uniform small compact centrally placed dots were seen in fibroadenosis (non neoplastic lesions), mostly uniform small compact but occasional large and irregular marginally located in infiltrating duct carcinoma. Mangala Simha et al (1996)⁽¹³⁾, found well defined, sharp dots in benign lesions and in malignant

lesions, the dots were often irregular, variable in size and shape and dispersed throughout the nucleus. Meehan et al (1994)⁽²³⁾ found large, irregular dots and small dots within the same nucleus in most malignant cells and occasionally in fibroadenoma and fibrocystic disease.

Thus the mean AgNOR count and its pattern of dispersion can be extremely useful in differentiating benign and malignant lesions of the breast.

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