Study OfAgNOR Count In FNAC of Breast Lesions

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Abstract: Background:ArgyrophilicNucleolar Organizer Region technique has a potential value in the diagnosis of malignancy and can be used in cases with equivocal and inconclusive cytological picture. The purpose of this study was to evaluate mean ArgyrophilicNucleolar Organizer Region count and ArgyrophilicNucleolar Organizer Region Pattern Assessment score in fine needle aspirates of breast lumps.Materials and Methods: The present study consists of 80 cases of AgNOR count done in fine needle aspiration cytology of various breast lesions in patients on O.P.D. basis or those admitted in Guru Govindsingh Hospital attached to Shri M.P. Shah Medical College, Jamnagar during the period between August '98 to May 2000. Fine Needle Aspiration smears were studied by conventional methods and silver staining for ArgyrophilicNucleolar Organizer Regions. Histopathologic diagnosis was taken as the gold standard. Results:ArgyrophilicNucleolar Organizer Region count and ArgyrophilicNucleolar Organizer Region Pattern Assessment score were helpful in differentiating benign from malignant lesions. Mean ArgyrophilicNucleolar Organizer Region count and ArgyrophilicNucleolar Organizer Region Pattern Assessment score were 2.63 ± 1.36 and6.26 ± 1.19 respectively in benign lesions while they were 8.42 ± 2.53 and 10.05 ± 2.22 respectively in malignant lesions.With few exceptions, cases with high counts had high scores. Conclusion: Mean ArgyrophilicNucleolar Organizer Region AgNOR count and Subjective ArgyrophilicNucleolar Organizer Region Pattern Assessment score provide useful information regarding cellular proliferation. Both count and score have comparable diagnostic potential but the latter is a more convenient and rapid method for ArgyrophilicNucleolar Organizer Region evaluation.[Dixit S et al NJIRM 2013; 4(1) : 98-104]

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Introduction: Nucleolar Organizer Regions (NOR) are the tools used by the cytogeneticists for the study of chromosomal disorders. It was noticed that NOR pattern in malignancy was different. After the development of the simple silver staining technique for the visualization of NORs at the optical level, scientists believed that NOR study using silver staining could be used for diagnosing malignancy[1].

The role of ArgyrophilicNucleolar Organizer Region (AgNOR) is already well established in the diagnosis of lymphoma, prostatic tumors and oral cavity tumors[2-4].

Several studies of breast lesions are also available. Smith and Crocker and subsequently Giri et al have evaluated this technique on a wide range of breast lesions, and showed significant differences between malignant and non malignant lesions when total AgNORs were counted[5,6]. Many studies have noted morphologic differences in the shape and distribution of AgNORs in benign and malignant cells[7,8]. It is difficult to accurately or reliably count the number of AgNORs aggregated within a cluster in a nucleolus because of their small size and their often apparent fusion or overlap; thus a high level of inter-observer disagreement is found[6].

Materials And Methods: A cross sectional study was carried out from December 2006 to April 2008 in the Department of Pathology,ShriM.P.Shah Medical College, Jamnagar. The study included 38 malignant and 72 benign cases. Patients presented with clinically palpable breast lumps and underwent fine needle aspiration followed by subsequent histopathologic examination. The FNA smears were studied by conventional methods and silver staining for AgNORs. Histopathologic diagnosis was taken as the gold standard.

Staining Procedure for AgNOR: Staining was carried out as per the method described byCrocker et al[7]. FNA smears were fixed in 90% propanol for at least one hour. Before staining, the smears were hydrated in deionised distilled water. The smears were then incubated in a silver colloidal solution

for 35-45 minutes in the dark at room temperature. Fresh silver colloidal solution was prepared by mixing one volume of two grams/100 ml gelatin in 1% formic acid solution to two volumes of 50% aqueous silver nitrate solution. The stained smears were then washed for three minutes in three changes of distilled water. Smears were dehydrated, cleaned, mounted in DPX and were examined under oil immersion (100X). AgNORs appeared as black dots within the nucleus.

Two parameters were taken into consideration while evaluating AgNORs:
1. Average number of AgNOR dots per cell (Mean AgNOR)
2. Subjective AgNOR pattern assessment (SAPA) scoring.

AgNOR Counting: Counting was performed using oil immersion . Altogether 100 cells were counted and the mean AgNOR count per nucleus calculated. SAPA score : Scoring was done according to the scoring system proposed by Meehan et al[9]. Five parameters were considered: estimated AgNOR dot count per cell, variation of satellite size and variation of satellite shape, variation in cluster size and variation in cluster shape. Using this subjective system, each case scored a minimum of 5 and a maximum of 15. After taking FNAC from breast and preparing smear, staining with silver nitrate according to AgNOR technique was done. Then AgNOR counting of each case was carried out by proposed method. The mean and standard deviation of each lesion was calculated. Statistical analysis was done according to standard method described by Dr. Mahajan[9].

Result: A study of 80 cases of various breast lesions and 10 normal breast tissues as control was undertaken.

The patients were admitted or had come on O.P.D. basis in Guru GobinsinghHospital affillated to Shri M.P. Shah Medical College, Jamnagar. Fine needle Aspiration Cytology samples from breast were taken from the period between August '98 to 2000.

Out of 80 cases, 12 cases of inflammatory breast lesions, 11 cases of benign proliterative (non neoplastic) lesions, 18 cases of benign neoplastic breast lesions and 39 malignant lesions were detected. Amongst benign proliferative (non-neoplastic) lesions, fibrocystic diseases comprised of 8 cases and fibroadenosis included 3 cases. Out of total 18 cases of benign neoplastic breast lesions, majority were fibroadenoma constituting 12 cases, next in number was phylloides tumour constituting 4 cases and minimum were that of lactating adenoma i.e. 2 cases. Total cases of inflammatory breast lesions were 12, which consisted of 6 cases of acute / chronic mastitis, 4 cases of fat necrosis and only 2 cases of abscess.

Malignant breast lesions comprised a total of 39 cases. Out of these, 12 were diagnosed as invasive duct carcinoma, 4 cases were of lobular carcinoma, medullary carcinoma constituted 2 cases, papillary carcinoma constituted a single case and maximum number consisted of lesions that were Not Otherwise Specified (NOS) i.e. 20 cases.Besides this, 29 cases were having lymph node metastasis and only 10 were without metastasis.

10 control cases are having range of 0.7 to 1.1 AgNOR with mean 0.9 and standard deviation of 0.14. While fibrocystic disease has a range of 2.0 to 3.0 AgNORs which is slightly higher than the range of fibroadenosis of 1.0 to 1.5 AgNORs.The mean and S.D.of fibrocystic disease are also more than that of fibrocystic disease are also more than that of fibroadenosis i.e. 2.50 +0.31 and 1.26 + 0.23 respectively.

Fibroadenoma had a range of 2.5 to 4.2 AgNORs, which is slightly higher than that of phylloides tumour which is 2.0 to 3.0 . Lactating adenoma had a range of 1.0 to 1.2 AgNOEs. The highest mean value was that of fibroadenoma, mean being 3. The next in frequency was phylloides with a mean value of 2.50 and lowest mean was that of lactating adenoma i.e. 1.1.

Table -1 shows that out of all malignant tumours, invasive duct carcinoma had highest range of

AgNOR count from 3.5 to 6.0 with a mean of 4.83. The highest mean value of AgNOR was 5.00, founin not otherwise specified lesions. Lobular carcinoma showed the lowest mean value of 4.0, while papillary carcinoma being a single caseshows no range.

TABLE 1 :AgNOR Count In Malignant Lesions.

No.	Lesion	Range of AgNOR Count	AgNOR count Mean + S.D.
1	Invasive duct carcinoma	3.5 – 6.0	4.83 + 0.84
2	Lobular carcinoma	3.5 – 4.5	4.00 + 0.43
3.	Medullary carcinoma	4.5 – 5.5	5.00 + 0.70
4.	Papillary carcinoma	4.4	4.40 + 0.00
5.	Not Otherwise specified	4.0 – 6.0	5.00 + 0.64

It is observed that acute / chronic mastitis had a range from 1.0 to 2.0 AgNORs which was highest in the group of inflammatory lesions.

Morphologically, inflammatory lesions had very few AgNORs, whereas benign proliferative and benign neoplastic breast lesions had relatively more number of AgNORs which was round, uniform and small in contrast to the AgNORs found in malignant neoplasms, which were usually irregular, of bizarre size and shape and were also clustered together.

Table2 shows the direct relationship of lymph node metastasis with mean AgNOR count, the mean count being significantly higher in cases with metastasis.

TABLE 2 :AgNOR Count In Relation With Lymph Node Status.

No.	Lesion	No. of cases	AgNOR count Mean + S.D.
1.	With metastasis	29	5.05 + 0.69
2.	Without metastasis	10	4.19 + 0.39

It is observed from Table 3 that cases of benign proliferative (non neoplastic) lesions and those of benign neoplastic lesions have more or less similar distribution in relation to AgNOR count. From both groups, the majority of cases fall in the range of 2 to 2.99, though a good number of cases of benign neoplastic lesions are in the range of 3.0 to 3.99 and 2 cases are having highest count in range of 4.0 to 4.99.

TABLE 3 :AgNOR Count - Case Frequency Distribution

No.	Lesions	AgNOR Count							Total No. of cases
		0 -0.99	1 – 1.99	2 – 2.99	3 – 3.99	4 – 4.99	5 – 5.99	6 – 6.99	
1.	Control	06	04	--	--	--	--	--	10
2.	Inflammatory	01	10	01	--	--	--	--	12
3.	Benign Proliferative (Non neoplastic)	--	03	07	01	--	--	--	11
4	Benign neoplastic	--	02	10	04	02	--	--	18
5.	Malignant	--	--	--	04	18	13	04	39

The frequency distribution of malignant cases is from the range of 3.0 onwards and there is more or less equal distribution in the range of 4.0 to 4.99 and 5.0 to 5.99, the former having few more cases than latter. the highest range 6.0 to 6.99 has only 4 cases.

It is also observed that there is an overlap of AgNOR count between benign neoplastic and

malignant cases in the range of 3.0 to 3.99 and 4.0 to 4.99 and thus typing the lesion solely on the basis of AgNOR count becomes difficult.The purpose of doing statistical analysis was to know whether the difference observed in the results were by coincidence or they were actually having significance.The test of significance of difference in means was applied.

Random selection of samples was done from the corresponding population. The sample showed homogeneity of variance.Results of benign neoplastic and malignant breast lesions were testedaccording to 'Z' test. Standard error (SE) =0.22, Z =9.81 and P< 0.001.

This suggests that the observed difference in mean value of benign neoplastic and malignant lesions is highly significant statistically. It is greater than 3 times S.E. hence biological and not by mere chance.

Results of malignant breast lessons with and without metastasis were also tested as per 'Z' test. Standard Error (SE) = 0.177; Z value = 4.85 ; p < 0.001

Thus, suggesting that the difference in mean AgNOR count of malignancy with and without metastasis is statistically significant as it is more than 3 times the S.E.

Discussion : Breast carcinoma behave differently in different individuals and the behavioural changes in the tumour determines the final outcome of the disease. Indicators of tumour behaviour are profilerative index and DNA content or ploidy8 . Different methods for assessing these indicators, such as breast cancer kinetic data which included immunohistochemical evaluation of nuclear antigens, flow cytometric DNA analysis etc. are time consuming, tedious procedures, which require costly equipments.. Hence, they cannot be used on a routine basis[9].

Over the last decade, silver staining of the AgNORs has become a widely used alternative method for assessing proliferative activity grade of malignancy in tumour pathology, since it is relatively less time consuming, simple procedure which is also less expensive and does not require specialized instruments. Many workers applied this technique to various tissues and lesions and found a consistent rise from benign to malignant transformation. Some workers did not include normal controls, while others excluded inflammatory lesions and studied only benign neoplastic, proliferative and malignant lesions. In present study, however, both control cases as well as inflammatory lesions were included.

In the present study the AgNOR techniques was applied to study the scores in breast tumours.

Giri et al[6] found it impossible to count reliably the number of AgNORs aggregated within a cluster in the nucleolus, because of their small size and fusion or overlap and suggested that quantification of total AgNORs may be prone to an unacceptable degree of observer variation. Hence, in their study, they enumerated all morphologically inseparable clusters as one in addition to those that were scattered individually through the nucleus.

Mean value in proliferative lesions in present study is 2.16 + 0.63 which is nearer to mean value of Giri et al[6]. Mean value in studies by Dervan et al[12] are more than the present study since they had counted the black dots as separate instead of consideringthem as a single aggregated dot. Whereas,in the present study, inseparable AgNORs were counted as a single dot. So the result of the present study is lower than their results.

Mean value in benign neoplastic lesions in present study to be 2.67 + 0.81 which is comparable to mean value of the study by Rajeevan et al[11],and lower than the mean values of Dervan et al[12] and Karmakar et al [13.]

Mean value of malignant lesions in present study to be 4.83 + 0.72 which is nearer to the values of Rajeevan et al [11] and much lower than the mean values of Dasgupta et al[14], Karmakar et al [13] and Dervan et al[12].

The discrepancy in AgNOR values in studies by different workers strongly points to the need for following a standard protocol if AgNORs are to be advocated for distinguishing between benign and malignant lesions.

Table 4: Comparision Of AgNOR Count In Benign Neoplastic And Malignant Breast Lesions.

No	LESIONS	No. of cases	Mean + S.D	P- Value
1	Benign neoplastic lesions	18	2.67 +0.81	p<0.001
2	Malignant lesions	39	4.83 + 0.72	Highly significant

From table 4, it is observed that the mean AgNOR count of malignant tumours is higher than benign ones and the observed difference in mean AgNOR count of both lesions is highly significant statistically.

Table 5 :AgNOR Count In Malignant Tumours In Relation To Lymph Node Status.

No	lESIONS	No. of cases	Mean + S.D	P- Value
1	Malignant tumours with metastasis	29	5.05 +0.69	p<0.001
2	Malignant tumours without metastasis	10	4.19 + 0.39	Highly significant

Table 5 shows that malignant tumour with metastasis have higher AgNOR scores than those with no lymph node involvement and the observed difference in mean value of both the groups is not coincidental but highly significant, statistically. Sivridis and Sims[9] in their study observed that in malignancies with 4 or more positive nodes, the mean AgNOR counts was significantly higher than in carcinomas with negative or 1-3 positive nodes. Agarwal et al[15], noticed mean AgNOR counts to be higher in tumours with more than 3 axillary lymph node involvement than in negative or less than 3 node involvement. Kumar et al[8] also observed a higher AgNOR count in patients with involvement of 4 lymph nodes compared to those with involvement of less than 4 nodes.

Thus, these observations establish a correlation of increased AgNOR value with a higher grade of malignancy. An overlap was observed in the present study in AgNOR count between benign neoplastic and malignant lesions in range of 3.0 to 4.99. Giri et al[6] also noticed an overlap between epitheliosis and intraductal carcinoma in the range of 2.0 to 3.0. This overlapping might be due to borderline lesions.

The morphology and distribution pattern of the silver stained dots is also important as a diagnostic and prognostic parameter, as observed in this study. Larger and irregular bizarre AgNORs were seen in malignant cases in contrast to the small, round and regular AgNORs in benign lesions. Similar morphologic alteration in pattern was also observed by Giri et al[6], Dervan et al[12], Sivridis and Sims[9] and a. Bankfalvi et al [10].

Sivridis and sims[9] had put forth a suggestion that this might be of potential value in predicting behaviour in breast carcinomas and would be very convenient for assessing aggressiveness in small incisional biopsy specimens, FNACs and imprint cytology.

The difference in pattern of silver binding nucleoli is described in a different context. Upon phytohaemagglutination stimulation, the first generation of transformed lymphocytes possess a single large nucleolus while proliferating cells of successive generations show multiple, small nucleoli. Alternatively in a relatively inactive cell the acrocentric chromosomes bearing AgNORs orientate in close apposition to each other forming a central, smooth nucleolus whereas in proliferating cell the chromosomal and therefore the AgNOR distribution remains disorganized resulting in formation of dispersed, multiple nucleoli. In an autonomously dividing cancer cell, this chromosomal disarray and the resultant abnormality of nucleolar contour might occur.

Though the AgNOR technique has its own limitations, it is easily performed on fine needle aspirates, is simple and inexpensive. It is helpful in distinguishing benign from malignant lesions, predicting high risk patients and also in indicating aggressiveness of tumour with prognostic implications.

Thus, the standardized AgNOR analysis and proper use of this method is a valuable tool and a very promising method for evaluation of cell kinetics and objective assessment of tumour differentiation in breast pathology and continues to find applications in numerous fields of research and diagnosis.

Conclusion : The number of AgNOR count gradually increases from normal breast tissue to the various lesions.Malignant lesions as compared to the benign lesions show a significant increases in AgNOR count and the count is in a higher range in malignant neoplasms having lymph node involvement.AgNORs in benign lesions are few, small and regular compared to numerous, larger and more variable size and share of AgNORs in malignant lesions.Thus, besides numerical alterations, morphologic changes can also be used as a prognostic parameter.Though there is a clear-cut distinction in the count between benign and malignant groups, some values from both groups lie in gray zone indicating overlapping.Statistical tests of significance like standard error, p-value and 'Z' test proves that the difference obtained in values of benign and malignant lesions and the malignancies with or without lymph node metastasis is biological and statistically significant and not coincidental.

Reference:

1. Khanna AK, Yadav SK, Dixit VK, Kumar M. AgNORcount and subjective AgNOR pattern assessment(SAPA) score in carcinoma of the pancreatic head including periampullarytumors. J Pancreas [Internet]. 2005;6(6):575–80. Available from: http://www.joplink.net/prev/200511/200511_04.pdf
2. Crocker J, Egan MJ. Correlation between NOR sizes and numbers in non-Hodgkins lymphomas. JPathol. 1988;156:233-9.
3. Contractor H, Ruschoff J, Hanisch T, Ulshofer B, Neumann K, Schultze-Seemann W, Thomas C.Silver stained structures in prostatic carcinoma:evaluation of diagnostic and prognostic relevanceby automated image analysis. Urol Int. 1991;46:9-14.
4. Khanna AK, Datta G, Kumar M. Correlation ofAgNOR with tumor size, stage of tumor, lymphnodestatus and grade of tumor in oral cancer. In: VermaAK, editor. Oral Oncology IV B. Bangalore, India:MacMillan India, 1995:25-30.
5. Khanna AK, Giri AK, Khanna A, Kumar M. Nucleolarorganizer region count and subjective AgNORpattern assessment (SAPA) score in skin tumors. JSurgOncol. 2001;78:273-8.
6. Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JCE. Silver-binding nucleolar organizerregions (AgNORs) in benign and malignant breastlesions: correlation with ploidy and growth phaseby DNA flowcytometry. J Pathol. l989;157:307-13.
7. Egan MJ, Crocker J. Nucleolar organizer regions inpathology. Br J Cancer. 1992;65:1-7.
8. Kumar Anand, Kushwaha Kumar Anand, Kumar Mohan, Gupta Saroj : ArgyrophilicNucleolar Organizer Regions. Their value and correlation with clinical prognostic factors in breast carcinoma. Journal of Surgical Oncology 1997; 65 : P 201-204.
9. Sivridis E. and Sims B. :Nucleolar Organizer regions new prognostic variables in breast carcinomas. J. Clin. Pathol. 1990 ; 43 : P 390-392.
10. Bankfalvi A., Ofner D., Schmid K. W., Schmitz K. J., Breukelmann D., Krech R., Bocker W. : Standardized in Situ AgNOR Analysis in Breast Pathology – diagnostic and Cell Kinetic Implications Pathology Res. Pract. 1999; 195 : P 219 – 229.
11. Rajeevan K/ Aravindan KP, and Chandrika K. : Value of AgNORs in fine needle aspiration cytology of breast lesions J. Pathol. Microbiol1995 ; 38 : P 17-24.
12. Dervan P. A., Gilmartin L. g., Loffus B. M. and Carnery D.N. : AgrytophilicNucleolar Organizer

Region counts correlate with Ki-67 scores. Am. J. Clin, Pathol. 1989 ; 92 : P 401 – 407.
13. Karmaker T., Radhika S., Gupta S. K. :Argyrophilicnucleolar organizer regioins(AgNORs) in breast lesions - a study on fine needle aspirates. Cytopathology 1995; 6 : P 5-13.
14. Dasgupta Anjali, Ghosh R.N., SarkarRanu, Laha R. N., Ghosh T. K., Mukherjee Chhanda : ArgyrophilicNucleolar Organizer Regions (AgNORs) in Breast Lesions. Journal of Indian Medical Association 1997 ; 95 : P 492 -494.
15. Agarwal P. K., MehrotraAnju, ChardraTulika : Diagnostic relevance of silver stained nucleolar organizer region (AgNORs) in benign and malignant breast lesions. Indian Journal of Experimental Biology 1995; 33: p 715-720.

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