Study of AgNOR count and SAPA score in fine needle aspirates of breast lumps

Dhakhwa R[1], Jha R[2], Sayami G[3], Shrestha HG[4]

[1]Lecturer, Department of Pathology, Kathmandu Medical College,
[2]Assistant Professor, Department of Pathology, Institute of Medicine,
[3]Professor, Department of Pathology, Institute of Medicine,
[4]Professor, Department of Pathology, Nepalese Army Institute of Health Sciences

Abstract

Background: Argyrophilic Nucleolar 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 Argyrophilic Nucleolar Organizer Region count and Subjective Argyrophilic Nucleolar Organizer Region Pattern Assessment score in fine needle aspirates of breast lumps.

Materials and Methods: A prospective, cross sectional study consisting of 110 patients (38 malignant and 72 benign) with clinically palpable breast lumps who underwent fine needle aspiration followed by subsequent histopathologic examination were included. Fine Needle Aspiration smears were studied by conventional methods and silver staining for Argyrophilic Nucleolar Organizer Regions. Histopathologic diagnosis was taken as the gold standard.

Results: Argyrophilic Nucleolar Organizer Region count and Subjective Argyrophilic Nucleolar Organizer Region Pattern Assessment score were helpful in differentiating benign from malignant lesions. Mean Argyrophilic Nucleolar Organizer Region count and Subjective Argyrophilic Nucleolar Organizer Region Pattern Assessment score were 2.63 ± 1.36 and 6.26 ± 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 Argyrophilic Nucleolar Organizer Region AgNOR count and Subjective Argyrophilic Nucleolar 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 Argyrophilic Nucleolar Organizer Region evaluation.

Key words: AgNOR, Fine needle aspirate, SAPA score.

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 were 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 Argyrophilic Nucleolar 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]. Meehan et al in a study of cytologic preparations of breast lesions, developed a Subjective AgNOR Pattern Assessment (SAPA) scoring system in which scores were assigned

Address for correspondence
Dr. Ramesh Dhakhwa
Lecturer, Department of Pathology,
Kathmandu Medical College, Kathmandu, Nepal
E-mail: rdhakhwa@gmail.com

keeping in mind the estimated number, size and shape of dots as well as clusters and their variation from cell to cell[9]. In the present study, both mean AgNOR count and SAPA scoring were used, and a comparison of mean AgNOR count with SAPA score in Fine Needle Aspiration Cytology (FNAC) smears of breast lumps was done.

Materials and methods

A cross sectional study was carried out from December 2006 to April 2008 in the Department of Pathology, Institute of Medicine. 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 by Crocker 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 Dextrene Polystyrene Xylene(DPX) and were examined under oil immersion (1000X). 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 at X 1000. 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. (Figure 1)

Statistical analysis

Diagnostic evaluation was done using SPSS version 15. AgNOR count and SAPA scores were examined for their diagnostic potential using different cutoff points and computing specificity and sensitivity based on which, Receiver Operative Curves (ROCs) were prepared.

Results

The AgNORs appeared as clustered dots. Representative areas with minimal cell overlap and no air-drying artifact were demarcated for counting in the silver stained smears. The AgNORs were visualized as black dots within the nucleus. Dots were defined as black homogenous silver precipitates with well-defined edges (Figure 2,3). The dots were scattered around the nucleus as satellites or grouped together as clusters. Overlapped dots with well defined edges were counted as greater than one when these appeared to be separate on viewing through all planes of focus.

The mean AgNOR count in benign breast lesions was 2.63 ± 1.36 while the SAPA score was 6.26 ± 1.19. The mean AgNOR count in malignant breast lesions was 8.42 ± 2.53 while the SAPA score was 10.05 ± 2.22 (Table 1 and 2). AgNOR staining showed many finely dispersed AgNOR dots within each nucleus, typical of malignancy. Three cases of breast carcinoma which were diagnosed as suspicious of malignancy on cytology showed AgNOR counts and SAPA score in the malignant range.

The area under the curve for AgNOR count was higher than for SAPA score in the ROC curve (Figure 4).The area under the curve for AgNOR count is highest for the value ≥6 (Figure 5, Table3). If the cutoff value for AgNOR count per nucleus is taken as six to designate malignancy, then the sensitivity is 89.5%; specificity, 88.9%; positive predictive value 82.2%; negative predictive value, 98.5% and the diagnostic accuracy is 95.5%.The area under the curve for SAPA score is highest for the value ≥8 in the ROC curve (Figure 6, Table 4). If the cutoff value of SAPA score is taken as eight to designate malignancy, then the sensitivity is 89.5%; specificity, 83.3 %; positive predictive value 73.9 %; and negative predictive value, 93.8% and the diagnostic accuracy is 85.5%.

Table 1: AgNOR count and SAPA score in benign lesions

Diagnosis	Number (%)	AgNOR Count	SAPA score
Fibroadenoma	34 (47.2)	2.6 ±1.54	6.09 ± 1.26
Gynaecomastia	17 (23.6)	2.23 ±1.09	6.59 ±1.0
Fibrocystic changes	7 (9.7)	2.71±1.38	6 ± 1.55
Fibroadenoma with fibrocystic changes	7 (9.7)	2.86 ± 1.21	5.86 ± 3.8
Chronic mastitis	3 (4.2)	3.3 ± 1.55	7.33 ± 1.55
Lactational adenoma	2 (2.8)	2	6
Tubular adenoma	1 (1.4)	3	7
Intraductal papilloma	1 (1.4)	5	7
Mean	72 (100)	2.63 ± 1.36	6.26 ± 1.19

Table 2: AgNOR Count and SAPA score in malignant breast lesions

Diagnosis	Number (%)	AgNOR Count	SAPA score
Infiltrating ductal carcinoma - NOS	32 (84.2)	8.31 ± 2.6	9.94 ± 2.2
Ductal carcinoma with mucinous component	1(2.6)	6	8
Infiltrating ductal carcinoma and ILC	1(2.6)	9	10
Invasive Papillary carcinoma	1(2.6)	9	12
Medullary carcinoma	2(5.2)	11.5 ± 0.7	13
Infiltrating lobular carcinoma	1(2.6)	7	8
Mean	38(100)	8.42 ± 2.53	10.05 ± 2.22

Table 3: Prediction for malignancy at different cut off values for mean AgNOR count

Mean AgNOR	Area under the curve	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Diagnostic accuracy
≥4	0.861	100	72.2	65.5	100	81.8
≥5	0.931	97.4	88.9	82.2	98.5	91.8
≥6	0.940	89.5	98.6	97.1	94.7	95.5
≥7	0.888	78.9	98.6	96.8	89.9	91.8
≥8	0.789	57.9	100	100	81.8	85.5

Table 4: Prediction for malignancy at different cut off values for SAPA score

SAPA score	Area under the curve	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Diagnostic accuracy
≥7	0.827	97.4	68.1	61.7	98	78.2
≥8	0.864	89.5	83.3	73.9	93.8	85.5
≥9	0.821	71.1	93.1	84.4	85.9	85.5
≥7	0.763	52.6	100	100	80.0	83.6

Discussion

The number of Argyrophilic Nucleolar Organizer Regions (AgNORs), their size and heterogeneity represent proliferative cellular activity, thus they can be used for detecting malignancy. Moreover, this technique is very simple and does not require special preservation or fixation of tissue. It can be performed in Fine Needle Aspiration (FNA) smears as well as in formalin fixed paraffin embedded sections[1].

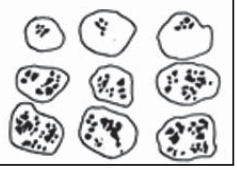
Parameter	Score	Illustration
Estimated number per cell		
Few (<5)	1	
Several (5-10)	2	
Many (>10)	3	
Variation satellite size and shape (score each)		
Uniform	1	
Moderate variation	2	
Marked variation	3	
Variation cluster size and shape (score each)		
Uniform	1	
Moderate variation	2	
Marked variation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

Fig. 1: Parameters used to calculate SAPA score

In this study, mean AgNOR count per nucleus and SAPA score in FNAC of malignant cases was 8.42 ± 2.53 and 10.05 ± 2.22 respectively as compared to benign cases, 2.63 ± 1.36 and 6.26 ± 1.19 respectively. The difference was statistically significant (P< 0.001) for both count and score. Three cases diagnosed as suspicious of malignancy on cytologic smears had AgNOR count and SAPA score in the malignant range.

The finding of a significant difference in AgNOR counts between benign and malignant breast lesions is not surprising, in view of the findings of other studies (Table 5). The counting method used in this study gave figures comparable to that cited by Khanna and Meehan but the figures considerably differed from those of Smith or Dervan[9-12]. The finding of significant differences in AgNOR might be because of different methods of counting and variable periods of incubation in silver nitrate[10]. In the present study, only clearly discernible dots were counted as separate and individual AgNORs. Attempts to estimate numbers of AgNORs in clusters based on relative sizes of satellite AgNORs or on the basis of vague dot outlines were avoided. Thus, some dots as large as clusters were counted as only one dot. The length of incubation was from 35-45 minutes.

The counting and scoring systems were of comparative diagnostic potential, although AgNOR count had a slightly greater value as evidenced from the ROC curve. However contradictory results were obtained in few of

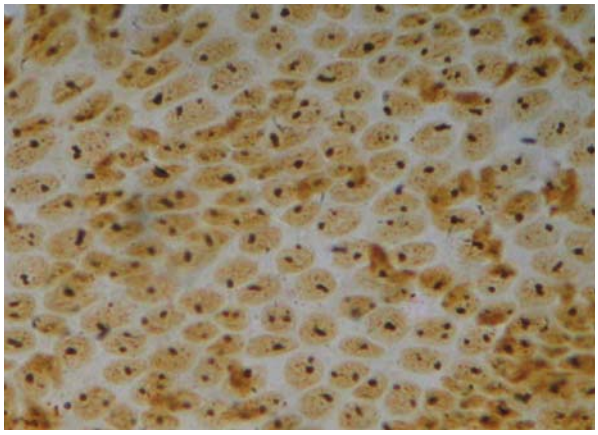


Fig. 2: Fibroadenoma: Mean AgNOR count 1.5; AgNOR 1, satellite 1+1, cluster1+1. Total SAPA score 5. (Silver Stain; magnificationX1000)

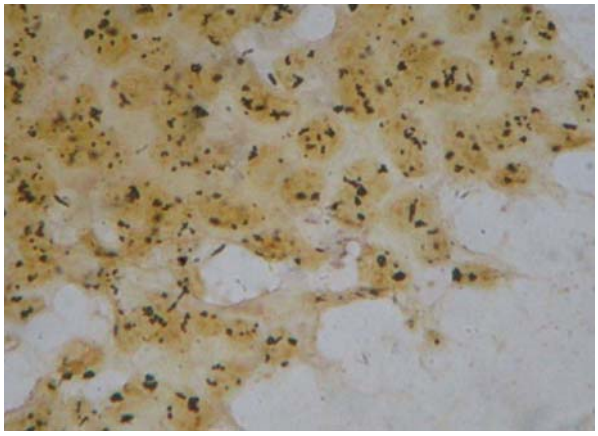


Fig 3: Ductal carcinoma: Mean AgNOR count 10, AgNOR 2, satellite 3+2, cluster 3+2. Total SAPA score 12. The smear is from an IDC-NOS, grade 3; tumor size 2 cm. (Silver Stain; magnification X 1000)

the cases. One case of carcinoma with low mean AgNOR count of five looked malignant with a SAPA score of nine. Similarly, one case of fibroadenoma had a mean AgNOR count of seven but SAPA score was five. Although the biologic significance of this finding, if any, is unknown, it indicates that consideration of both methods together may reveal better diagnostic accuracy.

A cut off level of six on AgNOR count per nucleus and eight on SAPA score was shown to have the greatest diagnostic potential. However, these cut off values are not absolute. A mean AgNOR count of five would have been considered malignant in Khanna's study[10] but is benign on present study. Similarly SAPA score of eight would be considered benign on Meehan's study[9] however, it is malignant in present study. Given the relatively wide variability of counts and SAPA score in

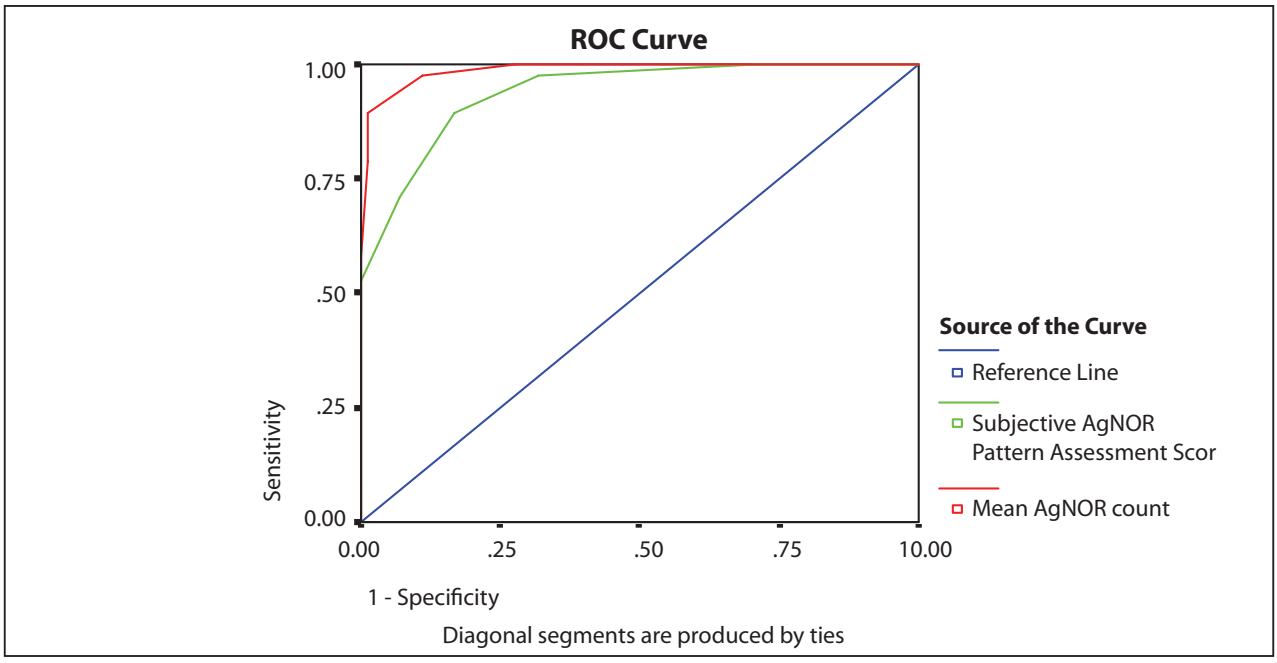


Fig 4 : ROC curve for AgNOR count and SAPA score

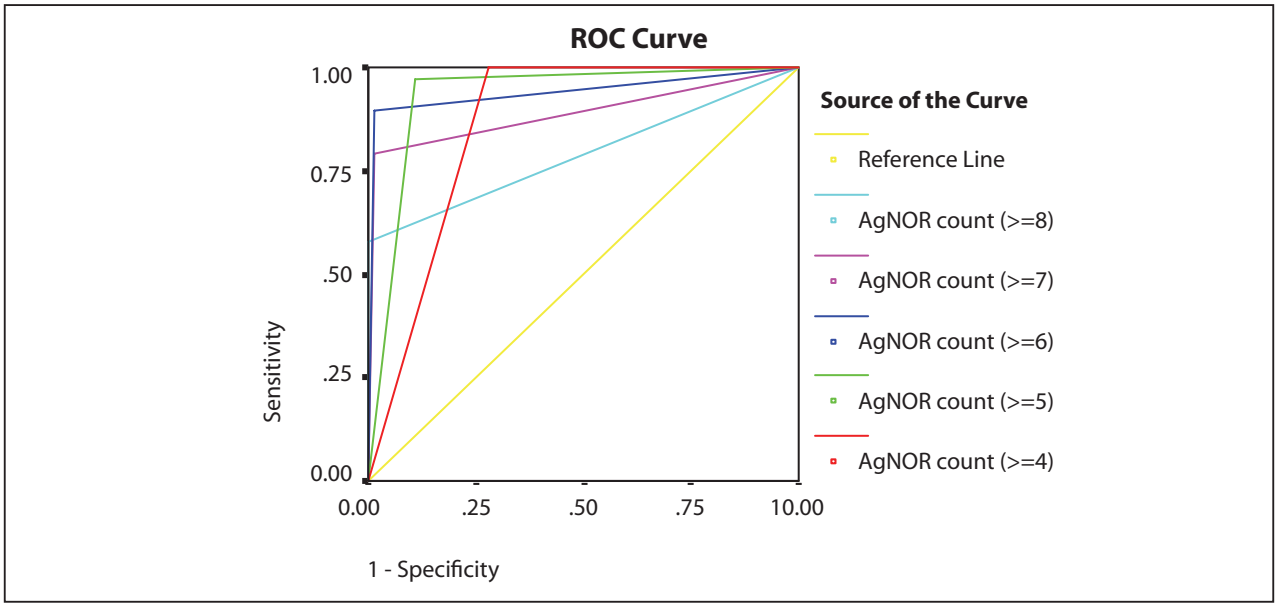


Fig. 5: ROC Curve for AgNOR Count at different cutoff values

other studies and the lack of clarity into the reason for this variability, each laboratory would likely have to establish its own cutoff levels to determine the optimum diagnostic use of AgNOR technique.

The application of the silver staining technique for NORs on FNA material has several distinct advantages over histologic sections. First using FNA, fresh tissue from the tumor is available for study[13]. Second, section thickness can produce variable AgNOR counts[14]. This factor is eliminated in smears obtained by FNA, and the AgNOR counts on smears are likely to more accurately represent the actual NORs present in the cell. Third, counting is easier and the appearance of the dots more easily discernible in FNAC smears as the smears are monolayer and the malignant cells easily detected from macrophages and stromal cells. However, tissue fluid or secretions or blood when present in the smear give the smear a dirty background which is disturbing in some of the smears[15].

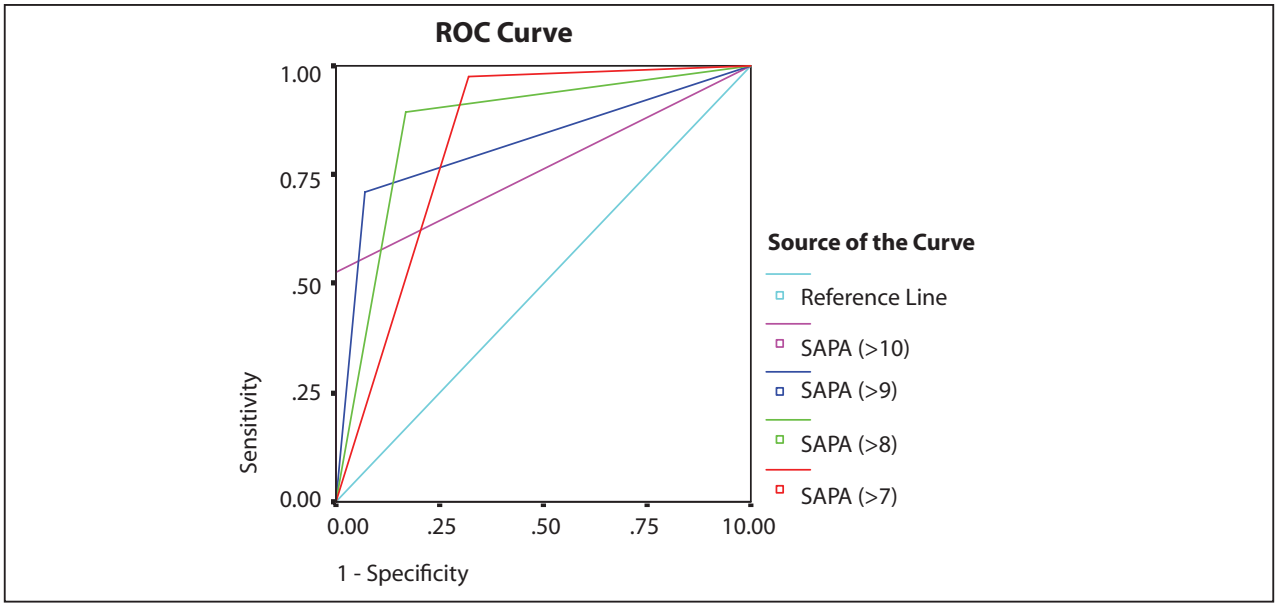


Fig 6: ROC Curve for SAPA score at different cutoff values

Conclusion

AgNOR count and SAPA score represent proliferating cellular activity and hence can be used for detecting malignancy. From a diagnostic point of view, AgNORs provide additional information, especially in cases that present difficulty in routine fine needle aspiration cytology. Subjective pattern assessment and AgNOR counting show comparable accuracy in distinguishing benign from malignant lesions, but may give contradictory results in some cases and are therefore more helpful when considered together. SAPA score is more convenient, reproducible and less time consuming than counting AgNOR dots.

References

1. Khanna AK, Yadav SK, Dixit VK, Kumar M. AgNOR count and subjective AgNOR pattern assessment (SAPA) score in carcinoma of the pancreatic head including periampullary tumors. 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. J Pathol. 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 relevance by automated image analysis. Urol Int. 1991;46:9-14.
4. Khanna AK, Datta G, Kumar M. Correlation of AgNOR with tumor size, stage of tumor, lymphnode status and grade of tumor in oral cancer. In: Verma AK, editor. Oral Oncology IV B. Bangalore, India: MacMillan India, 1995:25-30.
5. Khanna AK, Giri AK, Khanna A, Kumar M. Nucleolar organizer region count and subjective AgNOR pattern assessment (SAPA) score in skin tumors. J Surg Oncol. 2001;78:273-8.
6. Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JCE. Silver-binding nucleolar organizer regions (AgNORs) in benign and malignant breast lesions: correlation with ploidy and growth phase by DNA flow cytometry. J Pathol. l989;157**:**307-13.
7. Egan MJ, Crocker J. Nucleolar organizer regions in pathology. Br J Cancer. 1992;65:1-7.
8. Pich A, Chiusa I, Margaria E. Prognostic relevance of AgNORs in tumor pathology. Micron. 2000;31:133-41.
9. Meehan SM, Magee H, Carney DN, Dervan PA.The diagnostic value of silver nucleolar organizer region assessment in breast cytology. Am J Clin Pathol. 1994;101:689-93.
10. Khanna AK, Ansari MA, Kumar M, Khanna A. Correlation between AgNOR count and Subjective AgNOR Pattern Assessment Score in Cytology and Histology of Breast lumps. Anal Quant Cytol Histol. 2001 Dec;23(6):388 – 94.

11. Smith R, Crocker J. Evaluation of nucleolar organizer region-associated proteins in breast malignancy. Histopathology. 1988;12:113-25.
12. Dervan P, Gilmartin L, Loftus B, Carney D. Breast carcinoma kinetics: Argyrophilicnucleolar organizer region counts correlate with Ki67 scores. Am J ClinPathol. 1989;92:401-7.
13. Karmakar T, Radhika S, Gupta SK. Argyrophilic nucleolar organizer regions (AgNORs) in breast lesion – a study on fine needle aspirates. Cytology. 1999;6:5-13.
14. Crocker J, Boldy DAR, Egan M. How should we count AgNORs? Proposals for a standardized approach. J Pathol. 1989;185-8.
15. Basu A, Sanyal S, Bhattacharya A, Dasgupta S. A comparative study of silver binding nucleolar organizer regions (AgNORs) of breast lesions in histological sections and fine needle aspiration smears. J Indian Med Assoc. 1997 Aug;95(8):443-7.