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Significance of silver stained nucleolar organizer regions in benign breast diseases and breast cancer

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ABSTRACT

Silver stained nucleolar organizer regions (AgNOR's) in peripheral blood lymphocytes and biopsy specimens of benign and malignant breast tumor cases studied for a possible clinical relevance. Present study attempted to explore the role of number, size and nuclear diameter of AgNOR's in the peripheral blood lymphocytes (PBL's) in addition to the dispersion pattern and nuclear diameter in the biopsy specimens. For staining and destaining, method developed in our lab was used. Progressive increase in mean AgNOR counts in PBL's of breast cancer sample was found in comparison to benign and control group. In biopsy specimens overall NOR count showed a distinct histological type and stage wise correlation. Majority of the cells exhibited marked dispersion pattern (63.46%), in contrast to the cells from benign and control group which mostly showed simple dispersion pattern (43.5% and 79.7% respectively). Among dispersion pattern, marked dispersion showed a strong stage matched correlation. In benign breast tumor samples AgNOR's were of medium size and regular shape, while in breast cancer samples they were irregular and small. Thus the results highlight the diagnostic importance of number, shape and the dispersion pattern of AgNOR's in differentiating benign from malignant breast diseases.

Key words: Breast cancer, Fibroadenoma, Silver stain, Lymphocyte culture, Nucleolar organizer regions.

INTRODUCTION

Nucleolar Organizer Regions (NORs) are ribosomal DNA loops located on the short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22 [1]. Due their high affinity for silver ions they are also called as AgNOR. Using a silver staining technique, variability in the number of NORs from cell to cell has been demonstrated [2]. The biological significance of nucleoli and silver staining of mitotic chromosomes has been the subject of controversy for several years [3]. A quantitative relationship of AgNOR proteins has also been observed with several factors e.g. degree of cell maturity, cell cycle, proliferative activity and DNA ploidy [4].

In the beginning NOR's were silver stained using ammoniacal silver pretreatment step. By substituting aqueous silver nitrate (Ag) for the ammoniacal silver (As) pretreatment, a simplified technique, was developed. Ag-As technique further simplified by using an extended aqueous silver nitrate treatment alone[5]. The latter technique, known as Ag-I utilized an overnight incubation at 50ºC to achieve staining. A rapid one step technique was developed using two solutions [6]. This method needed warming up to 70ºC and good results for staining AgNORs

on chromosome preparations. This method also had the advantage of decreased background of silver precipitates, more uniform staining across the entire slide and increased shelf life.

Silver staining has been widely used, but the lack of a standard protocol and problems with background staining and silver precipitates make staining difficult [7]. At higher temperatures e.g., 70ºC less time is required than at room temperature. For optimal staining it is necessary to control various factors e.g. using of pure water and gelatin, clean plastic and glassware [8]. Considering the limitations in silver staining of NORs, Dhar et al. [9]introduced a new rapid AgNOR staining and destaining technique that has been used in the present study.

AgNOR studies in different types of cancers have aroused interest in the importance of AgNORs in breast cancer. Several studies have been conducted in the past to find out the importance of AgNORs, from that both positive and negative correlations has been reported [10]. AgNOR counts in breast carcinoma have been found to exceed significantly than those in benign lesions [11]. Diagnostic importance of AgNOR counts have also been suggested by many other researchers [11, 12]. Further, the metastatic potential of the cancer may be assessed by the AgNOR assay. Analysis of AgNOR by computer assisted system of image analysis, morphometric and other clinical parameters have show significant correlations with variations in NORs [13]. The subjective scoring of AgNOR size, shape and clustering indicate that the technique may tell more about the behaviour of malignant tumours than about benign versus malignancy [14]. The diagnostic and prognostic utility of AgNORs in breast cancer patients has received further supported by Hideo Kidogawa et al. [10]. There are only very few reports available on the study of lymphocytic AgNORs. Hence, the present study has been carried out to explore the role of number, size and shape of NORs in determining their diagnostic importance in differentiating benign from malignant breast lesions.

MATERIALS AND METHODS

Peripheral blood lymphocytes

Five milliliter of heparinized venous blood was collected under sterile conditions from healthy individuals (n=10), patients with fibroadenoma (n=31), patients with breast cancer (n=41). Blood culture was done using RPMI 1640 medium. After 48 hours of incubation colchicine was added to the culture at a final concentration of 5µg/ml. Cultures were further incubated for 2 hrs before terminating them for fixation and slide preparation. Cells were fixed in 3:1 methanol: acetic acid (v/v). Slides were prepared, dried and stored.

For staining, 50% silver nitrate, gelatin solution (prepared by dissolving 1g of gelatin in 49ml of distilled water) and 1ml of formic acid. The solution was stored in dark, and used within a week under refrigeration. For staining and destaining, method developed in our lab was used [9]. Slides were stained with 4 drops of silver nitrate and 2 drops of gelatin gently spread over the slide. Slides were gently warmed by passing to and fro few inches over the flame of Bunsen burner for 15-20 seconds till the slides turned golden yellow.

Biopsy Specimens

3µm thick paraffin sections of breast cancer biopsies and benign breast specimens were obtained from the pathology department and dewaxed in xylene. Tissue was rehydrated by passing it for 5 minutes each in descending grades of alcohol (absolute, 90%, 70%, 50%) and distilled water, and stained as described above.

Over stained slides were flooded with either 50% of hydrogen peroxide for one second or 25% of hydrogen peroxide for 15 seconds. The process was monitored under the microscope, and did not alter cellular architecture, composition an even restaining ability. The dissolved excess silver was washed away from the sections under running water. Standard protocols were followed for recording number, distribution of NORs and quantification of size and shape of AgNOR dots [8].

The following parameters were recorded using 100X oil immersion.

i) NOR count- mean count

ii) NOR size was measured using ocular micrometer(calibrated with stage micrometer). These dots were classified into 3 groups based on the basis of their diameter (small <1µm, medium 1-3 µm and large >3 µm).

iii) NOR shape: based on the shape classified into regular (with round or oval well defined margin) and irregular (with irregular serrated margin) dots. In each category the size (maximum diameter) of all dots were documented.

iv) NOR dispersion: the dispersion of NOR dots was classified as “simple, moderate and marked “depending on their scattering pattern within nucleus.

v) Nuclear diameter: measured using ocular micrometer at 100X times magnification.

Statistical Analysis

Data on mean NOR count, size wise distribution of dots and their dispersion pattern were tested using Oneway ANOVA. Wherever the test of homogeneity of variance showed high significance, the data were reanalyzed using square root transformation. For each type and stage, mean NOR count, standard deviation, 95% confidence interval, F-ratio and *P*-value were computed. The cumulative effect of age of patient with the type of breast disease and stage of cancer was assessed using Multivariant analysis (multiple ANOVA test).

RESULTS AND DISCUSSION

The present study has shown that AgNOR staining method can readily distinguish normal, bening and malignant tissues. This method has the potential clinico pathological value, especially in situations where tissue is insufficient for flow cytometry, such as small biopsies and limited needle aspirates. The AgNOR technique has also been used on chromosomal preparations to study the genetic disorders including trisomy 21 [15] and in leukemia [16] and other neoplasms. In clinical cytogenetics, AgNOR is useful to 1) to elucidate the morphology of chromosomal rearrangements, in particular to assess whether there has been gain or loss of genetic material, 2) to determine the chromosomal location of genes (in linkage studies) by providing a marker chromosome and 3) to determine the origin of chromosomal aberrations or cells in tissue culture. Studies on breast tissue suggested their diagnostic importance, to differentiate benign and malignant tumours [10]. Further the subjective scoring of AgNOR size, shape and clustering indicate that the technique may tell more about the behaviour of malignant tumours than about benign versus malignancy [14].

Histopahtological diagnosis of malignancy is routinely done by formalin fixation of the tissue followed by microscopic analysis of its stained section. However, these routine approaches may occasionally fail to precisely diagnose or provide adequate prognostic information [17]. It is therefore important to find new and reliable cellular markers that can overcome these limitations. The activity of nucleolar organizer regions differs from individual to individual and cell to cell. Quantification of AgNORs partly depends on the degree of dispersion of the relatively large number of AgNORs in the nucleus. Thus, the histological AgNOR count in tissues denotes a numerical index of dispersion rather than an absolute number. The AgNOR method is simple, reproducible and rapid, requiring no repeated antibody incubations as in immunohistochemical reactions. AgNOR staining has given encouraging results in some tumours, while in others it has been disappointing. It has been shown that hormonal treatment or viral infection alters the NOR frequency via gene amplification. Thus, while interpreting the results all confounding factors should be taken into consideration. Photographs of the present study are shown in figure 1.

Table 1: AgNORs counts in lymphocyte

Group	Sample size	Small size Mean ±S.D		Medium size Mean ±S.D		Large size Mean ± S.D	
1. Control	10	0.18	0.07	1.16	0.11	0.22	0.20
2. Fibroadenoma	31	0.08	0.08	1.41	0.41	0.40	0.25
3. Breast cancer (pooled from all stages)	41	0.31	0.45	1.50	0.39	0.46	0.36
Stages of breast cancer							
4. Stage I	10	0.17	0.10	1.29	0.16	0.66	0.24
5. Stage II	16	0.28	0.43	1.61	0.48	0.35	0.34
6. Stage III	06	0.73	0.84	1.58	0.54	0.33	0.17
7. Stage IV	09	0.33	0.26	1.51	0.25	0.51	0.52

Small sized- P< 0.05 (3v/s 2) Medium sized-P< 0.05 (3v/s 1)

In the present study, mean AgNOR count of blood lymphocytes in cancer patients (2.2/cell) was 1.2 times more in comparison to fibroadenoma and 1.4 times more in comparison to control group (Table: 1). However, no stage matched correlation was found. This non uniform correlation applied to all subgroups of AgNOR dots (i.e., small, medium and large). On closer inspection, the type wise analysis showed a progressive increase in the AgNOR count (from control to cancer vis a vis fibroadenoma), only for medium and large NOR dots. Thus, it is recommended that the analysis of numerical variation of NOR must be studied with reference to the size of NOR dots (Figure 2). However, irrespective of the size of NOR dots, mean NOR count was greater in malignant cells in comparison to normal cells, which supports the previous observations [18].

Table 2: Regular sized AgNORs counts in breast biopsy specimen cell

Group	Sample size	Small size Mean ± S.D		Med. size Mean ± S.D		Large size Mean ± S.D		TOTAL Mean ± S.D	
1. Control	10	0.68	0.17	1.25	0.44	0.04	0.01	1.98	0.59
2. Benign	49	1.84	1.32	1.08	0.54	0.01	0.00	2.94	1.63
3. Breast cancer	52	0.74	1.20	0.40	0.56	0.12	0.49	1.27	1.67
BENIGN									
4. Fibro	31	1.44	0.78	1.19	0.40	0.01	0.00	2.65	0.92
5. Fibro with EP	18	2.53	1.74	0.89	0.70	0.01	0.00	3.45	2.36
CANCER									
6. ID	09	1.02	0.83	0.73	0.46	0.21	0.28	1.96	1.28
7. IFL	11	0.75	1.23	0.49	0.75	0.03	0.03	1.28	1.69
8. IFD	32	0.66	1.30	0.28	0.47	0.13	0.61	1.07	1.75
Stages of breast cancer									
9. Stage I	07	0.27	0.30	0.80	0.51	0.27	0.30	2.33	1.22
10. Stage II	11	0.04	0.04	0.78	0.81	0.04	0.04	1.55	1.72
11. Stage III	13	0.02	0.03	0.25	0.29	0.02	0.03	0.95	1.28
12. Stage IV	21	0.18	0.76	0.17	0.36	0.18	0.76	0.97	1.89

Regular small sized P< 0.05 (2 v/s 3) (5 v/s 7, 8) (4 v/s 8), Regular medium sized P< 0.05 (3v/s 1, 2) (4 v/s 7, 8) (1, 5 v/s 8) (12 v/s 1, 9, 10), Regular large sized P< 0.05 (3v/s 2) (6 v/s 4)

Table 3: Irregular sized AgNORs counts in breast biopsy specimen cell

Group	Sample size	Small size Mean ± S.D		Med. Size Mean ± S.D		Large size Mean ± S.D		TOTAL Mean ± S.D	
1. Control	10	0.01	0.00	0.02	0.01	0.01	0.00	0.04	0.01
2. Benign	49	0.62	1.02	0.45	0.79	0.03	0.09	1.11	1.79
3. Breast cancer	52	2.89	2.14	1.67	1.07	0.22	0.25	4.78	2.93
BENIGN									
4. Fibro	31	0.35	0.70	0.22	0.41	0.01	0.02	0.60	1.02
5. Fibro with EP	18	1.09	1.32	0.86	1.10	0.05	0.15	2.01	2.43
CANCER									
6. ID	09	0.48	0.55	0.67	0.75	0.17	0.13	1.33	1.41
7. IFL	11	2.14	1.68	1.51	0.90	0.27	0.47	3.92	2.20
8. IFD	32	3.82	1.94	2.01	1.03	0.21	0.16	6.05	2.59
Stages of breast cancer									
9. Stage I	07	0.33	0.53	0.44	0.67	0.11	0.08	0.88	1.27
10. Stage II	11	1.29	1.22	1.13	0.78	0.30	0.45	2.73	1.73
11. Stage III	13	3.12	1.18	1.82	0.69	0.18	0.17	5.13	1.44
12. Stage IV	21	4.43	1.95	2.28	1.05	0.23	0.16	6.95	2.47

Irregular small sized P< 0.05 (3v/s 1,2), (8 v/s 1,4,5,6), (7 v/s 1,4), (12 v/s 1,9,10), (11 v/s 1,9,10), (10 v/s 1); Irregular medium sized P< 0.05 (3v/s 1,2), (8 v/s 1,4,5,6), (7 v/s 1,4), (12 v/s 1,9,10), (11 v/s 1,9) (10 v/s 1); Irregular large sized P< 0.05 (3 v/s 1,2), (8 v/s 1,4,5), (7 v/s 1,4,5), (6 v/s 1,4), (1 v/s 10, 11, 12); Irregular Total P< 0.05 (3 v/s 1, 2), (8 v/s 1, 4, 5, 6), (7 v/s 1, 4), (5 v/s 1), (12 v/s 1, 9, 10), (11 v/s 1, 9), (10 v/s 1, 9)

Table 4: Total AgNORs counts (Regular+Irregular) in breast biopsy specimen cell

Group	Sample size	Mean ± S.D		95% CONF. INT	F- Value
1. Control	10	2.02	0.59	1.59- 2.45	
2. Benign	49	4.06	1.27	3.70- 4.43	42.18
3. Breast cancer	52	6.06	1.99	5.51- 6.62	
BENIGN					
4. Fibro	31	3.25	0.70	3.00- 3.51	
5. Fibro with EP	18	5.46	0.67	5.12- 5.79	
CANCER					76.61
6. ID	09	3.31	0.19	3.16- 3.45	
7. IFL	11	5.21	0.89	4.61- 5.81	
8. IFD	32	7.13	1.61	6.55- 7.71	
Stages of breast cancer					
9. Stage I	07	3.23	0.11	3.12- 3.34	
10. Stage II	11	4.29	0.41	4.01- 4.56	123.50
11. Stage III	13	6.09	0.64	5.70- 6.48	
12. Stage IV	21	7.92	1.26	7.34- 8.49	

P< 0.05 (3 v/s 1,2) (2 v/s 1)(1 v/s 4,5,6,7,8) (4 v/s 7,8) (5 v/s 7,8) (6 v/s 8) (12 v/s 1,9,10,11) (11 v/s 1,9,10) (10 v/s 1,9) (9 v/s 1)

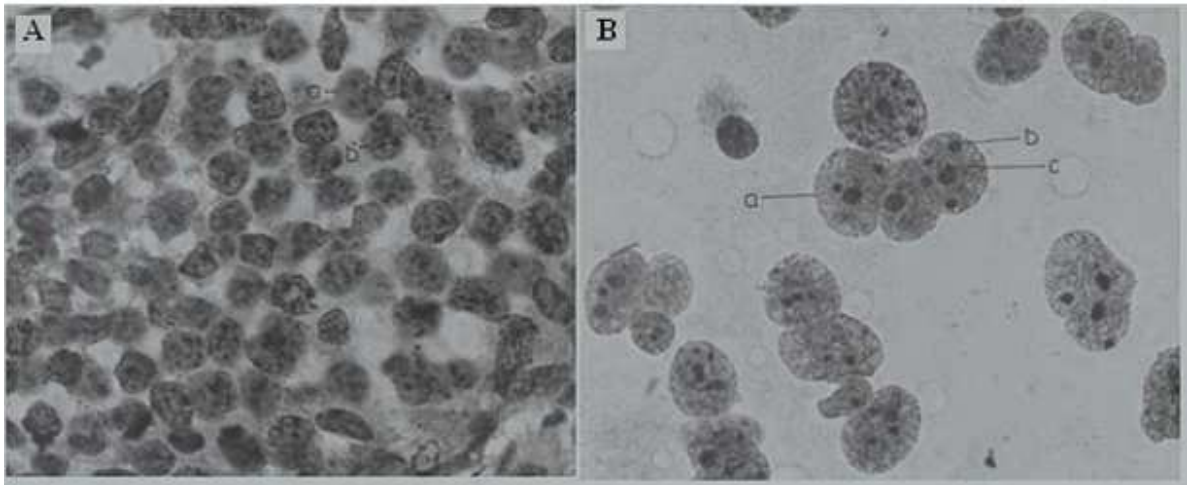

Figure 1: Silver stained AgNORs. In Tissues A- the markings a-Nucleus, b-AgNORs. In Blood lymphocytes B- the markings a- Nucleus, b- Medium sized, c- Large sized

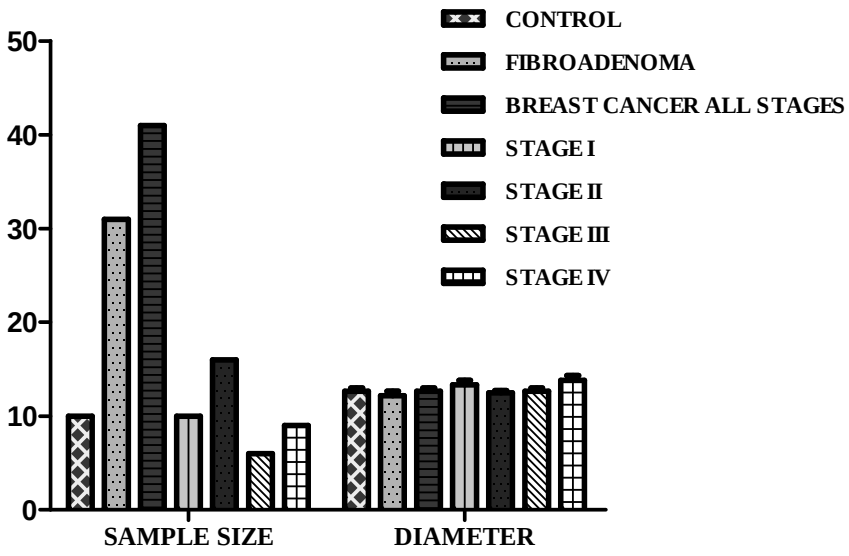

Figure 2: Nuclear diameter of lymphocytes

In tumours, majority of the cells exhibited marked dispersion (63.46%), in contrast to cells from benign and control group (Figure 3) which mostly showed simple dispersion pattern (43.5% and 79.7% respectively). However, analysis of benign groups showed more epitheliosis cells having moderate dispersion (46.3%), while simple dispersion was found in most of the fibroadenoma cells (49.5%). In cancer patients, most of the cells in all the three subgroups i.e., In situ Ductal (ID), Infiltrating Lobular (IFL) and Infiltrating Ductal (IFD) showed marked dispersion (i.e., 37.8%, 58.7% and 72.3% respectively). Of all the types of dispersions, marked dispersion showed a strong stage matched correlation. Number of cells showing marked dispersion increased 2.1 times from stage I to stage IV patients.

Present study attempted to explore the role of number, size and shape of NORs in determining their diagnostic importance in differentiating benign from malignant breast lesions. NORs were categorized into regular and irregular types, each comprising of small, medium and large dots. Overall, the NOR count increased two times from control to benign group and 1.5 times from benign to cancer patients. Within the benign category epitheliosis showed 1.7 times higher AgNOR count than fibroadenoma without epitheliosis. Among the various histological types of breast malignancies IFD formed the largest group, showing highest total AgNOR count (i.e; 7.13/cell),

while ID and IFL were 3.31/cell and 5.21/cell respectively. Further, a strong stage wise increase did not show a similar trend for all the shapes (i.e., regular and irregular) and size (i.e., small, medium and large) of NORs. A stage match increase in the frequency of NOR dots were observed in some cases of irregular dots (i.e; small and medium) and in none of the regular NORs irrespective of their size. In benign lesions AgNORs were of medium size and regular shape while in breast cancer cells AgNORs were often irregular, small sized. Results were tabulated in Table: 2- 4. Thus, the present study supports a strong diagnostic importance of the number, shape and the distribution pattern of the AgNORs in differentiating benign from malignant breast lesions [19]. The overall NOR count (regular and irregular; small, medium and large) showed a distinct histological type and stage wise increase (Table: 4). Thus, from the present study it is clear that the total NOR counts may not distinguish the different types of tumours. Thus, it is extremely important to classify NORs into various categories and study each group separately. Nuclear diameter in all biopsy samples were revealed in the figure 4. In this study NOR status in both tumour biopsy and blood samples were compared in order to assess their relative importance. A positive correlation in lymphocytes would qualify it as a supplementary parameter in diagnosing particular type or stage of tumour, and could even be proposed as a routine "non- invasive" parameter. The classification of NORs as small, medium or large is observer dependent and absence of standard guidelines, present findings cannot be compared unless similar categorization is followed. The reason for total irregular dots showing type wise correlation irrespective of their size is not clear. It is possible that this general increase observed in malignant tissue may be attributable to the proliferative activity of the cells [20], which might contribute towards the irregularity of the dots. In our study, an apparent increase in the mean AgNOR count in the breast cancer could be due to a) Active cell proliferation that leads to nucleolar dissociation leading to dispersion of AgNOR dots throughout the nucleus, b) Defect in nucleolar association resulting in AgNOR dispersion c) Increase in cell ploidy, resulting in prominence of otherwise inconspicuous AgNORs [21]. It has been generally found that quantity of interphase silver stained NOR proteins are strictly related to the cell proliferation, and grade of tumour.

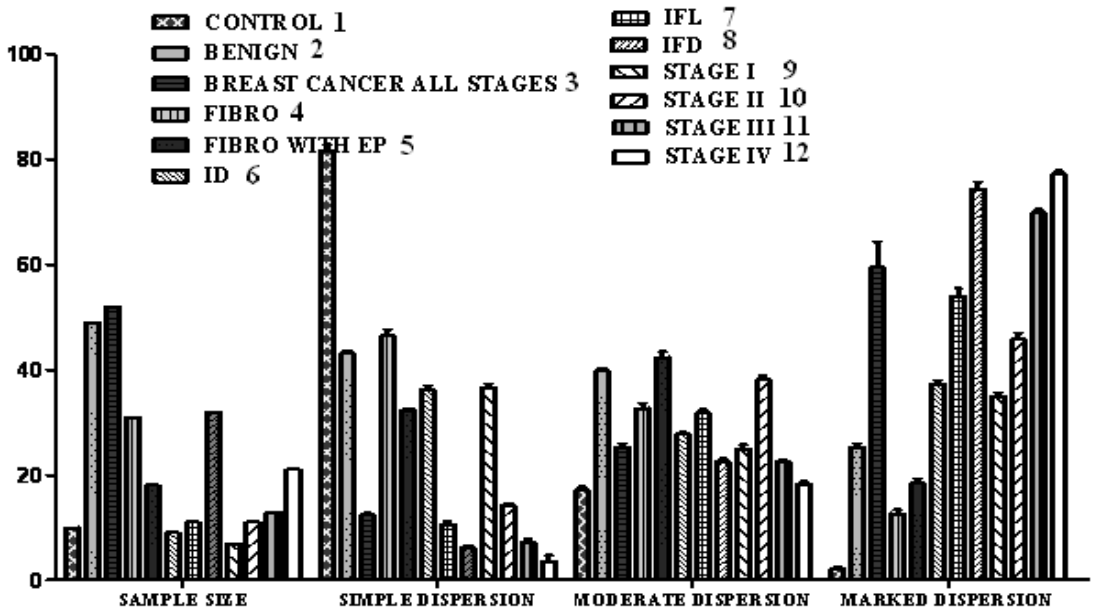

Figure 3 : Simple Dispersion (Percentage of cells)

P< 0.05 (1v/s 2, 3), (2v/s 3), (1v/s 4,5,6,7,8) (4&5 v/s 7,8), (1 v/s 9,10,11,12), (9 v/s 10,11,12), (10 v/s 11,12) ; Moderate Dispersion (Percentage of cells) P< 0.05 (2 v/s 1, 3), (5 v/s 1, 8), (10 v/s 1, 12); Marked Dispersion (Percentage of cells) P< 0.05 (3 v/s 1,2) (2 v/s 1) (8 v/s 1,4,5,6) (7 v/s 1,4,5) (6 v/s 1) (12 v/s 1,9,10)(11 v/s 1,9) (9,10 v/s 1).

In the present study, shortest staining time was used i.e., 20 seconds, which was standardized in our lab [9]. The chief consideration is that of background silver grain deposition. This has not been a problem in our experience except we have used less scrupulously clean plastic/ glass apparatus and pure water. Optimal results have been obtained with pure ethanol fixation which has been shown to be the ideal method for the demonstration of AgNORs in tissue slices. In this study, clearly discernible dots were counted as separate and individual AgNORs. Dots with

vague outlines were avoided. Perceived dot clarity is, however dependent upon fixation (alcohol 75% in this study, which gives optimum clarity), length of incubation in silver nitrate solution, and subjective impression of dot distinction, given that microscope examination is performed at 100X using oil immersion. Thus, all individually discernible AgNORs were enumerated while morphologically inseparable or clustered dots were not taken into account. AgNOR counts were of course, not absolute since NORs themselves are small compared with the section thickness. Quantification of total AgNORs in sections may be prone to high degree of observer variation. Given the relatively wide variability, each laboratory would likely to establish its own cutoff level to determine the optimum diagnostic use of the AgNOR technique. When the staining reaction is prolonged beyond the time for selective visualization of NORs, all the nucleolar structures are progressively stained, until the whole nucleolus appears homogenously stained by silver. It is therefore evident that different nucleolar structures have been stained and counted in various laboratories, and this has caused disagreement about AgNOR numbers reported in individual studies on the same neoplastic lesions. However, a major advantage of using the technique developed in our lab [9] was a total removal of these limitations. On heating as soon as color of the slide turned golden yellow, it indicated the endpoint of staining reaction. Thus, a possibility of over staining the slides was drastically minimized. In the present study, thickness equal to 3µm has been used in all the tumour biopsies to overcome some limitation. In order to improve the accuracy of recording NOR count, it is best to use automatic image analyzer [22].

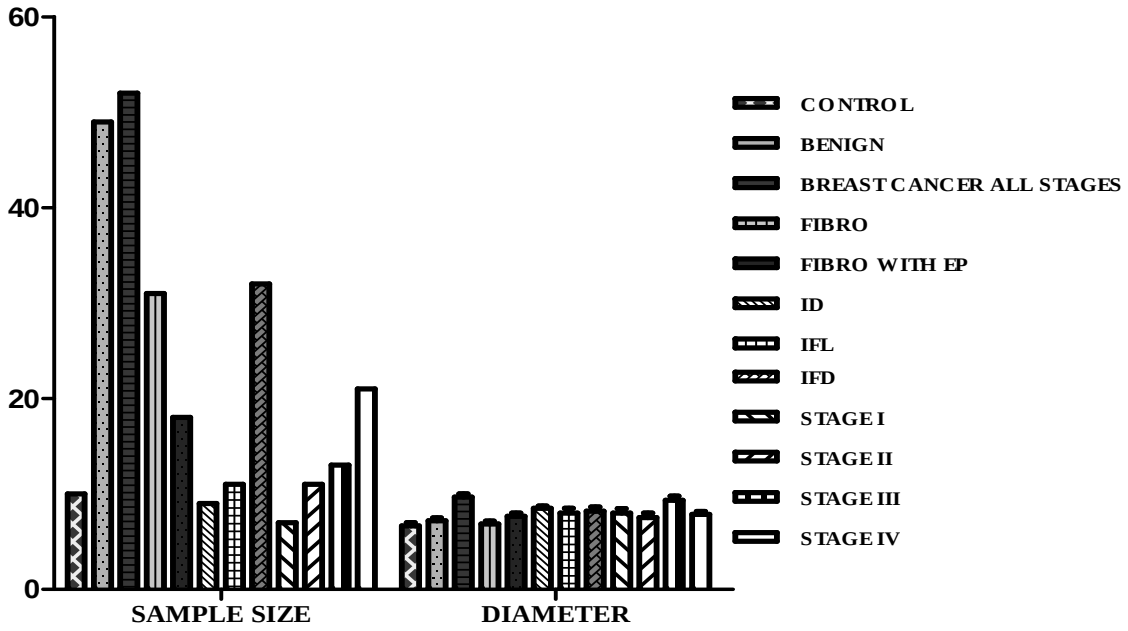

Figure 4: Nuclear diameter *P*<0.05 in (Breast cancer all stages v/s Control and Beningn), (IFD v/s Control, Fibro, Fibro with EP, ID), (IFL v/s Control, Fibro, Fibro with EP), (IDv/s Control, Fibro), (Stage II, III, IV v/s Control)

Nuclear size also plays a significant role in detection of malignant cells in a variety of cancers. Pleomorphism of cancer cell nucleus has been recognized as an important prognostic indicator in prostate cancer patients [23]. In the present study, it was not possible to measure nuclear roundness and nuclear volume. However, nuclear diameter was recorded both in lymphocytes and tumour cells. Lymphocytes showed non uniform variation in the diameter. Although an apparent stage wise (from stage I to stage IV) increase was found tumour cells, it was however statistically not significant. Thus, nuclear diameter was found to be unimportant in diagnostic evaluation of the tumour cells or blood lymphocytes.

The biological significance of nucleoli and mitotic silver staining has been the subject of controversy for several years. Equal amounts of argyrophilic proteins even in nucleoli with very low transcription rates have also been observed [24]. The significance of low AgNOR counts is also uncertain. Based on observed positive correlations of AgNOR counts with other markers of proliferation, such as Ki67 scores [25] and S-phase fractions [10], low AgNOR count may reflect lower proliferative activity. This idea is supported by studies of cells in culture showing

that higher AgNOR counts apparently shorter doubling times and greater proliferation rates than those with lesser amounts. From diagnostic point of view, AgNORs alone probably not useful in differentiating benign from malignant lesions, mainly because of the substantial false negative rate and an overlap in counts between the clear cut benign and malignant cases studied here. Although an overall increase in AgNOR count was observed in tumour and blood cells, it was necessary to analyze the role of age in contributing towards this increase. However, in the present study a distinct pattern between the age group and the distribution of AgNORs/cell could not be observed as the data showed a non uniform variation.

CONCLUSION

It might be speculated that in the coming years, the numbers, the shape and the distribution of AgNORs within the nucleus would become convenient in assessing aggressiveness in small incision biopsy specimens, needle aspiration cytology or imprint cytology. Given the close relationship of AgNOR quantity to proliferative state, the technique may tell us more about the behaviour of malignant tumours than about benignity versus malignancy. Subjective pattern assessment and AgNOR counting showed comparable accuracy in distinguishing normal from malignant lesions and are therefore helpful when considered together. However, the question still remains: how then, should we count AgNORs?, there are two approaches to this question. Firstly, all silver stained structures could be counted, but when lying in groups each cluster be treated as one structure. Secondly, where AgNORs can be seen separately within a nucleolus, each AgNOR could be counted as a unit, together with the smaller AgNORs seen outside the nucleolus. Both these techniques have a rational basis but are by no means mutually exclusive. Indeed, if the AgNOR count represents nucleolar disaggregation, which in turn reflects cellular activity, it is important to resolve AgNORs within the nucleoli. Proper attention should be paid to the staining timings and techniques involving shorter exposures should be preferably employed. In malignant tissues, the AgNORs become dispersed through the nucleus to a varying extent, enabling histologists to count them more readily. The counting of AgNORs is therefore, partly dependent upon the degree of dispersion or desegregation of the relatively large number of AgNORs in the nucleus. Thus, the AgNOR count in non malignant and malignant lesions denotes not only the absolute number of AgNORs but rather a numerical index of dispersion. This limitation does not, however, necessarily dilute the usefulness of AgNOR counts as discriminants of malignancy or transcriptional activity. In addition, the electron microscopic observations can also help in diagnostic pathology as fibrillar centers have been shown to be more numerous and smaller in cancers than their benign counterparts. However, our technique is less cumbersome than that of ultrastructural morphometry, since AgNORs can be studied in higher numbers and more rapidly than by means of electron microscopy. Furthermore, in the present study, AgNORs have been observed in the range of <1->3 microns in diameter, a more realistic measure of their size is made in 3 µm paraffin sections than in ultrathin sections. The use of computerized image analysis however, may be helpful in elimination of subjective variation in AgNOR counting. As with all the diagnostic models, the value of present findings should be tested in a prospective study.

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