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AgNOR and breast cancer. A study by image analysis

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Summary. The nucleolar organizer regions are used as a new marker in proliferation cell. AgNOR are loops of DNA actively transcribing to ribosomal RNA and they are associated with nonhistone proteins with strong argyrophile. In the present study we correlated the relationships between number and size of AgNOR measured by an automatic image analysis using CAS-100, in eleven breast carcinomas in different clinical stages and histological grades. We could identify the AgNOR with silver stain; the relationship between the number/size and histological grade/clinical stage shows that in normal breast we find one or two AgNOR but this number is larger when the carcinomas are less differentiated. When studied, the size is larger in the normal breast. We can make the same conclusions with the carcinomas which are lymph-node +.

Key words: Breast cancer, CAS-100, Image analysis, Nucleolar organizer regions, AgNOR

Introduction

The main problem that pathologists find in the diagnosis of a neoplasm, is to predict «what will be the clinical course of the lesion?. What is the prognosis?». A great number of prognosis factors have been implicated in breast cancer: clinical stage; histological type (Fisher et al., 1984); hormone receptors (Knight et al., 1977); and mitotic index, DNA aneuploidy (Eskelinen et al., 1989), etc. The study of the nucleolar organizer regions (NOR) demonstrate that they are loops of DNA actively transcribing to ribosomal RNA; they are associated with nonhistone proteins with strong argyrophile (Contractor et al., 1991). Their number and size seem to be related to cell proliferation, cell differentiation and malignant transformation, as the latest investigations have shown (Ruschoff et al., 1990).

During the last five years a silver-staining technique has been developed, demonstrating argyrophilic proteins associated with AgNOR. This procedure has been used by many pathologists because it can be applied to both formalin-fixed, paraffin-embedded tissue and cytological specimens. This new method has a potential advantage over immunohistochemical techniques like Ki67 where frozen or specially stored tissues is needed (Ruschoff et al., 1990). The proteins associated with AgNOR can be visualized with silver-staining as intranuclear black silver deposits. Recent studies (Dawson et al., 1991) have shown that it is possible to discriminate between benign and malignant tumors. However, the evaluation of AgNOR is only made by counting the silver-staining dots, which is at time-consuming procedure that leads to observer fatigue and consequent error. Therefore, it is necessary to use a method to obtain quantitative and objective data.

In this study we correlated the relationships between number and size of AgNOR-measured by an automatic image analysis using CAS-100- with histological grade and clinical state. We tried to find quantitative factors that may predict the prognosis of these neoplasms and to classify them into histological grades.

Materials and methods

Specimens

Eleven breast carcinomas in different clinical stages and histological grades and a normal breast as a control were used: 6 poorly differentiated; 2 moderately; and 3 well differentiated; 1 in T1, 9 in T2 and 1 in T3; 8 in No and 2 in N1; all cases are in Mx. They were fixed in formalin and paraffin embedded.

Silver stain for AgNOR

The technique to demonstrate AgNOR by a silver stain was described 15 years ago by Goodpasture et al., but in 1986 Ploton et al. (Ploton et al., 1986) introduced a modification in this technique which we could apply in paraffin-embedded and formalin-fixed tissues. Paraffin was dissolved by a bath of xylene and 100% ethanol.

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The sections were post-fixed in a 3:1 ethanol-acetic acid mixture and then rehydrated. The tissues were stained with a silver nitrate method for AgNOR protein sites, where the working solution is composed of 50% silver nitrate and gelatin solution. The sections were incubated in freshly prepared working solution for 45 mins at 28 °C. 2% thiosulphate sodium was used to take out the background and silver precipitate. They were then washed in distilled water, counterstained with fast red and dehydrated and mounted in Permount.

Digital Image Analysis

Cell measurement

Quantification of AgNOR was performed using the CAS-100 System. The image data were stored in the form of pixel optical density values. The conversion of light intensity values to optical density is based on previous standardization and calibration of the instrument. The cell measurement software included measurement of size, shape, sum density, average density and texture.

NOR analysis

Description some parts of the sofware: the point of gray-level thresholding and the choice of representative parameters. The previous report (Dawson et al., 1991) on the quantification of AgNOR dots define the NOR index as an absolute value, such as the number of NORs or NOR area, but we thought it may be more representative to use relative parameters such as N, A and N/A as an equivalent of AgNOR content of a given cell or tumour (Ruschoff et al., 1990). The morphological features that we measured for the same cell were: Number of AgNOR, OBSZ (object size), OBSH (object shape), OBSD (object sum density), OBAD (object average density), OBTX (object texture), OFFSET, N (= number of AgNOR/number of cells) and A (= OBSZ/number of AgNOR).

In order to be more objective, gray level threshold for binarization was only defined once for the whole slide. By choosing fields at random and avoiding non tumorous areas, we measured 50 cells in every case, with x 400 magnification, measuring the cells which were seen completely. Connective tissue and normal breast, which usually exhibited one single silver-stained dot, were measured and served as an internal control. Three typical cases in different histological grades were chosen: benign; poor; moderate and well differentiated; and we collected data of 100 nuclei from each case, obtaining the same morphological features.

The statistical analysis was performed with the statistical analysis system in this CAS-100, and we obtained the correlation matrix, inverse matrix, covariance matrix, bayesian classifier as a confusion matrix, Fisher histogram, Fisher linear discrimination and the histogram of each class (class 1 connective tissue, class 2 neoplastic cells) by the object size, separate and together for both classes.

Results

Staining procedure

The «black dots» can be identified with argyrophil proteins associated to AgNOR. When we reviewed different cases we could observe that the breast neoplasm had a higher number of «black dots» than the normal breast, where only one or two «black dots» were obtained (Fig. 1).

Table 1. Relationship between AgNOR, histological grade and clinical stage in brest cancer.

CASE No.	AgNOR				HG	CS	
	No.	N	A	N/A		T	LN
1	96	1.92	0.000483	3,950.91	3	2	-
2	84	1.68	0.000242	6,942.14	2	2	1
3	80	1.60	0.000875	1,828.57	1	3	0
4	134	2.68	0.000497	5,392.35	3	2	0
5	66	1.32	0.000315	4,190.47	3	2	1
6	57	1.14	0.002050	556.09	2	2	0
7	76	1.52	0.001260	1,206.34	1	1	0
8	65	1.30	0.001372	947.52	1	2	0
9	118	2.36	0.000168	14,047.61	3	2	0
10	52	1.04	0.001725	602.89	3	2	0
11	104	2.08	0.000867	2,399.07	3	2	0
Ctrl	79	1.58	0.001610	981.36	Ctrl	-	-

No.: number of AgNOR in every case; N: number of AgNOR/number of cells; A: size of AgNOR/number of AgNOR; T: tumor size; Ctrl: breast control; HG: histological grade; CS: clinical stage; LN: lymph node.

Table 2. Relationship between AgNOR and tumor size in breast cancer.

T	n	N	A	N/A
T1	1	1.520	0.001260	1,206.34
T2	9	1.723	0.000858	4,336.52
T3	1	1.600	0.000875	1,828.57
Ctrl	79	1.580	0.001610	981.36

Ctrl: breast control; n: number of cases; N: number of AgNOR/number of cells; A: size of AgNOR/number of AgNOR; T: tumor size.

Table 3. Relationship between AgNOR and lymphatic-node in breast cancer.

	n	N	A	N/A
N0	8	1.673	0.001138	2,978.59
N1	2	1.500	0.000278	5,566.30
Ctrl	79	1.580	0.001610	981.36

Ctrl: breast control; N0: Lymph-node - ; N1: lymph-node + ; n: number of AgNOR in every case; N: number of AgNOR/number of cells; A: size of AgNOR/number of AgNOR.

Relation between size, number of AgNOR, histological grade and clinical stage

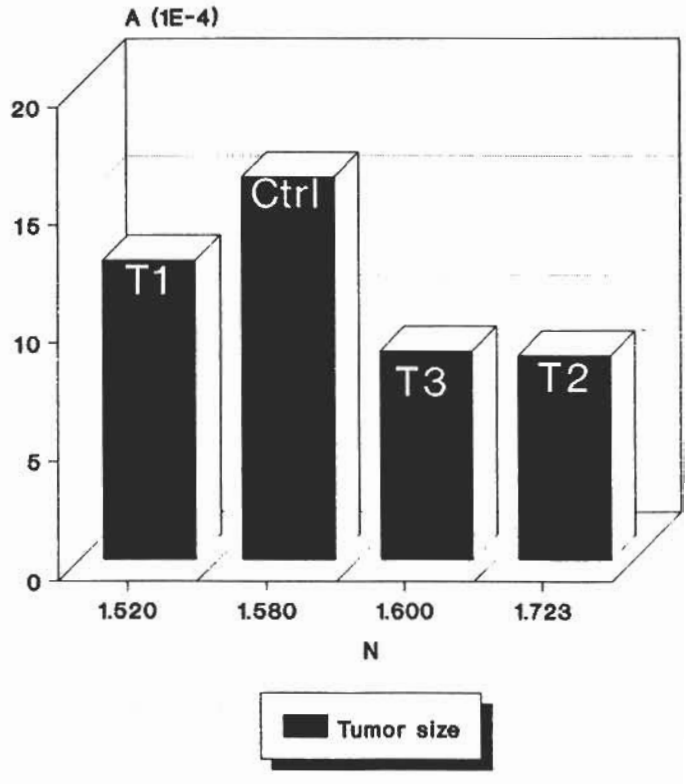
The size and number of AgNOR obtained with CAS-100 should be taken into account, but we did not use the absolute number. Instead we used the relative parameters N, A and N/A, and data of the clinical history of every case compared with the AgNOR (Table 1).

With the relation between tumor size and size/number of AgNOR we can see that in the normal breast and well-differentiated tumors, the number of AgNOR is less but with greater size than T2 and T3 (Graph 1) (Table 2). When the lymph-nodes were affected, the number of AgNOR was less than normal breast, and the quotient N/A (number of AgNOR by cells) was greater than

Table 4. Relationship between AgNOR and histological grade.

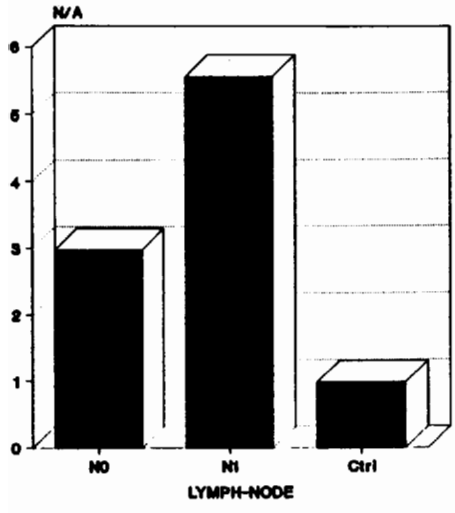
HG	n	N	A	N/A
3	6	1.90	0.0006763	5,097.16
2	2	1.41	0.001146	3,749.15
1	3	1.47	0.001169	1,327.47
Ctrl	1	1.58	0.001610	981.36

HG: histological grade; Ctrl: breast control; n: number of cases; N: number of AgNOR/number of cells; A: size of AgNOR/number of AgNOR.

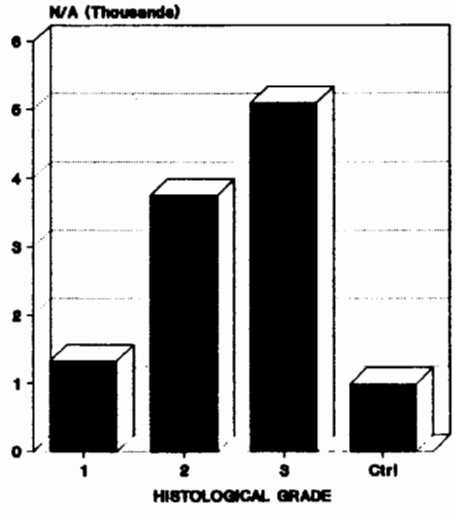


Graph 1. Tumor size and AgNOR (relationship between N and A).

Fig. 1. The «black dots» are the non-histone proteins with strong argyrophile associated with the AgNOR. Note the different numbers and size in each cell (arrows). x1,250



Graph 2. Lymph-node and AgNOR (quotient N/A).



Graph 3. Histological grade and AgNOR (quotient N/A).

normal breast and N0 (Table 3) (Grap 2).

There were a greater number of AgNOR in poorly-differentiated carcinomas, but they were smaller (Graph 3) (Table 4) and the N/A was higher in this type of carcinomas than in the well differentiated ones.

Discussion

AgNOR staining identifies NOR-associated proteins and NOR themselves (Ogura et al., 1992) and they could be detected easily and specifically by AgNOR staining. The use of AgNOR has the advantage that it can be used with the material that we obtained for routine process in a histological laboratory and we did not need special fixation, which Ki-67 needs, and the NOR-associated proteins have a longer half-life than other nuclear markers such as PCNA and Ki-67 (Ploton et al., 1986). Not all AgNORs are visible in normal histological sections, usually one or two may be present, free within the nucleus (Masai et al., 1992). The argyrophilia of the nucleolar AgNOR proteins is a good cytochemical marker of rDNA (Ruschoff et al., 1990). The AgNOR stainability gives information on the actual or potentially active substructures of the nucleolus and allows the study of their number, their spatial relationships within the nucleolus and also their behaviour during the phases of the cell cycle (Ploton et al., 1986). Different studies found positive relationships between Ki-67, and DNA index with the AgNOR counts; these results have led to speculation that the increased AgNOR counts might reflect increased cell proliferative activity, and there are several DNA flow-cytometry studies which confirmed AgNOR distribution with cell proliferation and differentiation (Contractor et al., 1991). The tumour proliferative activity is considered to be a good prognosis factor, and it is necessary to determine this because it reflects the potential biological aggressiveness and behaviour of the neoplasm (Ogura et al., 1992).

Controversial reports have been found that benign and malignant tumours could be easily discriminated by AgNOR: lung, reactive mesothelial cells from mesothelioma (WHO, 1982) and breast (Ruschoff et al., 1990; Eskelinen et al., 1991). Most studies have focused on counting the AgNORs in benign and malignant conditions, but some attempts have been made to apply this method in predicting the prognosis of cancers as well. The results involving cancer prediction have generally been disappointing (Ruschoff et al., 1990). The prediction of tumour progression and patient survival have significance in the management of human breast cancer; thus, a continuous search for the prognosis predictors is necessary and important.

Image analysis was applied to the problem of grading human breast carcinoma in order to obtain a reproducible objective grading of these tumors (Dawson et al., 1991). Automatic image analysis in the evaluation of AgNOR has the advantage over simply counting by eye as it involves the determination of AgNOR number and area. Crocker et al. (1988) found significantly larger and less frequent AgNOR in low-grade non-Hodgkin's lymphomas than in high-grade ones using the modified AgNOR technique of Ploton et al. (1986). Although it is still generally accepted that high-grade tumors exhibit numerous AgNORs compared to low-grade tumors, the method itself is still a point of discussion. The results in this study were very similar because they demonstrated an inverse correlation between the number and area of AgNORs with normal breast, whereas malignant tissue showed numerous but smaller AgNORs. AgNOR counts in cancer cells are higher than in normal or hyperplastic cells (Ogura et al., 1992; Ploton et al., 1992). The importance of the prognosis significance has been the subject of only few studies (Crocker et al., 1988; Sinn et al., 1989; Ruschoff et al., 1990), two of which

demonstrated the influence on survival in human tumors; but recently Crocker et al. (1988) and Trere et al. (1989) demonstrated that the number of AgNOR dots in one cell is an excellent index for the measurement of cell proliferation. In the same way, NOR staining with silver could also potentially appear as a prognosis index.

In breast cancer, a relationship between the AgNOR counts and malignancy has been reported (Sivridis and Sims, 1990), although the differences in the AgNOR counts between different malignancy grades have not been dramatic. This could also be confirmed in the present investigation, where the high grade tumours showed higher AgNOR counts than the low grade lesions. But in this study tumours with axillary lymph-node involvement (N1) at operation showed less and smaller AgNOR for cells than the tumours confined to the breast (N0). Eskelinen et al. have reported that AgNOR did not correlate with tumor size and lymph node status in breast carcinoma (Eskelinen et al., 1991), colon carcinoma, and myogenic tumors of the stomach (Sinn et al., 1989). No relationship was found between the number of AgNORs in primary tumor and presence of distant metastasis because these cases were not metastatic carcinomas. Our observations agree with Masai's study which showed a correlation of AgNOR with local size (Masai et al., 1992).

On the other hand Eskelinen et al. (1991) say that AgNOR counting does not add any significant information to the prediction of breast cancer even though the present findings clearly indicate that the mean number of AgNOR is directly related to the tumor size, lymph-node + and histological grade. Thus, it appears to be possible to use the mean number of AgNOR as an index of proliferative activity and is probably a good prognosis indicator (Ogura et al., 1992).

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