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QUANTIFICATION OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGIONS IN ESTROGEN RECEPTOR POSITIVE AND ESTROGEN RECEPTOR NEGATIVE DUCTAL BREAST CARCINOMAS

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Summary. *Argyrophilic nucleolar organizer regions (AgNORs) analysis of 20 estrogen receptor positive (ER+) and 16 estrogen receptor negative (ER-) invasive ductal breast carcinomas was performed. The quantification of AgNORs was determined by using morphometrical method in three ways: 1) mean number of AgNORs per nucleus counted on a 100 random nuclei, 2) mean number of AgNORs per nucleus counted on 200-300 nuclei in fields chosen by "the chess fields" method, and 3) mean number of AgNORs on the nuclear surface unit. A significant difference in mean number of AgNORs per nucleus and on unit of the nuclear surface between estrogen receptor positive (ER+) and estrogen receptor negative (ER-) invasive ductal breast carcinomas was observed ($p<0.001$). The mean number of AgNORs was higher in ER– than in ER+ invasive ductal breast carcinomas. Despite interobserver variability in counting AgNORs, comparison was completed as well as the evaluation of validity of three stated ways of quantification of nucleolar organizers. Our results suggest more rapid proliferative activity in ER- than in ER+ breast cancers.*

Key words: *Ductal breast cancer, argyrophilic nucleolar organiser regions (AgNORs), estrogen receptor, morphometry*

Introduction

Nucleolar organizer regions (NORs), segments of DNA closely associated with nucleoli, contain coding genes for ribosomal RNA and contribute to the regulation of cellular protein synthesis (1). Nucleolar organizer regions are closely associated with argyrophilic proteins, and a modification of a silver staining technique, long used by cytogeneticists, allows NORs to be visualized in conventional histologic sections, where they are called argyrophilic nucleolar organizer regions (AgNORs) (2). The increased number of AgNORs is the result of more active cellular proliferation, distorted nucleolar association, increased ploidy or greater transcription activity (3). Initially used as a parameter for the diagnosis of malignancy, the AgNOR parameter was found to be more useful for assessing the prognosis of cancer disease.

The AgNOR technique has been evaluated in breast lesions. A statistically significant difference in the mean number of AgNORs was found between normal, ordinary hyperplastic and neoplastic breast lesions (4). The colloidal silver staining of the nucleolar organizer regions - AgNORs has been used in distinguishing benign from malignant breast lesions (4,5,6). The increased number of AgNORs was found in breast cancer versus fibrocystic disease and fibroadenomas (5). Other studies have evaluated the prognostic significance of AgNOR counts correlating it with patients' outcome and with proliferation indices assessed by flow cytometry or immunohistochemistry (7-12). Furthermore, AgNOR count in benign and malignant breast lesions correlate with Ki 67 scores and provide significant kinetic information (1,8).

The breast is a target organ for estrogens and progesterone and these hormones control several functions of the normal and abnormal mammary epithelium including cell proliferation. Most of the actions of estrogens and progesterone are mediated via specific steroid receptors. The likelihood of a favorable response to ablative or additive hormonal therapy and aggressiveness of breast cancer are related to estrogen receptors (ER).

The aim of this study was to estimate mean number of AgNORs on nucleus and on unit of the nuclear surface in ER+ and ER- ductal breast carcinomas using morphometrical method.

Materials and Methods

Samples of 36 invasive ductal breast carcinomas diagnosed at Institute of Pathology in Niš from 1991 to 1992 were used for analysis.

I.R.M.A. method was performed for the determination of estrogen receptors (ER). ER were considered as positive, with value greater than 30 units/ml of cytosol. Breast cancers were divided into two groups according to ER: estrogen receptor positive (ER+) and estrogen

receptor negative (ER-). There were 20 ER+ and 16 ER– individual cases.

To demonstrate the AgNORs, the silver staining procedure described by Crocker et al. (1987) was performed on 5 μm thick paraffin sections (13). The silver colloid solution for staining of NOR was prepared by dissolving gelatin in 1% aqueous formic acid at a concentration of 2%. This solution was then mixed 1:2 volumes with 50% aqueous silver nitrate to obtain the working solution. The staining time was 45 min and silver reaction was carried out in the dark.

For quantification of Ag32NORs a 100× oil immersion objective and test system M 42 were used. The number of AgNORs was determined in three ways:

1. The mean number per nucleus counted on a 100 random nuclei by an eyepiece graticule;
2. The mean number per nucleus counted on 200-300 nuclei in fields chosen by "the chess fields" method, with the preliminary estimation of the needed field number for analysis with deviation not bigger than 10%.
3. The mean number of AgNORs on unit of the nuclear surface.

Statistical significances were obtained by ANOVA and t-test.

Results

In the first way, the numbers of AgNORs per nucleus are listed in Table 1. The mean number of AgNORs was higher in ER– than in ER+ invasive ductal breast carcinomas (Fig. 1).

Table 1. The mean number of AgNORs per nucleus counted on 100 accidental nuclei - the first way

Status ER	N	$\bar{x}$	SD	SE	p level
ER+	20	3.358	0.242	0.055	p<0.001
ER-	16	4.525	0.565	0.145	

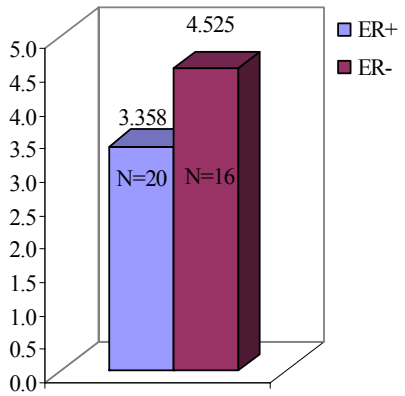

Fig. 1. The mean number of AgNORs per nucleus counted on a 100 random nuclei - the first way

In the second way, the numbers of AgNORs per nucleus are listed in Table 2. The mean number of AgNORs was higher in ER– than in ER+ invasive ductal breast carcinomas (Fig. 2).

Table 2. The mean number of AgNORs per nucleus counted on 200-300 nuclei in fields chosen by "the chess fields" method – the second way

Status ER	N	$\bar{x}$	SD	SE	p level
ER+	20	3.03	0.36	0.08	p<0.001
ER-	16	3.85	0.22	0.06	

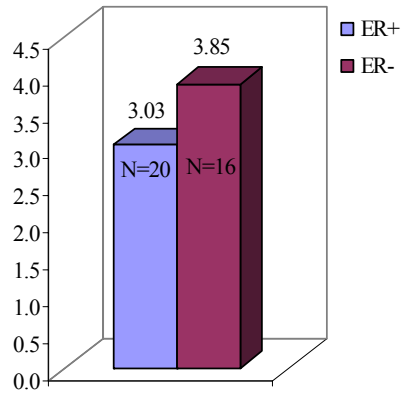

Fig. 2. The mean number of AgNORs per nucleus counted on 200-300 nuclei – the second way

In the third way, the numbers of AgNORs on unit of the nuclear surface are listed in Table 3. The mean number of AgNORs was higher in ER– than in ER+ invasive ductal breast carcinomas (Fig. 3).

Table 3. The mean number of AgNORs on unit of the nuclear surface – the third way

Status ER	N	$\bar{x}$	SD	SE	p level
ER+	20	0.019	0.005	0.001	p<0.001
ER-	16	0.029	0.006	0.002	

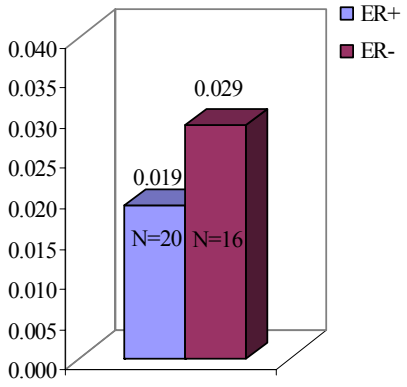

Fig. 3. The mean number of AgNORs on unit of the nuclear surface – the third way

A significant difference was found in mean number of AgNORs per nucleus and on unit of the nuclear surface between ER+ (Fig. 4) and ER- invasive ductal breast carcinomas (Fig. 5) in the three way of quantification, on the level of statistical significance $p < 0.001$.

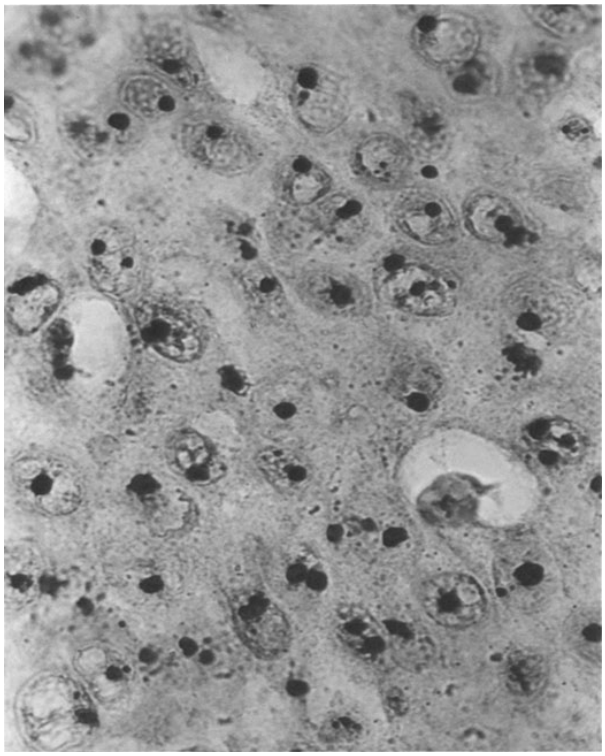

Fig. 4. Argyrophilic nucleolar organizer regions (AgNORs) in ER positive invasive ductal breast carcinoma. ×1000.

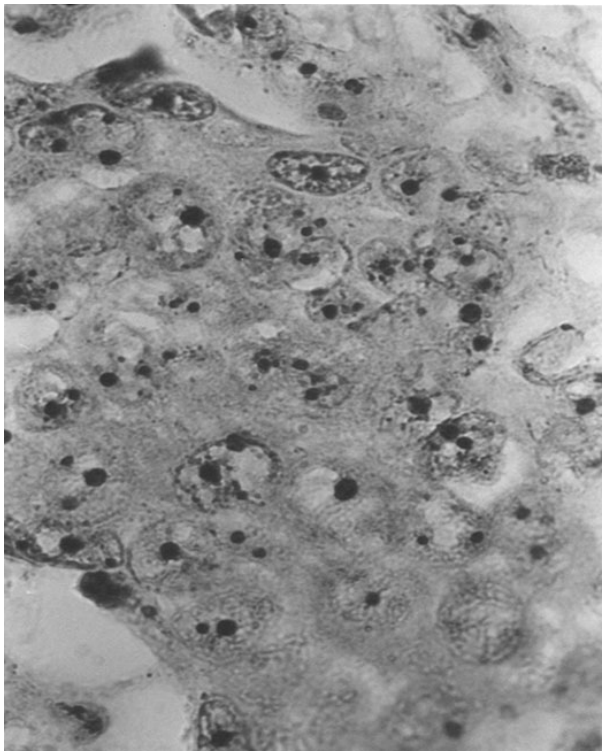

Fig. 5. Argyrophilic nucleolar organizer regions (AgNORs) in ER negative invasive ductal breast carcinoma. ×1000.

Discussion

The main prognostic factors of breast carcinoma are histological grade, tumor size, lymph node status and vascular invasion. Main predictors of therapeutic response are estrogen receptor and ERBb2 status. In the present study two groups of breast cancer patients were defined by estrogen receptor status.

The amount of argyrophilic nucleolar organizer regions (AgNORs) represents a cell kinetics parameter used in tumour pathology for prognostic purposes. The shorter the cell cycle, the greater the synthesis of rRNA for each time unit and, therefore, the quantity of AgNORs present in the nucleolus. Thus, the AgNOR value was thought to be a measure of the rate of cell proliferation. AgNOR expression is directly related to the rate of ribosome biogenesis, which has been recently shown to be controlled also by the tumour suppressor proteins pRb and p53. From the clinical point of view, cancers with changes in pRb and p53 status are generally more aggressive than those with normally functioning pRb and p53 pathways. Derenzini et al. (2004) found that the prognostic value of the AgNOR parameter depends on the status of the tumour suppressor proteins pRb and p53, and it cannot be ascribed to the relation between AgNORs and the cell proliferation rate (12).

During our research, an average number of AgNORs in cell nucleus of invasive ductal breast cancer was determined in relation to status of estrogen receptors. A statistically significant difference between ER+ and ER- invasive ductal breast carcinomas was observed. Similarly as the results reported by Raymond *et al*. (14), Guski *et al*. (15) and Günther *et al.* (16), the mean number of AgNORs, in our study, was inversely proportional to the status of estrogen receptors. This reflects biological and functional differences between ER+ and ER- breast cancers. AgNOR analysis bears a significant potential for characterizing cell kinetic and metabolic activity of breast lesions. This may provide us an insight into the biological background of breast carcinogenesis, differentiation and tumor progression and may also underlie the independent prognostic value of AgNORs in breast cancer (4).

Most commonly, the average number of AgNORs is determined per nucleus with the analysis of 100 randomly chosen nuclei (1,17). We consider that the estimation of AgNOR number for the nuclear surface unit of the examined cells, would render more precise and accurate data. This approach includes not only the number, but also the size of cell nucleus. In respect of the fact that AgNORs shows the different size and tendency to combine and aglomerate, it is very difficult to determine their number, and that is why we find it necessary to determine their quantity as well. During our research, an average number of AgNORs in cell nucleus of invasive ductal breast cancer was determined using morphometrical method as the mean number on nucleus and on the unit of nuclear surface; afterwards, the obtained results were compared. Despite interobserver variability

in counting AgNORs (results from the second way were lower than the results obtained from the first way), there was an agreement between results obtained in three ways of quntification of nucleolar organizers regions.

Up to now, more than a thousand papers have been published on the use of the interphase AgNOR measurement in tumor pathology. The AgNOR number is related to the level of ribosomal biogenesis and rapidity of cell cycle (18).

Conclusion

Our results suggest more rapid proliferative activity in ER- than in ER+ breast cancers.

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KVANTIFIKACIJA ARGIROFILNIH NUKLEOLARNIH ORGANIZATORA U ESTROGEN RECEPTOR POZITIVNIM I ESTROGEN RECEPTOR NEGATIVNIM DUKTALNIM KARCINOMIMA DOJKE

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Kratak sadržaj: *U ovom istraživanju analizirani su argirofilni nukleolarni organizatori (AgNOR) kod 20 estrogen receptor pozitivnih (ER+) i 16 estrogen receptor negativnih (ER-) invazivnih duktalnih karcinoma dojke. Kvantifikacija AgNOR-a je sprovedena morfometrijskom metodom na tri načina: 1) određivanjem prosečnog broja AgNOR-a po jedru ćelije brojanjem na 100 slučajno odabranih jedara, 2) određivanjem prosečnog broja AgNOR-a po jedru ćelije brojanjem na 200-300 jedara na svakom drugom vidnom polju i određivanjem prosečnog broja AgNOR-a na jedinicu površine jedra. Nađena je statistički signifikantna razlika u prosečnom broju AgNOR-a po jedru i po*

jedinici jedarne površine između ER+ i estrogen receptor negativnih ER- invazivnih duktalnih karcinoma dojke ($p < 0,001$). ER - karcinomi dojke imaju signifikantno veći broj AgNOR-a po jedru u odnosu na ER + karcinome. Izvršena je komaparacija sva tri načina kvantifikacije. Rezultati ovog istraživanja ukazuju na veću proliferativnu aktivnost u ER- u odnosu na ER+ karcinome dojke.

Ključne reči: *duktalni karcinom dojke, argirofilni nukleolarni organizatori (AgNOR), estrogeni receptori, morfometrija*