

PROGNOSTIC SIGNIFICANCE OF MORPHOMETRIC PARAMETERS OF NUCLEOLI AND NUCLEI OF INVASIVE DUCTAL BREAST CARCINOMAS

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The aim of this study was to evaluate associations between seven morphometric parameters of the nucleoli and nuclei of methyl green and pyronin Y (MG-PY) stained tumour cells of invasive ductal breast carcinoma with relapse-free survival (RFS) and overall survival (OS) time. Histological sections from 150 invasive ductal breast cancers were stained with MG-PY and the following parameters were evaluated by computer image analysis: the nucleolar area, long to short nucleolar axis ratio, nucleolar shape parameter assessing the degree of nucleolar roundness, long to short nuclear axis ratio, number of nucleoli in the nucleus and the percentage of the nuclear cross-section surface area occupied by the nucleoli. A statistically significant association between a nucleolar shape polymorphism and the number of nucleoli in the nuclei of tumour cells and the RFS but not OS was found in the entire group of patients as well as patients with axillary lymph node metastases. A higher polymorphism of nucleolar shape and a higher number of nucleoli in the nuclei of breast cancer cells were associated with decreased relapse-free survival ($p < 0.05$). The remaining morphometric parameters showed no statistically significant association with RFS or OS. The results indicate that morphometry of nucleoli in MG-PY stained histological sections can be useful in the analysis of associations between nucleolar parameters and prognosis of patients with invasive breast cancer.

Key words: breast carcinoma, morphometry, nucleus, nucleolus, prognosis.

Introduction

The main function of the nucleolus is to control ribosome biogenesis and cell proliferation, therefore the size of the nucleolus depends on the cell type, cell cycle phase and cell doubling time [1-5]. Apart from its main function, the nucleolus also plays an important role in other mechanisms involved in carcinogenesis and tumour progression such as for example regulation of the activity of components of the telomerase enzyme complex and stress-induced stabilization of p53 [6-10]. In histological sections stained with haematoxylin and eosin, the nucleolus is seen as a round and pink body because due to its protein content it stains with eosin. However, in

haematoxylin and eosin stained sections the nucleolus cannot always be detected. The nucleolus can also be visualized by electron microscopy or by a silver-staining procedure which visualizes a group of acidic proteins associated with nucleolar organizer regions so argyrophilic nucleolar organizer regions (AgNORs) can be analysed [11]. Finally, the nucleoli can be revealed in formalin-fixed, paraffin-embedded tissue using a relatively simple methyl green and pyronin Y (MG-PY) method [12]. Although nucleolar structural changes in tumour cells are an important element in the constellation of morphological indicators of malignancy, the diagnostic value of nucleolar size is low because of

overlapping of the value of the nucleolar size of malignant and benign lesions. However, nucleolar size revealed by AgNOR staining has been shown to predict the clinical outcome of breast cancers in the majority but not all studies [reviewed in 3, 13, 14]. The aim of this study was to evaluate the association of seven morphometric parameters of nucleoli and nuclei of MG-PY stained tumour cells of invasive ductal breast carcinomas, with relapse-free survival (RFS) and overall survival (OS) time.

Material and methods

Patients

A group of 150 patients with invasive ductal breast carcinoma was analysed in this study. The patients were subjected to mastectomy with axillary lymphadenectomy in the West Pomeranian Center of Oncology in Szczecin. No pre-operative treatment was employed. An adjuvant hormone therapy alone was administered in one case, chemotherapy alone in 29 cases and radiotherapy alone in 40 cases. The remaining patients received either a combination of two of the above therapeutic modalities or all three of them. Eight patients did not receive any postoperative treatment. In 13 cases, details on adjuvant treatment were not available. The mean follow-up

was 87.8 months (range: 2.3-148 months). Relapse-free survival was defined as the time interval between the date of surgery and the date of the first relapse. Relapses occurring between 4.3 months and 60 months were observed in 49 patients. A relapse was defined as a local-regional relapse (local relapses, metastases to supraclavicular lymph nodes), visceral or bone metastasis. A total of 109 patients survived 60 months after treatment, while 41 patients died between 2.3 and 60 months post operation.

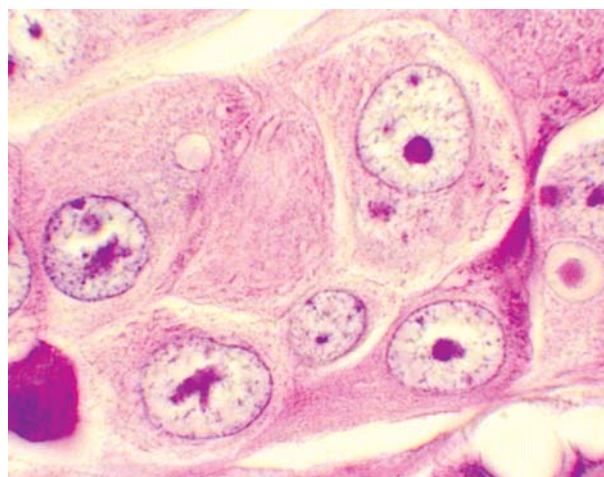


Fig. 1. Invasive ductal breast carcinoma. Note large polymorphic nucleoli (MG-PY stain, magnification 1000×)

Table I. Morphometric parameters of nuclei and nucleoli of breast cancer cells and percentage of patients with 60-month relapse-free survival in the whole study population, in the axillary node-negative (LN-) and node-positive (LN+) subgroups

PARAMETER	ALL PATIENTS (n = 134)		LN- PATIENTS (n = 41)		LN+ PATIENTS (n = 93)	
	[%]	p	[%]	p	[%]	p
ANCL						
> 1.53	70.8	NS	79.2	NS	65.9	NS
≤ 1.53	56.5		76.5		50.0	
L/B NCL						
> 1.73	63.6	NS	84.2	NS	55.3	NS
≤ 1.73	63.2		72.7		58.7	
SF NCL						
> 3.27	54.4	< 0.05	73.7	NS	49.9	< 0.05
≤ 3.27	72.7		81.8		68.3	
AN						
> 38.88	57.7	NS	75.0	NS	50.0	NS
≤ 38.88	69.7		80.9		64.4	
L/B N						
> 1.55	61.2	NS	76.9	NS	51.2	NS
≤ 1.55	65.7		80.0		61.5	
NCL/N						
> 1.91	53.7	< 0.05	73.7	NS	45.8	< 0.05
≤ 1.91	73.1		81.8		68.9	
NCLAX						
> 7.525	66.7	NS	76.0	NS	60.9	NS
≤ 7.525	60.9		81.3		53.9	

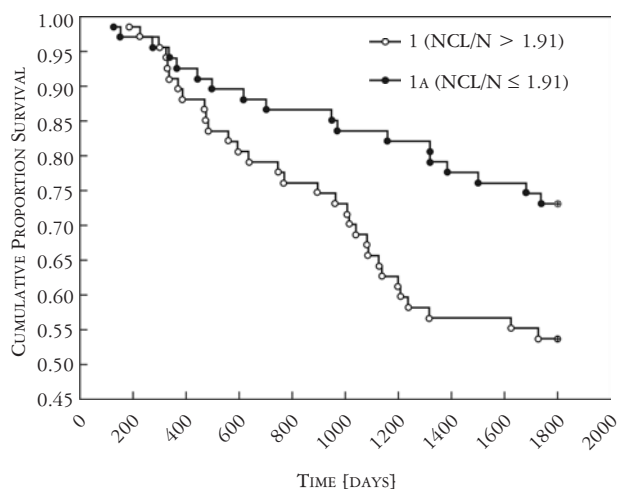


Fig. 2. Number of nucleoli in nuclei of tumour cells (NCL/N) and relapse-free survival of all patients with invasive ductal breast carcinoma (1 vs. 1A, $p < 0.05$)

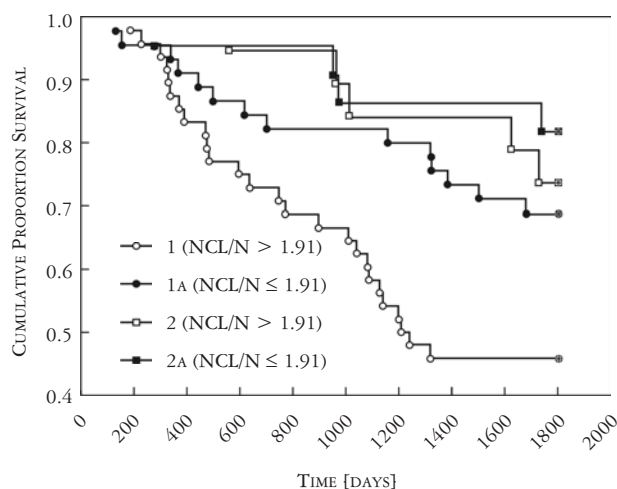


Fig. 3. Number of nucleoli in nuclei of tumour cells (NCL/N) and relapse-free survival of patients with axillary node-positive (1 vs. 1A, $p < 0.05$) and node-negative (2 vs. 2A, $p = \text{NS}$) invasive ductal breast carcinoma

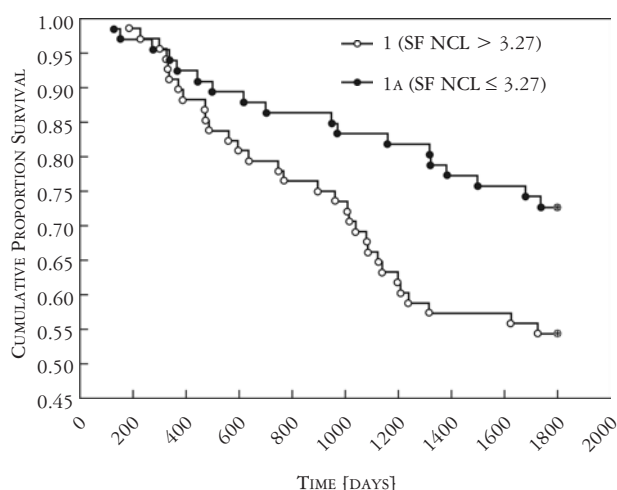


Fig. 4. Nucleolar shape (SF NCL) and relapse-free survival of all patients with invasive ductal breast carcinoma (1 vs. 1A, $p < 0.05$)

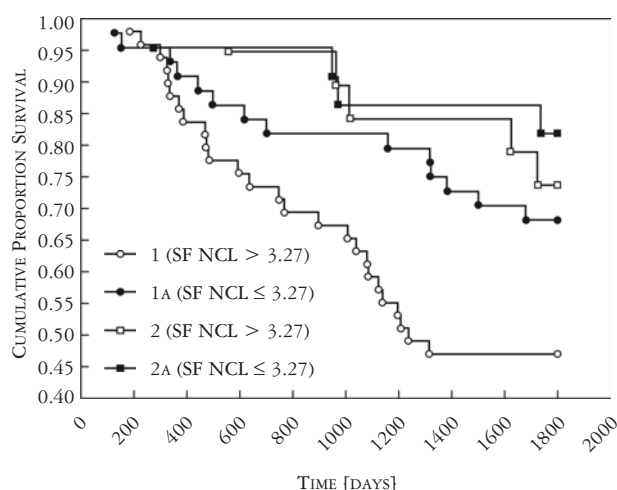


Fig. 5. Nucleolar shape (SF NCL) and relapse-free survival of patients with axillary node-positive (1 vs. 1A, $p < 0.05$) and node-negative (2 vs. 2A, $p = \text{NS}$) invasive ductal breast carcinoma

Histology and histochemistry

Tumour tissue was immediately fixed in 10% buffered formalin for approximately 24 hours and subsequently embedded in paraffin. Histological sections were stained with haematoxylin and eosin as well as with methyl green-pyronin Y. A histological type and histological grade of the tumour according to Bloom and Richardson [15] were determined on haematoxylin and eosin stained sections. For MG-PY procedure the Unna-Pappenheim method as modified by Trevan and Sharrock was used [12]. In MG-PY staining, a crimson nucleolus is seen in the green-blue nucleus, while the cytoplasm stains pink (Fig. 1).

Morphometry

In the sections stained with MG-PY, solid areas of neoplastic infiltration or single clusters of tumour cells without necrotic foci, constituted representative areas for morphometric analysis. In the analysed regions, the nuclei did not overlap because of the appropriate thickness of the sections. Using an oil immersion lens ($40\times$) an average number of 510 ± 263 nuclei and 1040 ± 765 nucleoli contained in those nuclei were measured in 3-4 subsequent microscopic fields. The measurements were performed using a Quantimet 600 S image analyzer (Leica, United Kingdom). The following parameters were measured: 1) *nucleolar surface area* expressed in μm^2 (ANCL), 2) long to short

nucleolar axis ratio (L/B NCL), 3) nucleolar shape parameter assessing the degree of nucleolar roundness (SF NCL), 4) *nuclear surface area* expressed in μm^2 (AN), 5) long to short nuclear axis ratio (L/B N), 6) number of nucleoli in the nucleus (NCL/N) and 7) percentage of the nuclear surface area occupied by the nucleoli (NCLAX).

The surface areas of the nucleoli and nuclei were defined as the cross-section surface areas seen under a light microscope. The SF NCL does not depend on the size and reflects the regularity of the nucleolus profile. For ideally round structures, the value of SF NCL equals one. Structures with the outlines that are not ideally round have a value of SF NCL that is greater than one. A higher shape polymorphism is associated with a higher SF NCL value.

We used the median value of the analysed parameters as the division criterion in the analysis of the associations between the morphometric parameters of the nucleoli and nuclei of invasive ductal breast carcinoma cells and the RFS and the OS. Breast carcinomas in which the ANCL, SF NCL, AN, NCL/N and NCLAX parameters fell below the median value were considered to be characterized by small nucleoli, a low nucleolar shape polymorphism, small nuclei, a low number of nucleoli in the nuclei and a small percentage of the nuclear area occupied by the nucleolus. On the other hand, breast carcinomas in

which the above parameters exceeded the median value were regarded as having large polymorphic nucleoli, large nuclei, a high number of nucleoli in the nucleus and a high percentage of the nuclear area occupied by the nucleolus. Carcinomas in which the L/B NCL and L/B N parameters were below the median value were considered as having round nucleoli and nuclei, while tumours in which the L/B NCL and L/B N parameters exceeded the median value were regarded as having elliptic nucleoli and nuclei.

Statistics

The univariate association between the nucleolar and nuclear morphometric parameters and the RFS and the OS was presented on the basis of Kaplan-Meier survival curves. The difference between the curves was determined using the Wilcoxon test.

Results

Relapse-free survival

A statistically significant association between the nucleolar shape polymorphism and the number of nucleoli in tumour cell nuclei and the percentage of patients with 60-month RFS were found in the entire group of patients and the subgroup with metastases to the axillary lymph

Table II. Morphometric parameters of nuclei and nucleoli of breast cancer cells and percentage of patients with 60-month overall survival in the whole study population, in the axillary node-negative (LN-) and node-positive (LN+) subgroups

PARAMETER	ALL PATIENTS (n = 150)		LN- PATIENTS (n = 44)		LN+ PATIENTS (n = 106)	
	[%]	p	[%]	p	[%]	p
ANCL						
> 1.53	71.8	NS	88.5	NS	71.9	NS
≤ 1.53	73.3		77.8		73.3	
L/B NCL						
> 1.73	77.26	NS	85.0	NS	77.2	NS
≤ 1.73	68.95		83.3		68.7	
SF NCL						
> 3.27	66.7	NS	80.0	NS	66.7	NS
≤ 3.27	78.6		87.5		78.7	
AN						
> 38.88	68.0	NS	80.9	NS	68.0	NS
≤ 38.88	77.3		86.9		77.3	
L/B N						
> 1.55	71.6	NS	82.1	NS	71.6	NS
≤ 1.55	73.6		87.5		73.6	
NCL/N						
> 1.91	66.2	NS	84.2	NS	66.2	NS
≤ 1.91	78.9		84.0		78.9	
NCLAX						
> 7.525	69.3	NS	85.2	NS	60.3	NS
≤ 7.525	76.0		82.4		74.1	

nodes. A higher degree of the nucleolar shape polymorphism and a greater number of nucleoli in tumour cell nuclei were associated with a decreased percentage of patients with 60-month RFS, with a decrease amounting to 18% and 20%, respectively (Table I). Kaplan-Meier survival curves confirmed that in the entire group and in the subgroup with metastatic axillary lymph nodes, a high number of nucleoli and a high degree of the nucleolar shape polymorphism were associated with worse RFS as compared to the patients with carcinomas characterized by a low number of nucleoli and low nucleolar shape polymorphism (Figs. 2-5). The remaining morphometric parameters, either in the entire group or in the subgroups with and without axillary lymph node metastases, did not show statistically significant associations with a 60-month RFS (data not shown).

Overall survival

No significant associations were observed between the analysed morphometric parameters of the nucleoli and nuclei of carcinoma cells and the percentage of patients who survived 60 months, both in the entire group and in the subgroups with and without axillary lymph node metastases (Table II).

Discussion

We have observed a statistically significant association between the RFS and the degree of the nucleolar shape polymorphism and the number of nucleoli in tumour cell nuclei. The results of a prognostic significance of nuclear and nucleolar morphometry are conflicting. Based on the morphometric assessment of 65 cases of breast carcinoma, larger and more polymorphic nucleoli were observed in deceased females as compared to surviving patients [16]. It has also been shown that breast carcinomas with more elliptic nuclei were associated with a lower incidence of relapses [17]. Baak *et al.* [18, 19] regarded tumour cellularity, mitotic index and nuclear shape as the most valuable prognostic morphometric parameters. In the cytological material originating from 98 breast carcinomas, Van Diest *et al.* [20] demonstrated a statistically significant association between the nucleolar surface area, percentage of nuclei devoid of nucleoli, total number of nucleoli in the nuclei of tumour cells and the RFS. The number of nucleoli in 100 nuclei was suggested as an independent prognostic factor [20]. Longer OS and longer RFS of patients with the so-called "small nuclear type" of breast cancer as assessed in cytological specimens stained with May-Grunwald-Giemsa solution, were

demonstrated [21]. Similar results with respect to the survival time were obtained by Van Bogaert *et al.* [22]. By electron microscopy, Lloreta *et al.* [23] observed that in axillary node negative patients, the number of nucleoli in the nuclei of tumour cells and the number of cells in which the nucleoli were in contact with the cellular membrane were independent prognostic factors predicting the relapse occurrence. Thus, the evaluation of nucleolar parameters by AgNORs morphometry of breast cancer cells appeared to be useful in predicting the prognosis in many studies [reviewed in 3]. On the other hand, no association between the number of AgNORs and prognosis was found [25, 26], and Lipponen *et al.* [27] suggested that the surface area of AgNORs had no greater prognostic value than the factors known to date. Lack of correlation of nuclear morphometry with progression of node negative stage II breast cancers was reported [28].

In summary, our results indicate an association between the nucleolar shape polymorphism and the number of nucleoli in tumour cells examined by morphometry in MG-PY stained histological sections and the RFS of patients with invasive ductal breast carcinoma. However, the assessment of the prognostic value of these parameters assessed on MG-PY stained sections requires further investigations performed in a larger group of patients, and a comparison with other known prognostic factors in breast cancer.

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