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Maspin and c-erbB-2 expression in correlation with microvessel density in invasive ductal breast cancer

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Abstract: Maspin is a unique member of the serpin family involved in regulation of cell migration, apoptosis and angiogenesis in breast and prostate cancers. In this study maspin expression in comparison with c-erbB-2 (HER2/neu) oncogene expression and microvessel density was investigated. The examined material included specimens of primary invasive ductal breast cancer derived from 69 patients. They were analyzed immunocytochemically to assess maspin and c-erbB-2 expression, as well as microvessel density using endothelium marker CD31. In the studied cancers, maspin expression in cancer cells was detected in more than half of the cases (50.73%). Although statistically insignificant ($p=0.27$), maspin expression showed decreasing tendency with the increase of tumor grade. C-erbB-2 oncogene expression was observed in 78.26% of the examined cancers. Statistically significant positive correlation was found between c-erbB-2 expression and tumor grade ($p<0.005$). Analysis of the dependence between maspin and c-erbB-2 expression exhibited statistically significant inverse correlation ($p<0.001$). Mean microvessel density (MVD) of the studied cancers was 71.64 (SD=19.36). MVD decreased with the increase of maspin expression, whereas in the cases showing c-erbB-2 overexpression MVD was clearly higher. Both correlations were statistically significant ($p<0.005$). In conclusion, it could be stated that increase in maspin expression is associated with weaker expression of c-erbB-2 oncogene and lower microvessel density, which implies a significant role of maspin in tumor biology. However, the exact mechanism of maspin action (including its potential role in angiogenesis), as well as the assessment of its prognostic significance in breast cancer require further studies.

Key words: Maspin - Breast cancer - c-erbB-2 - Microvessel density (MVD) - CD31

Introduction

Maspin is a 42 kDa protein belonging to a big family of serine protease inhibitors (serpins). It exhibits strong structural homology with other members of this family, such as plasminogen-activator inhibitors 1 and 2 (PAI-1 and PAI-2), and α_1-antitrypsin. For the first time, maspin was identified in normal mammary gland in myoepithelial cells, and later in breast cancers [31, 39], hence its name (**ma**mmary **s**erine **p**rotease **in**hibitor).

Little is known about physiological role and the mechanism of maspin functioning.

Numerous studies suggest that a suppressive function of maspin in cancers results in the inhibition of their invasiveness and metastasis. The probable mechanism underlying this process includes the limitation of cell motility through the inhibition of the cascade of extracellular plasminogen activation [2, 4, 19, 32]. Besides, maspin has been shown to participate in angiogenesis inhibition [38], and in sensitizing cells to proapoptotic factors [11]. Therefore, maspin can potentially contribute to the inhibition of cancer development through various mechanisms.

Data concerning the role and clinical significance of maspin in human breast cancer are equivocal. Some experiments have demonstrated inverse correlation between the decrease in maspin expression, and increase in breast cancer malignancy and poor clinical course of the disease [5, 15, 39]. However, other studies suggest that strong maspin expression is a poor prognostic factor in breast cancer [34].

Little information has been accumulated about correlation between maspin expression and classical prognostic factors in breast cancer, such as tumor stage, estrogen and progesterone receptors, or c-erbB-2, and p53 expression. Moreover, the obtained results are often contradictory [3, 10, 15, 22].

C-erbB-2 (HER2/neu) is a transmembrane glycoprotein belonging to the family of epidermal growth factor

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receptors (EGFRs) with intrinsic tyrosine kinase activity. Its over-activation in multiple cancers promotes proliferation of cancer cells and induces their resistance to apoptosis. Numerous clinical and experimental data imply positive correlation between c-erbB-2 overexpression, and breast cancer progression and invasiveness [21]. Signaling pathway triggered by c-erbB-2 controls expression of various factors associated with cancer progression and angiogenesis, such as transforming growth factor - TGFα, vascular endothelial growth factor - VEGF, plasminogen activator inhibitor-1 (PAI-1), and trombospondin (TSP) [7].

Angiogenesis plays an important role in cancer progression and metastasis. Cancer growth, invasiveness, and capability of metastasizing requires the presence of new blood vessels. Multiple reports indicate that determination of tumor microvessel density (MVD) may be a significant prognostic factor of a cancer disease course. Studies conducted on breast [24, 35], colorectal [9, 33], prostate [14] and endometrial [13] cancers strongly support the notion that MVD is a significant and independent prognostic factor in these diseases.

The location of tumor vessels can be detected on paraffin sections by immunocytochemical methods with specific antibodies directed against endothelial cells. In this study, the expression of CD31 antigen (PECAM1) was determined, because it is regarded as the most sensitive immunocytochemical marker of endothelium [26].

The aim of the present study was to evaluate the correlation between maspin and c-erbB-2 expression as related to microvessel density measured by CD31 expression in primary invasive breast cancer.

Materials and methods

The experiments were conducted on 69 specimens of primary invasive ductal breast carcinoma obtained from Lower Silesian Oncology Center in Wroclaw. Carcinoma specimens were graded according to the histological criteria due to the modified method of Bloom and Richardson [8] by two independent pathologists. Clinical characteristics of the patients is shown in Table 1.

Formalin-fixed paraffin-embedded tissue was immediately sectioned. Four μm sections were mounted on Superfrost slides (Menzel Glaeser, Germany), dewaxed with xylene, and gradually rehydrated. Activity of endogenous peroxidase was blocked by 30 min incubation in 1% H_2O_2. Immunocytochemical reactions were performed using the following antibodies:

(1) monoclonal mouse antibodies against maspin (clone G167-70; Pharmingen, USA), 1:1000;

(2) monoclonal mouse antibodies against c-erbB-2 (clone 353-10; DakoCytomation, Denmark), 1:100, and

(3) monoclonal mouse antibodies against CD31 (DakoCytomation, Denmark), 1:100.

The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation, Denmark). Antigen retrieval was performed by heating in a microwave oven at 800 W in citrate buffer (pH 6.0) for 10 min. The tested sections were incubated with the antibodies for 1 h at room temperature. Subsequently, incubations were performed with biotinylated secondary antibodies (15 min at room temperature) and with streptavidin-biotinylated peroxidase complex (15 min at room temperature) (LSAB+, HRP, DakoCytomation, Denmark). DAB (DakoCytomation, Denmark) was used as a chromogen (7 min at room temperature). All the sections were counterstained with Meyer's hematoxylin. In all cases, negative controls were included in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation, Denmark).

Table 1. Clinicopathologic features of the studied patients with invasive ductal breast cancer

All patients: 69 (100%)		
Age	mean: 53.75 yrs; range: 33-77 yrs	
	≤50	30 (43.48%)
	51-60	21 (30.43%)
	>60	18 (26.09%)
Grade	G1	19 (27.54%)
	G2	23 (33.33%)
	G3	27 (39.13%)
Stage	3a	18 (26.09%)
	3b	51 (73.91%)

To estimate maspin staining intensity, a semiquantitative scale was used, in which points were scored according to the reaction intensity level as well as the percentage of positive cells. The percentage of positive cells was rated as follows: 0 points: 0-5%; 2 points: 6-50%; 3 points: >50%. The staining intensity was rated as follows: 1 point: weak intensity; 2 points: moderate intensity; 3 points: strong intensity. The points for expression and percentage of positive cells were added. Tumors were categorized into four groups: negative - 0 (≤5% cells stained regardless of intensity), weak expression - 1 (2-3 points), moderate expression - 2 (4-5 points), and strong expression - 3 (6-7 points). The slides were also examined for the presence of maspin-positive myoepithelial cells in tumor areas.

The assessment of immunocytochemical reaction demonstrating c-erbB-2 expression was performed according to FDA (Food and Drug Administration) recommendations. The specimens were evaluated as follows: no staining or membrane staining in less than 10% of tumor cells - 0; faint and partial membrane staining in more than 10% of tumor cells - 1; moderate complete membrane staining in more than 10% of tumor cells - 2; strong complete membrane staining in more than 10% of tumor cells - 3. Values 2 and 3 were considered to reflect overexpression of c-erbB-2 antigen.

The assessment of microvessel density was performed according to the method of Weidner (1995). Areas with the highest density of vessels showing expression of CD31 antigen were described as "hot spots". In these areas, at low magnification, sites of similar shape and size were selected, in which vessels were counted. Vessel counting was performed under the Olympus BH2 microscope in three separate areas within one chosen "hot spot". Clusters of stained endothelial cells (observed in cross-section as separate foci) with arrangement indicating that they belonged to one vessel were counted as such. Microvessel density was defined as a sum of the results from three analyzed areas within the "hot spot". Vessel counting was performed at × 400 magnification in the field of 0.18 mm^2.

Intensity of immunocytochemical reactions was evaluated independently by two pathologists. In case of divergences, the evaluation was repeated using double-headed microscope.

Statistical analysis of the obtained results was performed using Statistica 98 PL software (Statsoft Poland). When required χ^2 test and ANOVA test were applied. $P<0.005$ was considered statistically significant.

Results

Maspin expression

In normal tissue of mammary gland adjacent to cancerous sites, maspin expression was limited only to myoepithelial cells surrounding ducts and secretory units (Fig.1a). In tumor areas, maspin expression, besides myoepithelial cells, was present also in cancer cells (Fig. 1b, c), or occurred exclusively in cancer cells (Fig. 1d). Both in myoepithelial and cancer epithelial cells maspin expression was detected in the cytoplasm and nuclei. No cells were detected that would show nucleus-limited maspin expression paralleled by the lack of cytoplasmic expression.

All of the studied specimens of grade G1 (n=19) showed maspin-positive myoepithelial cells. In grade G2 (n=23), 19 specimens exhibited maspin-positive myoepithelial cells (82.6%), whereas in grade G3 (n=27) no myoepithelial cells showing maspin expression were found.

The examined carcinoma specimens varied in maspin expression. No expression (0) was found in 34 specimens (49.27%), weak expression (1) in 9 specimens (13.04 %), moderate expression (2) in 16 specimens (23.18%), and strong expression (3) in 10 specimens (14.49%).

At the particular differentiation stages maspin expression showed the following mean values: G1 - 1.53 (SD = 1.26); G2 - 1.04 (SD = 1.02); G3 - 0.67 (SD =1.07). Statistical analysis with χ^2 test failed to show correlation between the intensity of maspin expression and tumor grade ($\chi^2 = 2.64$, $p = 0.27$). Nevertheless, a clear tendency can be observed indicating the association between stronger maspin expression and higher differentiation level of the cancer. In G3 grade, percentage of the cells showing no maspin expression was the highest (70.37%), more than twice as high as in G1 grade (31.60%). Conversely, percentage of the cells exhibiting strong maspin expression was the least in G3 grade (7.40%) as compared to G1 (31.60%) and G2 (8.7%) (Fig. 2a).

C-erbB-2 expression

The product of immunocytochemical reaction indicative of the expression of c-erbB-2 antigen was present on the cell surface of cancer cells (membrane staining) (Fig. 1e). Among 69 examined human ductal breast carcinomas, 15 specimens (21.74%) failed to show c-erbB-2 -staining (0), 16 specimens (23.19%) exhibited weak staining (1), 18 specimens (26.01%) showed moderate staining (2), and 20 specimens (28.98%) exhibited strong staining (3).

For the specific tumor grades, the level of c-erbB-2 antigen expression showed the following mean values: G1 - 0.68 (SD=0.75); G2 - 1.83 (SD=0.83); and G3 - 2.11 (SD=1.88).

The least differentiated G3 group contained the highest percentage (55.5%) of cancer cells showing strong immunostaining (3) in comparison with more differentiated cancer cells, especially highly differentiated G1 group that did not contain any cells with strong immunostaining. Conversely, G1 group contained the highest percentage of cells (47.38%) showing no c-erbB-2 expression, whereas G3 group contained much lower percentage of such cells (18.52%) (Fig. 2b).

Inverse correlation between c-erbB-2 expression and differentiation stage (tumor grade G1-G3) was statistically significant ($\chi^2 = 16.75$, $p<0.005$).

Correlation between maspin and c-erbB-2 expression

In the further analysis, association between c-erbB-2 oncogene and maspin expression in cancer cells was studied. After comparison of the mean values exhibiting the intensity of c-erbB-2 expression between four groups showing different levels of maspin expression (0-3), the following relations were found: the specimens showing no maspin expression (0) exhibited high (2.39) c-erbB-2 expression, the specimens showing weak maspin expression (1) also exhibited high (2.50) c-erbB-2 expression, the specimens showing moderate maspin expression (2) exhibited moderate (1.35) c-erbB-2 expression, and the specimens showing strong maspin expression (3) exhibited weak (0.72) c-erbB-2 expression.

Inverse correlation was found between c-erbB-2 oncogene and maspin expression. Within 43 specimens exhibiting no or faint maspin expression (0 and 1), 8 specimens (18.61%) showed no or faint c-erbB-2 expression (0 and 1), whereas 35 specimens (81.39%) showed overexpression of c-erbB-2 (2 and 3). Within the group of 26 specimens exhibiting moderate or strong maspin expression (2 and 3), in 23 specimens (88.46%) no or faint expression of c-erbB-2 was observed (0 and 1), whereas only in 3 specimens (11.54%) c-erbB-2 oncogen showed overexpression (2 and 3) (Fig. 2c). These correlations were statistically significant ($\chi^2 = 22.29$, $p<0.001$).

Microvessel density (MVD) in relation to maspin and c-erbB-2 expression

All the studied specimens were immunopositive for CD31 antigen. Immunostaining was observed only in endothelial cells of the vessels (Fig. 1f). Mean MVD of the studied cancers calculated from three counts was 71.64 (SD=19.36).

MVD decreased with the increase in maspin expression (Fig. 3a). Mean MVD values in the groups with different intensity of maspin expression (0-3) were as

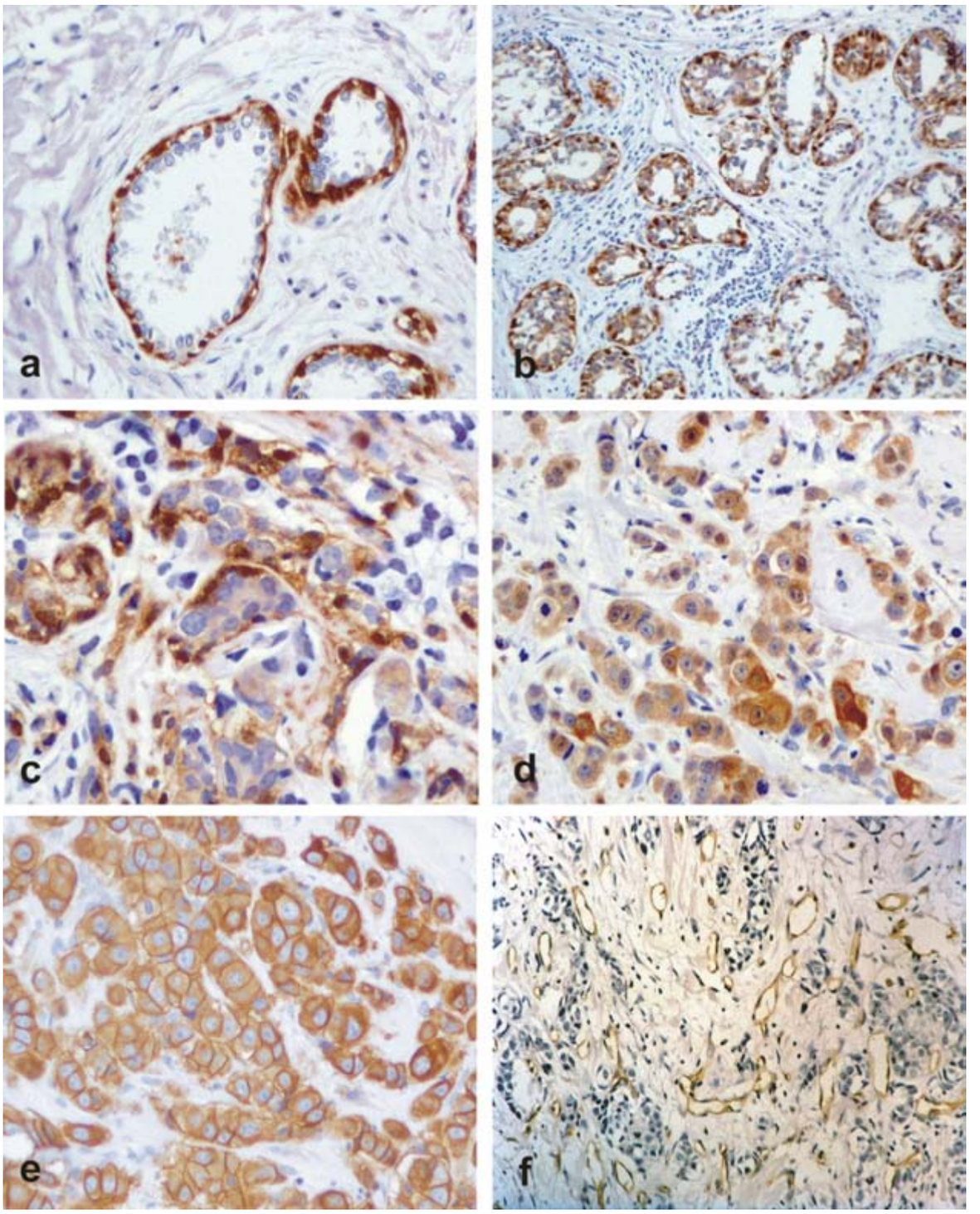

Fig. 1. Immunocytochemical demonstration of maspin (**a, b, c, d**), c-erbB-2 oncogene (**e**), and microvasculature (**f**) in primary invasive breast cancers. In the areas of normal mammary gland tissue, maspin is present only in myoepithelial cells (**a**). In the cancerous areas, maspin is also present in cancer cells (**b, c**) or occurs exclusively in cancer cells (grade G3) (**d**). Micrograph **e** shows breast cancer cells exhibiting c-erbB-2 oncogene overexpression. Micrograph **f** demonstrates blood vessels with CD31-stained endothelium. Magnifications: **a** - × 400, **b** - × 200, **c** - × 600, **d** and **e** - × 400, **f** - × 200.

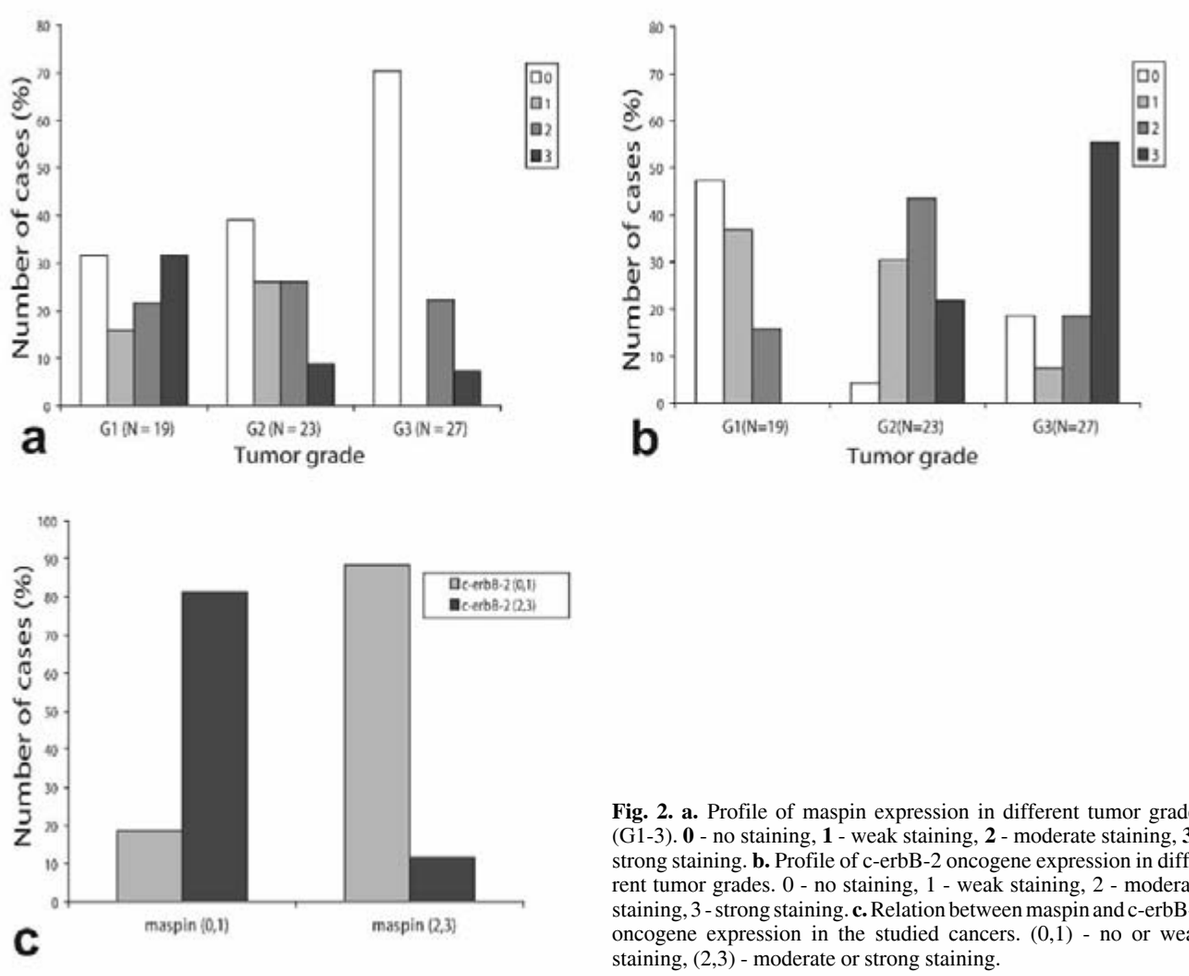

Fig. 2. a. Profile of maspin expression in different tumor grades (G1-3). **0** - no staining, **1** - weak staining, **2** - moderate staining, **3** - strong staining. **b.** Profile of c-erbB-2 oncogene expression in different tumor grades. 0 - no staining, 1 - weak staining, 2 - moderate staining, 3 - strong staining. **c.** Relation between maspin and c-erbB-2 oncogene expression in the studied cancers. (0,1) - no or weak staining, (2,3) - moderate or strong staining.

follows: 0 - 94.00 (SD=14.19), 1 - 89.10 (SD=13.46), 2 - 65.39 (SD=13.79,), 3 - 51.39 (SD=2 .63). These differences showed statistical significance (ANOVA, $F=28.47$, $p<0.005$).

There was also a correlation between MVD and c-erbB-2 antigen expression. Mean MVD values in four groups differing in c-erbB-2 expression (0-3) were as follows: 0 - 54.60 (SD=9.74), 1 - 55.80 (SD=10.75), 2 - 75.61 (SD=13,36), 3 - 97.80 (SD=7.57). These differences were statistically significant (ANOVA, $F=55.55$, $p<0.005$); especially MVD in the groups with c-erbB-2 overexpression (2 and 3) was clearly higher in comparison with the groups with weak c-erbB-2 expression (0 and 1) (Fig. 3b).

Discussion

More than a half of the studied specimens of human ductal breast carcinoma (50.73%) exhibited maspin expression in cancer cells. In spite of the lack of statistical significance, the results show tendency indicating that weaker maspin expression is associated with lower degree of histological differentiation. However, despite the highest percentage of specimens showing no maspin expression within the least differentiated group (G3) (above 70%), nearly 30% of the specimens exhibited moderate or strong maspin expression in cancer cells (2 or 3), whereas there were no cases with weak staining (1).

Little information is available on the relationships between maspin expression in mammary cancer cells and prognostic factors, as well as clinical data in the course of breast cancer disease. What makes this problem even more unclear, the reported findings are often contradictory. Statistically significant correlations between maspin expression and tumor grade were observed in prostate cancer [17] and oral squamous cell carcinomas [37], as well as in animal models of breast cancer [27]. Investigations conducted on breast cancer cell lines and on primary breast carcinomas showed that the decrease in maspin expression was associated with the transition of cancers *in situ* to invasive form and that

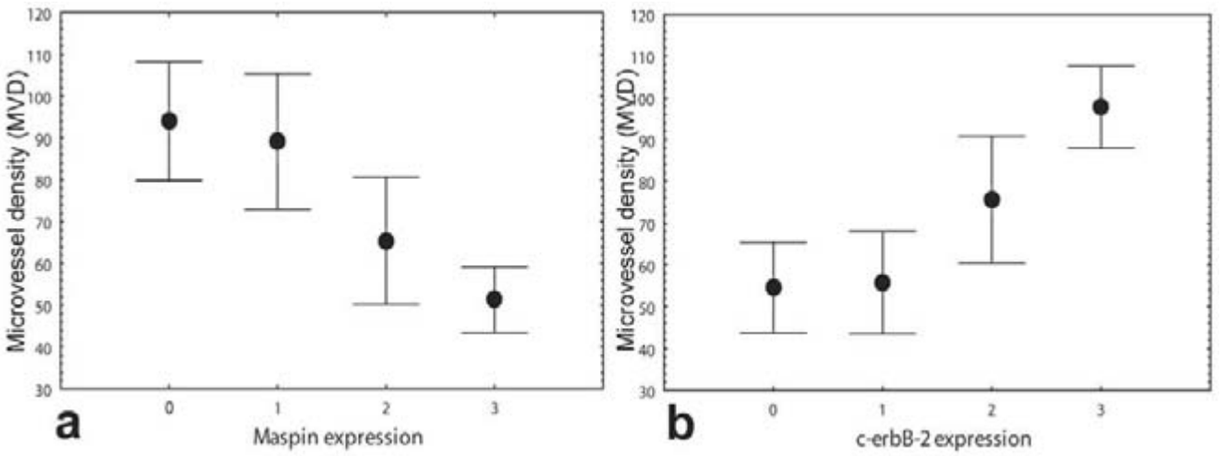

Fig. 3. Microvessel density (MVD) of the studied cancers in comparison with maspin and c-erbB-2 oncogene expression. **a** - MVD *versus* maspin. **b** - MVD *versus* c-erbB-2 oncogene.

the lack of expression was associated with highly metastatic carcinomas [5, 15, 39]. Therefore, in the light of some investigations, low maspin expression is potentially a poor prognostic factor [10, 15, 16]. On the other hand, Umerika *et al.* [34] and Bièche *et al.* [3] obtained opposite results; they reported that maspin overexpression was associated with poor prognosis of breast cancer disease, as well as with short survival rates of patients. Similar results were obtained by Kim *et al.* [12], who observed the highest percentage of maspin-positive cells within low-differentiated breast carcinomas, and high maspin expression proved to be a poor prognosis factor.

The discrepancies concerning the significance of maspin in breast cancer prognosis can result from differences in experimental design, including different material - various cancer types used in studies, as well as different methods applied for the estimation of maspin expression.

A separate issue concerns maspin expression in myoepithelial cells present within the tumor area of the studied cancers. In our material undifferentiated carcinomas (G3) did not show the presence of maspin-positive myoepithelial cells, whereas in G1 grade such cells were found in all the examined cases.

In normal mammary gland, only myoepithelial cells are able to synthesize maspin. Since maspin is one of the most sensitive and specific markers of myoepithelial cells [28], it remains unclear whether during transformation to more aggressive cancer form, myoepithelial cells undergo atrophy within tumor tissue, or whether they loose their ability to synthesize maspin.

Interaction between myoepithelial and cancer cells is one of the significant local mechanisms regulating tumor development [10]. Due to their location, myoepithelial cells create a natural barrier separating cancer cells from connective tissue stroma and blood vessels, what hinders progression of cancers from *in situ* to the invasive form. Numerous recent experiments suggest that myoepithelial cells inhibit cancer progression through the inhibition of epithelial cells proliferation, apoptosis induction and angiogenesis inhibition [23]. The majority of the suppressor functions ascribed to myoepithelial cells overlaps biological activities of maspin in tumor biology. Maspin is secreted to the surface of myoepithelial cells [25], and paracrinally affects the neighboring epithelial cells, as well as the endothelium of the adjacent blood vessels.

These observations suggest that maspin expression in cancer cells is initiated in response to the atrophy of myoepithelial cells or the cessation of maspin synthesis in these cells.

Data obtained in this study indicate inverse correlation between maspin and c-erbB-2 expression in breast cancer cells. On the basis of immunocytochemical analysis it is difficult to conclude whether this correlation results from a direct dependence or reflects an antagonistic involvement of both factors in a common metabolic processes such as angiogenesis regulation. Since maspin is present mainly on the cell surface [25, 30], it may directly affect the activity of c-erbB-2 receptor, thus modulating metabolic processes triggered by this receptor. However, the molecular mechanism of this relationship has not been investigated and further, more detailed studies are needed to verify this hypothesis.

The analysis of microvessel density of the studied cancers suggests that the increase in maspin expression is associated with a significant reduction of microvessel density. Similar results were obtained in experimental studies where exogenous maspin significantly reduced the number of blood vessels in prostate tumors implanted into athymic mice, as well as completely blocked neovascularization of cornea induced *in vivo* by bFGF [38].

Unlike in the case of maspin, microvessel density increased with the increase in c-erbB-2 expression. Moreover, it was clearly higher in the cancers showing c-erbB-2 overexpression (2 and 3). These observations are in agreement with other studies exhibiting that c-erbB-2 overexpression in cancer cells promotes neoangiogenesis through the release of angiogenic cytokines (VEGF, Ang-2) [1].

The mechanism of angiogenesis inhibition by maspin is not fully elucidated. Some data suggest that maspin bound by endothelium may directly modify cell response to receptor-mediated processes regulating angiogenesis, similarly as in the case of CD 36 effect on trombospondin activity [6].

In vitro and *in vivo* studies suggest that maspin is an efficient inhibitor of angiogenesis induced by bFGF, as well as by cancer cell line LNCaP secreting VEGF [38]. Activated c-erbB-2 oncogene increases the secretion of VEGF by cancer cells [1, 20], which results in angiogenesis induction. It might be proposed that maspin would inhibit angiogenesis by affecting c-erbB-2 receptor and blocking the signaling pathway leading to VEGF secretion.

Nevertheless, the interdependence between maspin and c-erbB-2 expression might be more complicated and involve other processes associated with breast cancer development such as regulation of the expression of urokinase-type plasminogen activators and their receptors (uPA, uPAR), which would affect cell motility and adhesiveness. Moreover, the interplay between the two factors may modulate sensitivity of cancer cells to pro- and antiapoptotic agents [11, 18, 21].

In conclusion, it can be assumed that maspin expression is associated with fainter c-erbB-2 expression, as well as decreased microvessel density in breast cancers. It implies a significant role of maspin in breast cancer development. Therefore, a deeper insight into the mechanisms underlying mutual relationships between maspin expression, c-erbB-2 expression and neoangiogenesis would allow better understanding of a complex process of cancer development. However, more studies are needed to elucidate the exact maspin function in this process, as well as its potential prognostic significance.

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