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Stromal myofibroblasts in breast cancer: relations between their occurrence, tumor grade and expression of some tumour markers

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Abstract: It is suggested that tumour stromal myofibroblasts exert an unfavourable effect on the biology of breast cancer. We are aware of only a single study which examined relationships between manifestation of myofibroblasts in the stroma of breast cancer and clinicopathological data of the patients. The present study was aimed at estimation of the effect exerted by myofibroblasts present in the tumour stroma on principal pathological parameters and on expression of Ki67 , P53 and HER-2 proteins in the group of the most frequent breast cancers, the ductal cancers. In paraffin sections of 60 ductal breast cancers (20 cases in G1, 20 in G2 and 20 in G3), immunohistochemical reactions were performed to detect expression of smooth muscle actin (SMA) in order to visualize myofibroblasts, Ki67, P53 and HER-2. The studies demonstrated that the most numerous myofibroblasts were present in G3 cases and they were the least frequent in G1 cases (P=0.02). Positive correlations were observed between the presence of myofibroblasts in tumour stroma and expression of Ki67 and HER-2 in breast cancer cells in the entire group (P <0.001 and P=0.001, respectively), in G2 cases (P=0.003 and P=0.03) and in G3 cases (P=0.01 and P=0.03). Considering that the higher grade, Ki67 and HER-2 are thought to represent unfavourable prognostic factors, the elevated content of myofibroblasts in tumour stroma is probably typical for cases with worse prognosis. (www.cm-uj.krakow.pl/FHC)

Key words: Myofibroblasts - Breast cancer - Grade - Ki67 - P53 - HER-2

Introduction

Breast cancer is the most common malignant tumour of females in the western world, being responsible for about 32% of the estimated new female cancer cases. The incidence of breast cancer remains high, and the clinical courses are highly variable. For years, a growing incidence of the disease has been documented and the risk of dying of breast cancer continues to increase in consecutive cohorts of generations [5]. Therefore, in numerous centres intense efforts develop to detect new prognostic factors which would permit to intensify the therapy in high risk cases. Moreover, such prognostic factors might provide an interesting topic for studies on new therapeutic approaches. Conventional prognostic and predictive markers for breast cancer are stage of the advancement (nodal status, tumour size and presence of the metastases), tumour grade and tumour size [4]. Expression of receptors for female sex hormones [10] and HER-2 is also of importance [4]. In addition, expression of the proliferation-associated antigen, Ki67 and of the suppressor protein, P53 are frequently estimated in order to establish the dynamics of tumour growth [12]. Significance of several other proteins and of their expression in breast cancer cells continues to stimulate studies worldwide. Until now, however, only expression of receptors for

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female sex hormones and of HER-2 have found application in a routine diagnosis of breast cancer [4].

In recent years, numerous publications have described effects of tumour-associated tissues on the dynamics of neoplastic disease. Invasion of tumour cells is slowed down by myoepithelial cells and basal membranes [9]. The immune system has been found to protect the body from neoplastic cells. On the other hand, tumour cells produce, *i.a.* metalloproteinases and growth factors which may facilitate invasion of tumour cells. Tumour-associated vessels provide pathway for spread of tumour cells [6]. The interactions between tumour cells and stromal myofibroblasts remain, however, poorly recognized. Myofibroblasts produce several factors which may stimulate proliferation of cancer cells and facilitate their infiltration. They have been found to secrete, *i.a.*, insulin-like growth factor-2 (IGF-2) and hepatocyte growth factor (HGF) [2]. Recognition of relationships between myofibroblasts and breast cancer cells may facilitate definition of new prognostic factors and provide targets for novel therapeutic approaches.

The present study was aimed at examination of relationships between the presence of myofibroblasts in tumour stroma and principal pathological parameters as well as expression of Ki67, P53 and HER-2 proteins in a group of most frequent breast cancers, the ductal cancers.

Materials and methods

Patients. Immunocytochemical analysis was performed retrospectively on tissue samples collected for routine diagnostic purposes. Based on histology (invasive ductal breast cancer) and grade (equal groups for each grade), 60 patients with primary invasive breast cancer diagnosed in the years 1999 to 2000 in the Lower Silesian Centre of Oncology (Wroclaw, Poland) were included in the study. The mean age of the patients was 55.62 years ± 9.86 SD (age range: 43 to 72 years). Every grade (G1, G2 and G3) was represented by 20 cases. In 34 patients stage 2a, in 22 patients stage 2b and in 4 patients stage 3 of the tumour was diagnosed. Fragments sampled from the studied tumours were fixed in 10% buffered formalin and embedded in paraffin. In every case, hematoxylin and eosin stained sections were examined by two pathologists. The stage of tumours was assessed according to TNM classification system [8]. Tumour grade was estimated according to Bloom-Richardson, according to the modification of Elston and Ellis [3] (Table 1).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections (4 μm) were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene, and gradually rehydrated. Activity of endogenous peroxidase was blocked by 30 min incubation in 1% H_2O_2. The studied sections were boiled 15 minutes in Target Retrieval Solution (DakoCytomation, Poland) , in a microwave oven at 250 W. Then, immunohistochemical reactions were performed using monoclonal antibodies detecting smooth muscle actin (SMA, clone 1A4, DakoCytomation, Poland) diluted 1:200, Ki67 (clone MIB-1, DakoCytomation, Poland) diluted 1:100, P53 (clone DO-7, DakoCytomation, Poland) diluted 1:100 and polyclonal anti-HER-2 antibodies (DakoCytomation, Poland) diluted 1:350. The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation, Denmark). The sections were incubated with antibodies for 1 h at room temperature. Subsequent incubations included biotinylated secondary antibodies (15 min, room temperature) and streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP, DakoCytomation, Denmark). NovaRed (Vector Laboratories, UK) was used as chromogen (10 min, at room temperature). All sections were counterstained with Meyer's hematoxylin. In every case, control reactions were included, in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation, Denmark).

Table 1. Characteristics of patients and tumours

Characteristics		No. (%)
Patients		60 (100)
Age (mean 55.62 years ± 9.86 SD)	≤50	5 (8.3)
	50-60	32 (53.3)
	>60	23 (38.3)
Tumour grade	1	20 (33.3)
	2	20 (33.3)
	3	20 (33.3)
Tumour stage	2a	34 (56.7)
	2b	22 (36.7)
	3	4 (6.6)
Histology	Invasive ductal breast cancer	60 (100)

Scoring of immunostaining results. Myofibroblasts visualized by SMA expression were assessed semiquantitatively with the use of a simplified scale based on the percentage of myofibroblasts in the population of tumour stromal cells (0 - no myofibroblasts, 1 - <10% of myofibroblasts in tumour stroma, 2 - 10-30% of myofibroblasts in tumour stroma, 3 - >30% of myofibroblasts in tumour stroma). Expression of P53 and Ki67 was quantitated by calculating the percentage values of immunopositive cells. For evaluation of HER-2 reactivity, the DakoCytomation scoring system was used (0 = negative; + = partial membranous; ++ = complete membranous, weak; +++ = complete membranous, strong). The intensity of immunohistochemical reactions was assessed independently by two pathologists; in doubtful cases a re-evaluation was performed using a double-headed microscope. In each case we analyzed 6 microscopic fields at × 200 magnification.

Statistical analysis. Statistical analysis of the obtained results was conducted using Statistica 98 PL software (Statsoft, Poland). At the first stage of the calculations, the presence of myofibroblasts was compared in individual groups (G1, G2, G3) using Kruskal-Wallis rank ANOVA test. Subsequently, relationships were examined (employing the same test) between the presence of myofibroblasts and expression of the studied variables in all groups together (G1 to G3) and in each group (G1, G2 and G3).

Results

Immunostaining

Detection of SMA yielded reaction of a cytoplasmic localization and of variable intensity in individual cases. SMA was present in myofibroblasts (Fig. 1), myoepithe-

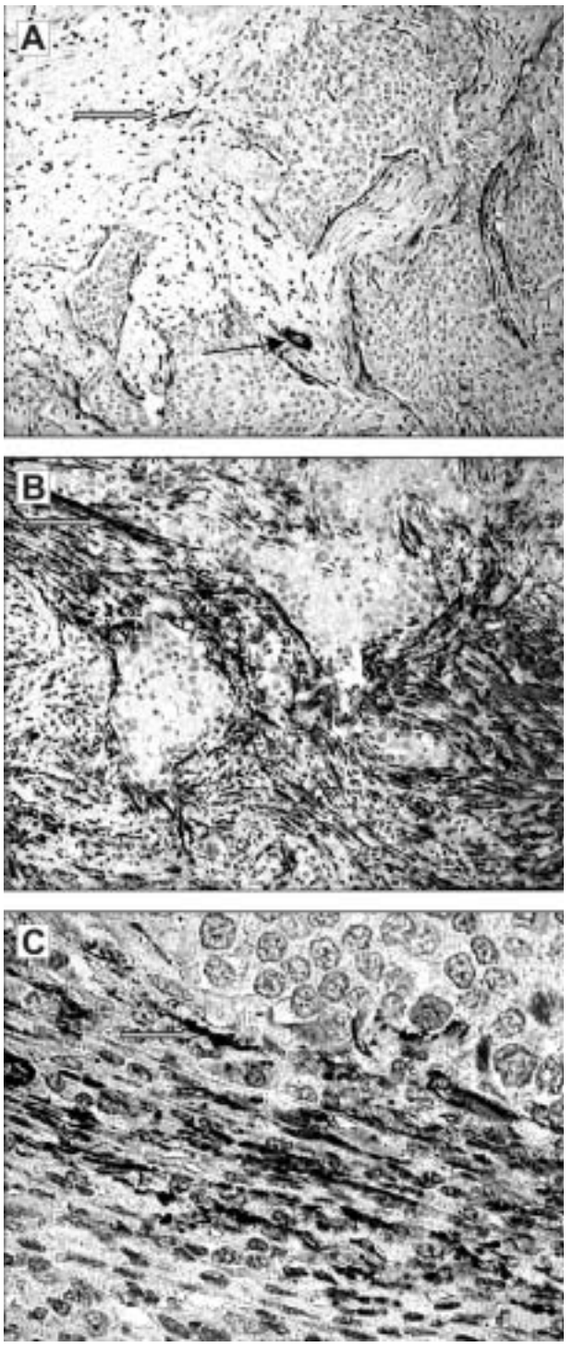

Fig. 1. Immunohistochemical localization of smooth muscle actin in breast cancer tumors. **A.** Reaction in individual myofibroblasts (<10%, score: 1) in tumour stroma (gray arrow) and in blood vessel (black arrow). × 200. **B.** Reaction in numerous myofibroblasts (>30%, score: 3) in tumour stroma (gray arrow). × 200. **C.** Reaction in numerous myofibroblasts (>30%, score: 3) in tumour stroma (gray arrow). × 400.

lial cells and in muscles of blood vessels. According to the applied scale, the mean manifestation of myofibroblasts amounted to 2.01±0.69.

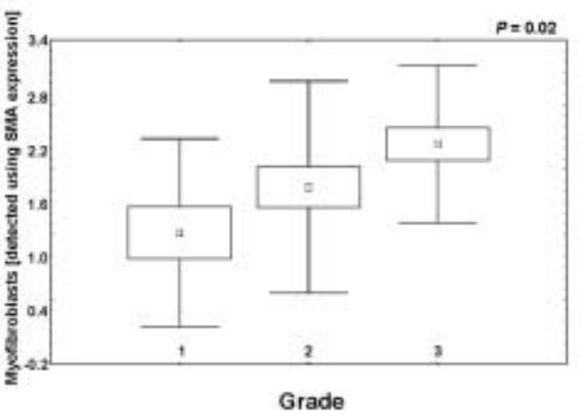

Fig. 2. The presence of myofibroblasts in tumour stroma related to tumour grade (Kruskal-Wallis rank ANOVA test). Myofibroblasts are the most numerous in stroma of G3 tumours.

Application of antibodies against P53 or Ki67 yielded in tumour cells colour reaction of nuclear localization and of a variable intensity in individual cases. The mean percentage of P53-positive and Ki67-positive breast cancer cells was 40.71±26.69 and 28.56±17.7, respectively.

In the case of HER-2, membrane reaction was obtained of various intensity in individual cases, localized in the tumour cells. The mean reaction intensity in DakoCytomation 0-3 score was 1.84±0.74.

Relationships between the presence of myofibroblasts and histopathological parameters

The analysis performed using the Kruskal-Wallis rank ANOVA test demonstrated no relationships between the presence of myofibroblasts in tumour stroma and stage or age of the studied patients (P=0.67 and P=0.54, respectively). Nevertheless, a significant positive correlation was disclosed between the presence of myofibroblasts in tumour stroma and grade of the studied tumors (P=0.02, Fig. 2). Cases of higher grade manifested augmented content of myofibroblasts in tumour stroma.

Relationships between the presence of myofibroblasts and expression of P53, Ki67 and HER-2

Using also the Kruskal-Wallis rank ANOVA test, relationships were examined between the presence of myofibroblasts in the tumours and expression of P53, Ki67 and HER-2 in individual grades (G1, G2 and G3) or in the entire group (G1-3). The test demonstrated no significant relationships in the G1 group (P>0.05, Table 2). In none of the examined groups could a relationship be disclosed between the presence of myofibroblasts in the

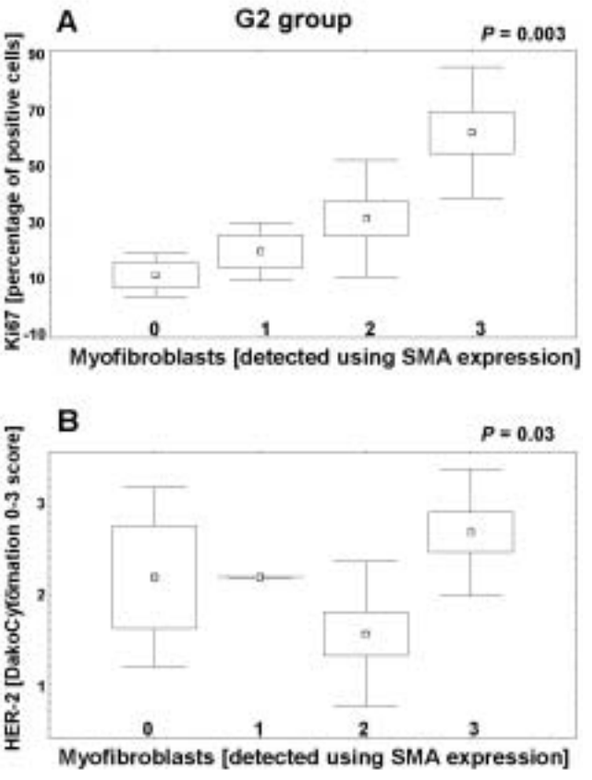

Fig. 3. In G2 group, cases with the highest content of myofibroblasts exhibit: **A.** Higher content of cancer cells with Ki67 expression; **B.** Higher expression of HER-2 w in cancer cells (Kruskal-Wallis rank ANOVA test).

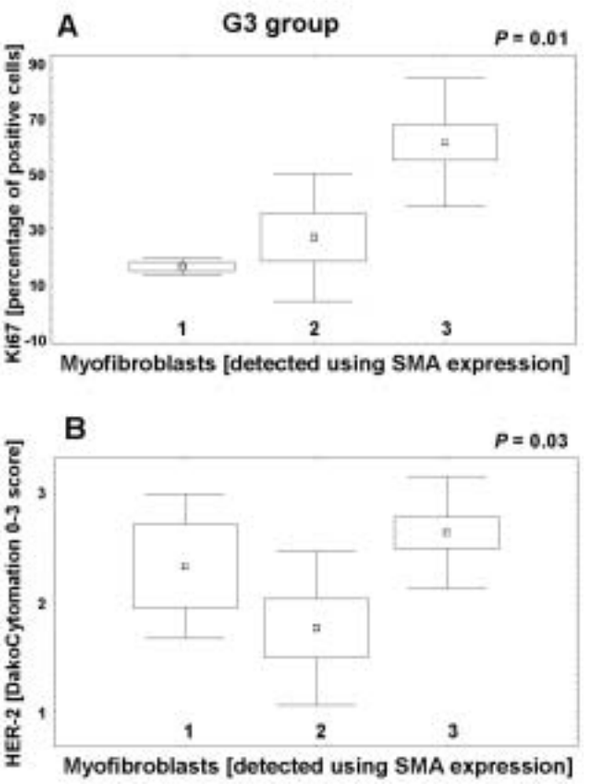

Fig. 4. In G3group, cases with the highest content of myofibroblasts exhibit: **A.** Higher content of cancer cells with Ki67 expression; **B.** Higher expression of HER-2 in cancer cells (Kruskal-Wallis rank ANOVA test).

tumour stroma and expression of P53 in cancer cells ($P>0.05$, Table 2). However, in groups G2 (Fig. 3), G3 (Fig. 4) and in the entire studied material (G1-3, Fig. 5) a significant positive correlation was noted between the presence of myofibroblasts in tumour stroma and expressions of Ki67 and HER-2 in cancer cells ($P<0.05$, Table 2). In cases with the highest content of myofibroblasts in tumour stroma, the most pronounced expression of Ki67 and HER-2 was also observed in cancer cells.

Discussion

The present study demonstrates the significance of myofibroblast presence in tumour stroma, as detected by smooth muscle actin (SMA) expression [1]. We have documented that myofibroblasts are present in tumour stroma in a proportion of breast cancers. This result has confirmed results of other authors. Yazhou *et al.* [11] investigated the relation between the presence of myofibroblasts in tumour stroma and clinico-pathological variables in the breast cancer patients. In the present study we have shown that cases of higher grade demonstrate higher content of myofibroblasts in tumour stroma. Similarly to Yazhou *et al.* [11], we have noted no relationship between manifestation of myofibroblasts and age of the studied patients. Also, in line with the data of the cited authors, the content of myofibroblasts in tumour stroma has not been related to stage of the studied tumours.

We have also examined potential relationships between manifestation of myofibroblasts in tumour stroma and expression of Ki67 , P53 and HER-2 in breast cancer cells. Until now, only the relationship between myofibroblast content and expression of Ki67 in breast cancer cells has been investigated [11] and no significant relationship has been detected. In the present study, however, cases with higher content of myofibroblasts in tumour stroma have been found to contain higher proportions of cancer cells with Ki67 expression. Since myofibroblasts, both in tumours and in healing processes, produce numerous growth factors [2], we suggest that myofibroblast-released growth factors might stimulate proliferation of breast cancer cells.

In this study, no relationship has been documented between the presence of myofibroblasts in tumour stroma and expression of P53 in breast cancer cells. However, cases with higher content of myofibroblasts have

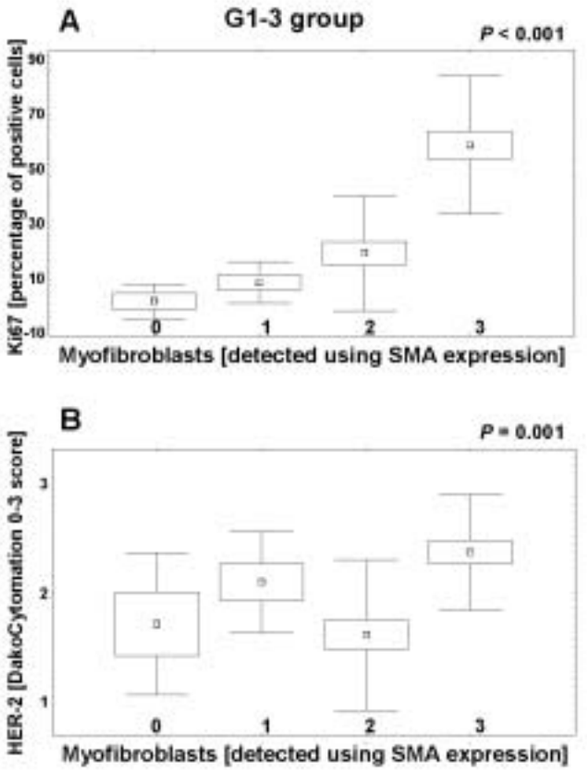

Fig. 5. In the entire material (G1-3), cases with higher content of myofibroblasts exhibit: **A.** Higher proportion of cancer cells with expression of Ki67; **B.** Higher expression of HER-2 in cancer cells (Kruskal-Wallis rank ANOVA test).

been found to exhibit more intense expression of HER-2 in breast cancer cells. Myofibroblasts may secrete insulin-like growth factor-2 (IGF-2) and hepatocyte growth factor (HGF) [7]. They represent ligands for receptors of the Epidermal Growth Factor Receptor group, which includes, *i.a.*, HER-2. The ligands, most probably in a paracrine way, stimulate expression of HER-2 in breast cancer cells. Since HER-2 represents one of the best recognised negative prognostic factors [4] we suggest that the presence of myofibroblasts in tumour stroma may be linked to unfavourable prognosis in breast cancer patients.

In summary, the present investigations have demonstrated that breast cancer cases with higher content of myofibroblasts in tumour stroma are characterised by higher grade of the tumour, more intense proliferation of neoplastic cells and more intense expression of HER-2 in cancer cells. To our knowledge, this paper describes for the first time a positive correlation between the presence of myofibroblasts in tumour stroma and intensity of Ki67 and HER-2 expression. Taking into account that higher grade, Ki67 and HER-2 are regarded to represent negative prognostic indicators, the intensified content of myofibroblasts in tumour stroma may be typical for cases with a worse prognosis. The significance of myofibroblast presence in the stroma of breast cancers should be investigated in detail, in order to detect new prognostic indicators and novel targets for therapeutic approaches.

Table 2. Correlations between the presence of myofibroblasts in tumour stroma (SMA) and expression intensity of the studied antigens in breast cancer cells in the entire group (G1-3) and in the G1, G2 and G3 groups (Kruskal-Wallis rank ANOVA test).

Studied pair of variables	Grade	*P* value
SMA and P53	G1-3	0.5399
	G1	0.2625
	G2	0.2521
	G3	0.3957
SMA and Ki67	G1-3	<0.001
	G1	0.1111
	G2	0.0031
	G3	0.0114
SMA and HER-2	G1-3	0.0011
	G1	0.2083
	G2	0.0299
	G3	0.0349

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