The effect of chemotherapy on Ki-67, Bcl-2 and Bak expression in primary tumors and lymph node metastases of breast cancer

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Received December 29, 2006; Accepted March 19, 2007

Abstract. The aim of this study was the analysis of Ki-67, Bcl-2 and Bak expression in primary tumor and axillary lymph node metastases of breast cancer as well as an attempt to assess preoperative chemotherapy influence on the mentioned markers with regard to changes in the morphological appearance of the primary tumor and its metastases. Immunohistochemical examinations of Ki-67, Bcl-2 and Bak expression were conducted on sections collected from 135 patients treated surgically on invasive ductal breast cancer. Sixty-four of these patients were administered preoperative chemotherapy, whilst on 71 patients the surgery was performed without initial chemotherapy. In the group of patients without preoperative chemotherapy positive correlation in Ki-67 and Bcl-2 expression between primary tumors and lymph node metastases ($p<0.0001$, $r=0.707$; $p<0.0001$, $r=0.604$, respectively) was observed. In the group of patients after chemotherapy positive correlation between primary tumors and lymph node metastases in case of Bcl-2 and Bak proteins ($p<0.04$, $r=0.424$; $p<0.02$, $r=0.478$, respectively) was observed. It was also found that preoperative chemotherapy has an influence on the expression of proteins connected with proliferation and apoptosis and thus, it can influence neoplastic process biology. It does not have any significant impact on the proapoptotic Bak protein expression either in primary tumor or in lymph node metastases of breast cancer. However, it is related to lower expression of antiapoptotic Bcl-2 protein ($p<0.0005$) and of Ki-67 proliferation marker ($p<0.03$) in primary tumors, which indirectly indicates a beneficial influence of preoperative chemotherapy on the primary tumor. Concurrently, the influence of neoadjuvant therapy on lymph node metastases seems to be relatively small, which can limit its effectiveness.

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Key words: breast cancer, primary tumor, lymph node metastasis, preoperative chemotherapy, apoptosis, proliferation, apoptosis, Ki-67, Bcl-2, Bak

Introduction

The most characteristic feature of neoplasm growth is an uncontrollable growth of a certain group of cells. It is not subject to control mechanisms and it leads to emaciation of the organism or/and its death. The development and progression of neoplasm, also breast cancer, depends on the survival of genetically changed cells. Mutual proportions of the factors which stimulate and inhibit processes of proliferation and apoptosis influence neoplastic cell survival in both primary tumor and metastasis foci. Apoptosis inhibition and abnormal Bcl-2 expression can contribute to genesis and accumulation of genetic disorders leading to breast cancer progression and metastases. Thus, it seems that Bcl-2 over-expression and disturbances of the apoptosis process are mechanisms through which breast cancer achieves metastasis ability (1-4). Probably, Bak and Bax proteins are also connected with the regulation of apoptosis process in breast cancer; however, studies of the role of these proteins are relatively few (3-6). There is also evidence that the cytotoxic effect of antineoplastic drugs is related to the intensity or induction of apoptosis process.

Ki-67 antigen is present in all proliferating cells (normal and neoplastic) and its evaluation allows determining growth fraction of cellular population in a relatively easy way (7). Previous studies proved the hypothesis that in breast cancer the Ki-67 index is an independent prognostic factor in both patient survival assessment and disease recurrence (8,9). Ki-67 index can also be used as a predictive factor of neoplastic cell response to certain types of therapy. Thus, while planning individual antineoplastic therapy, it seems that evaluation of Ki-67 expression ought to be one of many essential parameters taken into account.

During routine histopathological diagnostics the biological parameters evaluation is usually performed on primary tumor cross-section. It is considered that the effect of chemo- or hormonotherapy on lymph node metastases of breast cancer should be similar to the effect exerted on primary tumors. However, both our observations and other reports indicate that there are differences in response to systemic therapy between primary tumor and lymph node metastases of breast cancer. The cause of such a situation might be heterogeneity in expression of different biological factors between primary tumor cells and neoplastic cells in metastasis foci (10-14).

The aim of our study was the analysis of Ki-67, Bcl-2 and Bak expression in primary tumor of breast cancer and its

axillary lymph node metastases as well as an attempt to assess preoperative chemotherapy influence on the expression of the mentioned markers with regard to changes in morphological appearance of primary tumor and metastases connected with chemotherapy effect.

Materials and methods

Patients. The examinations were conducted on 135 patients aged 30-82 years (mean age, 54.4). Sixty-four of these patients were administered preoperative chemotherapy, whilst on 71 patients the surgery was performed without initial chemotherapy. In order to make the treatment group uniform the analysis enrolled only the cases of invasive ductal breast cancer in the pT1 and pT2 stages as well as the G2 and G3 stages. Before the operation on patients with chemotherapy, the Ansfield program (cyclophosphamide, prednisone, 5-fluorouracil, metotrexate and vincristine) or CMF program (cyclophosphamide, metotrexate, and 5-fluorouracil) were routinely applied. As the aim of the present study was not the analysis of the influence of particular types of preoperative chemotherapy, the subgroups of different types of treatment were not separated.

In 19 cases of patients who underwent chemotherapy, the immunohistochemical assessment of the examined marker expression was impossible due to severe damage of the primary tumor. Thus, in further analyses, including statistical evaluation, a group of 45 invasive ductal breast cancers was included, in which immunohistochemical assessment of examined markers was possible, despite using preoperative chemotherapy. In the group of cancers without preoperative chemotherapy (n=71) the presence of regional lymph node metastases was diagnosed in 35 cases (49.3%). Analysis of cancers after preoperative chemotherapy (n=45) revealed regional lymph node metastases in 30 patients (66.7%).

Methods. For immunohistochemical studies we selected 2 representative sections from each case of the primary tumors and 1-4 involved lymph nodes with the largest metastatic foci. The quantity of selected nodes depended on the number of involved nodes in specific cases. Immunohistochemical studies were performed as described previously (4,10,12), using antibodies: monoclonal mouse MIB-1 antibody (Ab) (Dako, Denmark) at 1:100 dilution; monoclonal mouse Bcl-2 Ab (Dako) at a 1:50 dilution and goat polyclonal Bak Ab (SCBt, USA) at a 1:200 dilution. The dilution of used antibodies was determined to obtain only specific immunostaining. A streptavidin-biotin-peroxidase complex technique was used to reveal antibody-antigen reactions (LSAB kit, Dako). Staining was routinely developed using 3,3'-diaminobenzidine as a chromogen (Dako). Slides were counterstained with haematoxylin. In negative controls the primary antibodies were omitted. Two independent pathologists analyzed by light microscopy immunostaining of each marker in 10 different tumor fields and the mean percentage of tumor cells with positive staining was assessed. The sections were classified as positive when ≥10% of cells expressed the studied antigens.

Statistical analysis. The Spearman test was used to analyze correlations among studied proteins. The differences in Ki-67, Bcl-2 and Bak expression in patients with and without preoperative chemotherapy were evaluated using the Mann-Whitney U test. Statistical significance was assumed at $p<0.05$.

Results

Evaluation of Ki-67, Bcl-2 and Bak expression in primary tumor and lymph node metastases of breast cancer without preoperative chemotherapy. A positive reaction to Ki-67 antigen was observed in nuclei of neoplastic cells (Fig. 1a and b) as well as in cells located in lymph node germinal centra which constituted additional positive control of the carried out immunohistochemical reactions. In neoplastic cells the Ki-67 expression was observed in 71.8% of primary tumors and 74.3% of lymph node metastases.

Positive immunohistochemical reaction to Bcl-2 protein was found in neoplastic cell cytoplasm (Fig. 1e and f), inflammatory infiltrate cells and in lymph node lymphocytes, which were treated as an additional positive control of the conducted immunohistochemical examinations. In neoplastic cells the Bcl-2 expression was observed in 83.1% of primary tumors and 85.7% of lymph node metastases.

Expression of Bak protein was observed only in neoplastic cell cytoplasm (Fig. 1i and j). It was noted in 70.4% of primary tumors and 94.3% of lymph node metastases.

Evaluation of Ki-67, Bcl-2 and Bak expression in primary tumor and lymph node metastases of breast cancer after preoperative chemotherapy. Ki-67 expression in neoplastic cells was observed in 57.8% of primary tumors and 63.3% of lymph node metastases. In all cases nuclear localization of reaction was observed (Fig. 1c and d).

Bcl-2 (Fig. 1g and h) and Bak (Fig. 1k and l) expression in neoplastic cells was observed in 60.0% and 62.2% of primary tumors and 66.7% and 90.0% of lymph node metastases, respectively.

Analysis of mutual relations of Ki-67, Bcl-2 and Bak expression in breast cancer without preoperative chemotherapy (in primary tumor and metastases). Ki-67 expression in primary tumors positively correlated with expression of this protein in lymph node metastases ($p<0.0001$, $r=0.707$; Table I). Analogical relation was observed in case of Bcl-2 protein ($p<0.0001$, $r=0.604$; Table I). However, such correlation was not observed in Bak protein expression. Negative correlation between Ki-67 and Bcl-2 was observed between primary tumors and lymph node metastases ($p<0.009$, $r=-0.449$; Table I).

In the group of primary tumors a negative correlation between Ki-67 and Bcl-2 was noted ($p<0.0001$, $r=-0.445$), whilst Ki-67 expression positively correlated with Bak expression ($p<0.03$, $r=0.226$; Table III). Also in lymph node metastases a negative correlation between Ki-67 and Bcl-2 was found ($p<0.04$, $r=-0.369$, Table IV). In primary tumors as well as in lymph node metastases no statistically significant correlation between Bcl-67 and Bak was observed.

Analysis of mutual relations of Ki-67, Bcl-2 and Bak expression in breast cancer after preoperative chemotherapy (in primary tumor and metastases). Bcl-2 expression in primary tumors

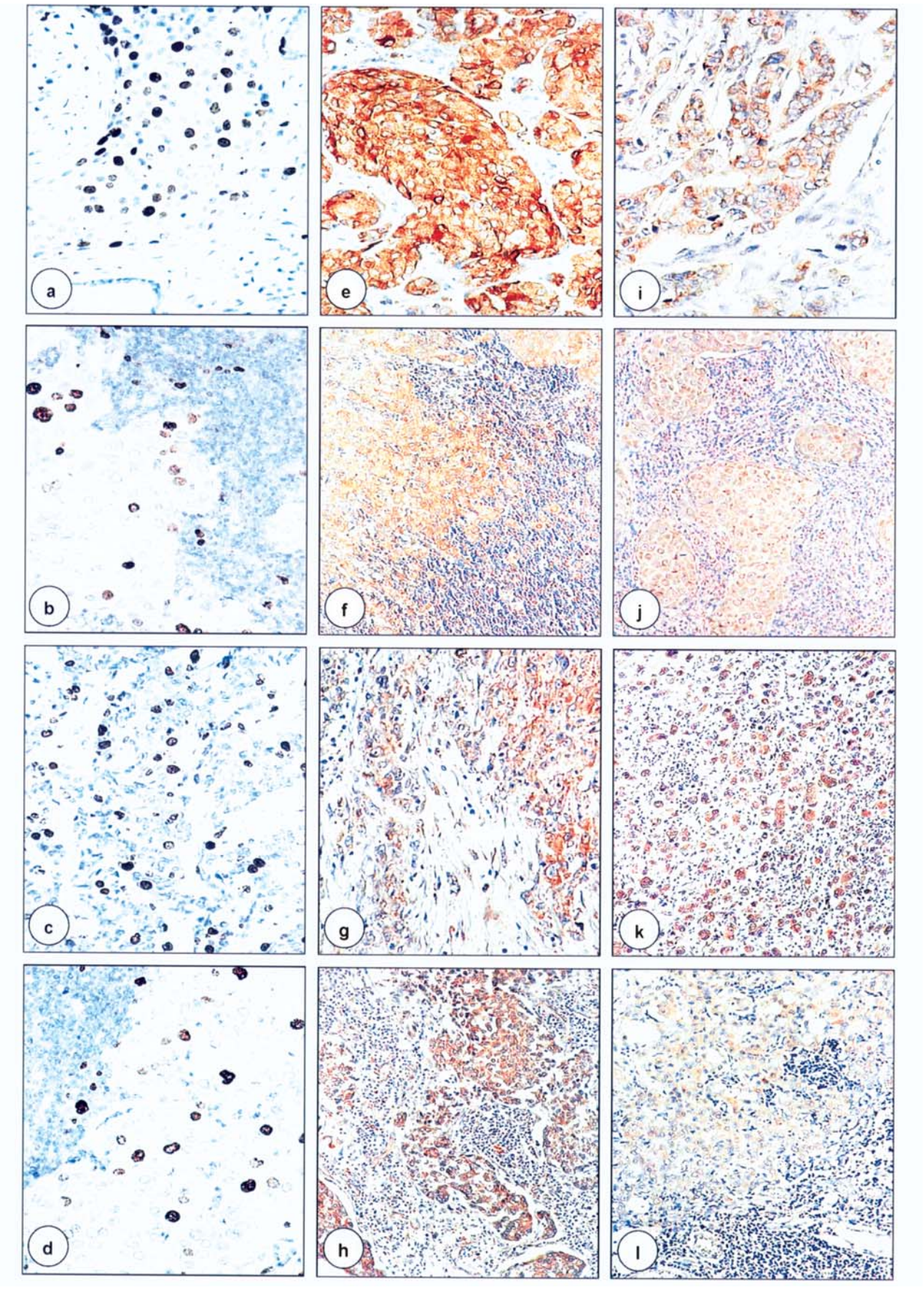

Figure 1. Immunohistochemical demonstration of Ki-67, Bcl-2 and Bak expression in human breast cancer. Nuclear staining for Ki-67 in primary tumor (a and c) and lymph node metastases (b and d) of breast cancer without (a and b) and after (c and d) preoperative chemotherapy. Cytoplasmatic staining for Bcl-2 was observed in primary tumor (e and g) and lymph node metastases (f and h) of breast cancer without (e and f) and after (g and h) preoperative chemotherapy. Bak expression in primary tumor (i and k) and lymph node metastases (j and l) of breast cancer without (i and j) and after (k and l) chemotherapy. The influence of preoperative chemotherapy on Ki-67, Bcl-2 and Bak expression as described in Results. The original magnification for a-d, e and i was x200; for f-h and j-l was x100.

Table I. Evaluation of correlation between Ki-67, Bcl-2 and Bak expression in primary tumors (PT) without preoperative chemotherapy and Ki-67, Bcl-2 and Bak expression in lymph node metastases (LNM).

Compared markers		p	r
PT, n=35	LNM, n=35		
Ki-67	Ki-67	**p<0.0001**	0.707
Ki-67	Bcl-2	N.S.	-0.046
Ki-67	Bak	N.S.	0.013
Bcl-2	Ki-67	**p<0.009**	-0.449
Bcl-2	Bcl-2	**p<0.0001**	0.604
Bcl-2	Bak	N.S.	-0.063
Bak	Ki-67	N.S.	0.126
Bak	Bcl-2	N.S.	0.085
Bak	Bak	N.S.	-0.076

Bold type, statistically significant.

Table II. Evaluation of correlation between Ki-67, Bcl-2 and Bak expression in primary tumors (PT) after preoperative chemotherapy and Ki-67, Bcl-2 and Bak expression in lymph node metastases (LNM).

Compared markers		p	r
PT, n=30	LNM, n=30		
Ki-67	Ki-67	N.S.	0.022
Ki-67	Bcl-2	N.S.	-0.328
Ki-67	Bak	**p<0.05**	-0.382
Bcl-2	Ki-67	N.S.	-0.136
Bcl-2	Bcl-2	**p<0.04**	0.424
Bcl-2	Bak	p=0.089	-0.333
Bak	Ki-67	N.S.	0.020
Bak	Bcl-2	N.S.	-0.284
Bak	Bak	**p<0.02**	0.478

Bold type, statistically significant.

from patients after preoperative chemotherapy positively correlated with this protein's expression in lymph node metastases (p<0.04, r=0.424; Table II). Analogical relation was observed in Bak protein (p<0.02, r=0.478; Table II), whilst no such correlation was observed in Ki-67 expression. Negative correlation between Ki-67 and Bak was observed between primary tumors and lymph node metastases (p<0.05, r=-0.382; Table II).

Table III. Evaluation of correlation between Ki-67, Bcl-2 and Bak expression in primary tumors (PT) before and after preoperative chemotherapy.

	Compared markers		p	r
PT without	Ki-67	Bcl-2	**p<0.0001**	-0.445
chemotherapy,	Ki-67	Bak	**p<0.03**	0.266
n=71	Bcl-2	Bak	N.S.	-0.154
PT after	Ki-67	Bcl-2	**p<0.007**	-0.410
chemotherapy,	Ki-67	Bak	N.S.	-0.055
n=45	Bcl-2	Bak	N.S.	-0.095

Bold type, statistically significant.

Table IV. Evaluation of correlation between Ki-67, Bcl-2 and Bak expression in lymph node metastases (LNM) of breast cancer without and after preoperative chemotherapy.

	Compared markers		p	r
LNM without	Ki-67	Bcl-2	**p<0.007**	-0.410
chemotherapy,	Ki-67	Bak	N.S.	-0.055
n=35	Bcl-2	Bak	N.S.	-0.095
LNM with	Ki-67	Bcl-2	p<0.085	-0.331
chemotherapy,	Ki-67	Bak	N.S.	-0.165
n=30	Bcl-2	Bak	N.S.	-0.169

Bold type, statistically significant.

In primary tumors a negative correlation between Ki-67 and Bcl-2 was observed (p<0.007, r=-0.410; Table III), whilst in lymph node metastases we noted only a trend toward negative correlation between Ki-67 and Bcl-2 (p=0.085, r=-0.331; Table IV). No statistically significant correlation between other examined markers was observed (Ki-67 and Bak as well as Bcl-2 and Bak).

Comparison of Ki-67, Bcl-2 and Bak expression between breast cancer with and without preoperative chemotherapy (in primary tumor and metastases). In order to determine the direct influence of preoperative chemotherapy on Ki-67, Bcl-2 and Bak expression, we also compared the mean percentage of cells with positive immunohistochemical reaction to these proteins between primary tumors and between lymph node metastases in patients with and without preoperative chemotherapy (Tables V and VI). It should be accentuated that obtained results concern only those cases of cancers after chemotherapy, in which evaluation of analyzed markers was possible. It was shown that in the examined group of cancers, despite partial damage of neoplastic cells, preoperative chemotherapy had no statistically significant influence on Bak expression in either the primary tumor (Table V) or the

Table V. Comparison of Ki-67, Bcl-2 and Bak expression in primary tumors (PT) between the group of breast cancer without preoperative chemotherapy and the group after chemotherapy.[a]

Compared markers	PT without chemotherapy (Mean ± SD) n=71	PT after chemotherapy (Mean ± SD) n=45	p
Ki-67	30.1±25.2	20.5±19.6	**p<0.03**
Bcl-2	61.1±25.1	43.2±29.4	**p<0.0005**
Bak	47.5±26.2	40.5±24.8	N.S.

[a]Mean percentage of cells revealing positive immunohistochemical reaction ± standard deviation, SD. Bold type, statistically significant.

Table VI. Comparison of Ki-67, Bcl-2 and Bak expression in lymph node metastases (LNM) between the group of breast cancer without preoperative chemotherapy and the group after chemotherapy.[a]

Compared markers	LNM without chemotherapy (Mean ± SD) n=35	LNM after chemotherapy (Mean ± SD) n=30	p
Ki-67	26.7±22.7	22.3±20.9	N.S.
Bcl-2	59.1±25.7	49.4±32.6	N.S.
Bak	65.5±22.4	59.5±22.6	N.S.

[a]Mean percentage of cells revealing positive immunohistochemical reaction ± standard deviation, SD.

lymph node metastases (Table VI). At the same time, Ki-67 and Bcl-2 expression in primary tumors after preoperative chemotherapy was considerably lower ($p<0.03$ and $p<0.0005$, respectively; Table V) comparing with primary tumors without preoperative chemotherapy. On the other hand, no statistically significant influence of chemotherapy on Ki-67 and Bcl-2 expression in lymph node metastases was observed (Table VI).

Discussion

Both macroscopic and microscopic evaluations of the degree of advancement and histological malignancy require taking into account possible changes caused by chemotherapy. Rasbridge *et al* (16) evaluated the influence of different types of preoperative chemotherapy on neoplastic cell morphology and primary tumor proliferation. They showed that in some tumors after chemotherapy the degree of histological malignancy changed comparing to the evaluation before chemotherapy. In some patients after chemotherapy the primary focus was not found (or only the pre-invasive cancer foci were found) and thus, further diagnostics of breast cancer was impossible. In cases with considerable regression of breast cancer, Rasbridge *et al* (16) observed fibrosis within surrounding tissues and in the connective tissue stroma of primary tumor. They suggested that histological evaluation of neoplasm after preoperative chemotherapy, including defining the histological malignancy degree, would be erroneous due to the occurrence of retrograde changes. In the study by Aktepe *et al* (17) the influence of preoperative chemotherapy on lymph node metastases and on lymph nodes without diagnosed metastases was evaluated. The most common changes were fibrosis and hyalynization of the nodes. In the nodes only vacuolization of the cytoplasm of some neoplastic cells was observed. Our observations also indicate a minor influence of preoperative chemotherapy on the morphology of neoplastic cells in metastasis foci.

Cytogenetic studies provided, among others, information that lymph node metastases, comparing to primary focus, are much less heterogenic, mainly due to the fact that only a few cellular clones in the primary tumor are able to metastasize (18,19). It appears that genetic heterogeneity of primary focus, which increases the neoplastic cell ability to adapt to various environmental conditions, might be the underlying cause of the development of the immunity of breast cancer metastasis cells against chemotherapeutics. On account of this, defining the degree of differences between neoplastic cells present in primary tumor and metastasis foci seems to be an important issue in the understanding of neoplasm biology, clinical oncology and development of new methods of antineoplastic therapy.

Preoperative chemotherapy influence on Ki-67, Bcl-2 and Bak expression in primary tumor of breast cancer is poorly understood (16,20-23). Moreover, there is a lack of studies on the preoperative chemotherapy influence on the expression of these markers in lymph node metastases.

The present study showed a positive correlation between Ki-67 expression in breast cancer and Ki-67 expression in regional lymph node metastases in patients without preoperative chemotherapy as well as lack of such relation in the group of patients after preoperative chemotherapy. In our previous study (24) expression of Ki-67 in matched pairs of primary tumors without preoperative chemotherapy and in lymph node metastases was compared. Despite almost identical percentage values of Ki-67-positive primary and metastasis tumors, important differences were found in the analysis of particular tumor-metastasis pairs. Only in ~70% of tumor-metastasis pairs, the Ki-67 expression was the same in primary tumor as in corresponding lymph node metastases.

In the present study, we showed that mean percentage of Ki-67 of positive neoplastic cells was significantly statistically lower in primary tumors after chemotherapy compared to tumors without chemotherapy, whilst no significant differences were observed in lymph node metastases. Also, Bottini *et al* (20) showed the Ki-67 expression decrease in primary tumors after preoperative chemotherapy. However, the authors did not show statistically significant influence of preoperative chemotherapy on the ER, PgR, Bcl-2 and P53 expression. They suggested that the difference in Ki-67 expression before and after chemotherapy as well as lack of influence on P53, Bcl-2 and ER expression are caused by the cytotoxical influence of chemotherapy, which results in the decrease of tumor size. They also suggested that high proliferous activeness in tumor after chemotherapy might be a characteristic of a more malignant cancer phenotype and might require modification of the adjuvant therapy type. Ki-67

expression changes found by Bottini *et al* (20), despite statistical importance, were considerably small. Thus, the observed decrease in primary tumor size, might be attributed to both lowered proliferation and intensified apoptosis.

Bcl-2 protein, apart from generally known as apoptosis inhibiting, might also inhibit cell cycle progression by blocking cells entering the S-phase and keeping cells in the G0 phase of the cycle (25). Antiproliferous results of Bcl-2 protein effects in epithelial cells might be lost in the course of breast cancer development (26). Makris *et al* (21) noted the lack of changes in the expression of antiapoptotic Bcl-2 protein in patients who underwent neoadjuvant therapy. Concurrently, they showed the decrease of Ki-67 and ER expression. Rasbridge *et al* (16) also evaluated the influence of preoperative chemotherapy on proliferous activeness of breast cancer as well as on Bcl-2 protein expression. They did not find any statistically significant changes. On the other hand, Sato *et al* (23) observed a significant decrease of Bcl-2 protein expression in breast cancers with preoperative chemotherapy (respectively: 67% tumors before and only 46% after chemotherapy were Bcl-2 positive). The authors suggested that the Bcl-2 expression decrease was caused by the fact that chemotherapy stimulates apoptosis processes and has a damaging influence on neoplastic cells. In the present study, the Bcl-2 expression was found in 60% of primary tumors and in 66.7% of lymph node metastases from patients after preoperative chemotherapy and, respectively, in 83.1% and 85.7% of patients without chemotherapy. No statistically significant differences in Bak proapoptotic protein expression were observed. This may indicate that by intensifying neoplastic cell apoptosis, chemotherapy causes relative dominance of proapoptotic proteins in them.

Contrary to the group of patients without chemotherapy, in the group after chemotherapy a negative correlation between Ki-67 expression in primary tumor and Bak expression in lymph node metastases was shown. It appears that the observed difference results from considerable decrease of Ki-67 expression in primary tumors after chemotherapy as well as from lack of influence of the applied treatment on the examined markers' expression in lymph node metastases. Pohl *et al* (22) evaluated the influence of preoperative chemotherapy with the use of the CMF model on Ki-67 expression as well as proteins regulating cellular cycle (p21Waf1, p27Kipl, p53 and cyclin D3). The authors showed decreased Ki-67 expression and increased expression of proteins regulating cellular cycle. They also suggested that increased expression of these proteins may lead to cellular cycle inhibition and resistance to drugs acting in particular phases of its cycle.

Mitochondrial pathway and the so-called death receptors connected with cell surface are the main tracts leading to programmed cell death (27). There is an accepted assumption that the mitochondrial pathway plays a main role in responding to the applied antineoplastic treatment and the proteins from the Bcl-2 family are of fundamental importance. However, in the study by Sjöström *et al* (28) no relation between Bcl-2 expression in breast cancer and response to chemotherapy was found. Low expression of Bcl-2 was related to a shorter progression time of neoplasm and shorter total survival time of patients.

Our previous studies suggested the possibility of diversified response of primary tumor and metastases of breast cancer to preoperative chemotherapy (15). The results of the present study prove these conjectures and indicate that preoperative chemotherapy influences the expression of proteins connected with proliferation and apoptosis and thus, can influence the biology of the neoplastic process. It has no significant influence on proapoptotic Bak protein expression in primary tumor of breast cancer, but it is related to the lower expression of the antiapoptotic Bcl-2 protein and Ki-67 proliferation marker, which indirectly indicates its positive effects on the primary tumor. Concurrently, the influence of neoadjuvant therapy on lymph node metastases seems to be relatively small, which can limit its effectiveness.

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