

Correlation between metallothionein (MT) expression and selected prognostic factors in ductal breast cancers

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Abstract: Our study aimed at examining significance of metallothionein (MT) expression in ductal breast cancers by determination of a relationship between expression of MT protein (MT-1/2) and selected prognostic factors, including grade of histological differentiation (G), expression of Ki-67 proliferative antigen, expression of estrogen receptors (ER) and progesterone receptors (PgR) and expression of HER-2 receptor. Material for the studies involved 54 samples of invasive ductal breast cancer, manifesting malignancy grades of G1-G3. In paraffin sections of examined tumours immunohistochemical reactions were performed using specific antibodies directed to MT, Ki-67, ER, PgR or HER-2. Intensity of MT-specific immunohistochemical reactions was measured using the semiquantitative IRS scale of Remmele. Intensity of colour reactions targeted at Ki-67, ER, PgR was evaluated scoring proportions of positive cells, while HER-2-specific reactions were evaluated in the scale of 0-3 points. The lowest level of MT expression was detected in breast cancer cases of G1 malignancy grade (G1 vs G3 $p=0.020$). A positive correlation between MT and Ki-67 antigen expression ($r=0.32$, $p=0.019$) was disclosed. Moreover, MT expression exhibited negative correlations with expression of ER ($r=-0.35$, $p=0.008$) and PgR ($r=-0.27$, $p=0.046$). No relationships could be detected between expression of MT and expression of HER-2 ($r=0.12$, $p=0.37$). The obtained results suggest that MT expression might be helpful in prognostic evaluation of ductal breast cancers.

Key words: metallothionein, breast cancer

Introduction

Metallothioneins (MT) represent intracellular proteins of 6-7 kDa molecular weight, widely manifested both in plant and animal kingdom. They consist of 61-68 amino acids, among which around 30% are cysteine residues [1,2]. In humans four classes of the proteins were identified, including MT-1, MT-2, MT-3 and MT-4, containing the total number of ten functional isoforms. Expression of MT-1 and MT-2 manifest variable intensity in all tissues and organs of the body. The highest concentration of the proteins was detected in the liver, pancreas, intestine and kidneys [1]. MT-3 and MT-4 are tissue specific and their expression in normal conditions is manifested, respectively, in neurons of central nervous system and in squamous epithelia of

skin and alimentary tract [2,3]. MT-coding genes are located on chromosome 16q13, and their expression may be induced by various factors, including heavy metals, hormones, cytokines, growth factors, organic compounds and free radicals [4-7].

The unique property of MT, which significantly determines their role in the body, involves the ability of binding heavy metals ions, such as zinc, copper, mercury, lead or cadmium. MT are responsible, first of all, for maintenance of metal homeostasis and detoxication processes [2,8,9]. Reversible binding of zinc ions causes that MT affect activity of several zinc-dependent proteins and enzymes. In this manner MT control several intracellular processes, including proliferation, apoptosis and cell differentiation [10].

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Abbreviations: MT - metallothionein; Ki-67 - proliferative antigen; ER - estrogen receptor; PgR - progesterone receptor; HER-2 - human epidermal growth factor receptor 2; G - grade of histological differentiation; IRS - immuno-reactive score

Moreover, MT, due to high content of cysteine residues in their molecules exhibit strong anti-oxidative properties. In addition to glutathione and anti-oxidative enzymes, they constitute one of the principal elements of cell protection against free radicals and effects of oxidative stress [3,8].

Studies performed till now demonstrated augmented synthesis of MT in various tumours of either epithelial or mesenchymatic origin [3,10,11]. It is suggested that role of the proteins in carcinogenesis is linked to their involvement in processes of cells proliferation and differentiation [12,13]. There have been proposed various mechanisms of MT influence on proliferation of cells and progression of tumours. MT provide a direct source of zinc for enzymes engaged in processes of replication, transcription and proteins biosynthesis, taking place in intensely proliferating neoplastic cells. This has been confirmed not only by elevated level of MT in the region of hyperplasia but also by protein translocation from cytoplasm to cell nucleus at the phase of DNA synthesis (S phase) in proliferating cells [3,14,15]. Moreover, in *in vitro* experiments MT was shown to be capable of controlling zinc ion accessibility and, in this way, of modulating activity of transcription factors, including Sp1 and TFIIA, engaged in cell cycle control [1,10]. Interaction with p53 protein, the product of a suppressor gene, seems to be very important for development of a tumour. MT was demonstrated to be capable of removing zinc ions from p53 protein molecules, leading to changes in their spatial structure and inactivation. This, similarly to mutations within p53, results in an uncontrolled cell proliferation [16–20]. The other suggested mechanism by which MT may increase proliferative potential of neoplastic cells involves their protection from apoptosis. There was described a specific interaction of MT with p50 subunit of NF- κ B transcription factor, which results in inhibition of apoptosis [21,22].

Our study aimed at examining significance of MT expression in ductal breast cancers by determination of a relationship between MT protein (MT-1/2) and selected prognostic factors, including grade of histological differentiation (G), expression of Ki-67 proliferative antigen, expression of estrogen receptors (ER) and progesterone receptors (PgR) and expression of HER-2 receptor.

Material and methods

Tissues. The investigated material included 54 samples of invasive ductal breast cancer, obtained from patients subjected to surgery in the Lower Silesia Centre of Oncology in Wrocław in 2004–2007. The mean age of the patients was 63.65 ± 12.84 years. In each case the diagnosis was confirmed by histopathology. The grade of histological differentiation (G) was estimated according to Bloom-Richardson scale [23]. The group of studied breast cancers contained highly differentiated tumours (G1) – 11 cases, moderately

differentiated tumours (G2) – 27 cases and poorly differentiated tumours (G3) – 16 cases.

Immunohistochemistry. Expression of MT (MT-1/2), Ki-67, ER, PgR and HER-2 was examined immunohistochemically in paraffin sections. Tumour samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Four-micrometer sections were mounted on Superfrost Plus slides (Menzel Gläser, Germany), dewaxed with xylene and gradually rehydrated. Activity of endogenous peroxidase was blocked using 3% hydrogen peroxide. Immunohistochemical reactions were performed using specific antibodies: MT-specific monoclonal mouse antibody, clone E9 (DakoCytomation, Denmark) at dilution 1:100, Ki-67-specific monoclonal mouse antibody, clone MIB-1 (DakoCytomation, Denmark) at dilution 1:100, ER-specific monoclonal mouse antibody, clone 1D5 (DakoCytomation, Denmark) at dilution 1:75, PgR-specific monoclonal mouse antibody, clone PgR 636 (DakoCytomation, Denmark) at dilution 1:100. Incubation with the antibodies was conducted overnight, at 4°C. Except of the MT-specific reaction, in all remaining cases the sections were boiled in Antigen Retrieval Solution (DakoCytomation, Denmark). Following incubation with secondary biotinylated antibodies (Biotinylated Link, DakoCytomation, Denmark), reactions with the streptavidin-biotinylated peroxidase complex (LSAB+ System-HRP, DakoCytomation, Denmark) were performed. The peroxidase substrate of 3,3'-diaminobenzidine (DAB+ Chromogen, DakoCytomation, Denmark) was used as a chromogen. All the sections were counterstained with haematoxylin. Expression of HER-2 was examined using HercepTest™ kit containing appropriate rabbit polyclonal antibody, according to the procedure recommended by the manufacturer (DakoCytomation, Denmark).

The intensity of MT expression was evaluated using the semi-quantitative IRS (*immuno-reactive score*) scale according to Remmele, which took into account the intensity of the colour reaction and the percentage of positive cells [24]. The final score represented a product of points given for individual traits and ranged between 0 and 12 (Table 1). Expression of Ki-67 antigen, estrogen receptors (ER) and progesterone receptors (PgR) was evaluated scoring percentage of cells manifesting nuclear colour reaction. In every case the appraisal was performed in five representative microscope fields (hot-spots, magnification $\times 200$) using the software for computer-assisted image analysis, analysis 3.2. (Germany). In evaluation of HER-2 receptor expression the scale was employed which took into account intensity and character of membranous colour reaction and percentage of cells manifesting such a reaction [25] (Table 2). Intensity of immunohistochemical reactions was evaluated independently by two pathologists.

Statistical analysis. The obtained results were subjected to statistical analysis using Statistica 7.1 software (StatSoft, Poland). For normality of the distribution the Kolmogorov-Smirnov test was employed. Correlation between expression intensities of selected markers was examined using Spearman rank correlation index. Significance of differences in expression of studied markers was examined using U test of Mann-Whitney. The results were regarded statistically significant at $p < 0.05$.

Results

Expression of metallothionein (MT) was demonstrated in 53 cases of ductal breast cancer (98.15%). In all the cases a cytoplasmic or a nuclear-cytoplasmic reaction was noted (Fig. 1A, 1B). Most of the examined tumours manifested different distribution of MT and differences in intensity of the reaction. The mean intensity of reaction in IRS scale was 4.48 ± 2.72 pts.

Table 1. Evaluation of MT expression using IRS scale [24].

Percentage of positive cells	Points	Intensity of colour reaction	Points
No positive cells	0	No colour reaction	0
<10% positive cells	1	Weak colour reaction	1
10–50% positive cells	2	Moderate colour reaction	2
51–80% positive cells	3	Strong colour reaction	3
>80% positive cells	4		

Expression of Ki-67 antigen was detected in all studied cases. The colour reaction of a variable intensity manifested the nuclear localization (Fig. 1C). The mean percentage of positive cells was $41.18\% \pm 25.93\%$.

Expression of estrogen receptors (ER) was demonstrated in 40 cases (74.07%) (Fig. 1D). The mean percentage of positive cells was $53.35\% \pm 40.16\%$.

Presence of progesterone receptors (PgR) was documented in 37 cases (68.52%) (Fig. 1E). The mean percentage of positive cells was $36.85\% \pm 41.50\%$.

Expression of HER-2 receptor was detected in 42 cases (77.78%). The colour reaction of a variable intensity demonstrated a membranous location (Fig. 1F). The mean intensity of the reaction in the applied scale of appraisal was 1.48 ± 1.06 pts.

The lowest expression of MT was found in the highly differentiated breast cancers, G1. In the worse differentiated breast cancer cases, G2 and G3, the level of MT was slightly higher and manifested an increasing trend (G1 vs G3, $p=0.020$) (Fig. 2).

In order to examine the relationship between expression of MT and other studied markers, Spearman's rank correlation index was used. Results of the analyses demonstrated a moderately positive correlation between expression of MT and Ki-67 antigen ($r=0.32$, $p=0.019$) (Fig. 3). Moreover moderately negative correlations were detected between MT expression and expression of ER receptors ($r=-0.35$, $p=0.008$) (Fig. 4) and PgR receptors ($r=-0.27$, $p=0.046$) (Fig. 5). On the other hand, no relationship could be disclosed between MT expression and expression of HER-2 ($r=0.12$, $p=0.37$).

Discussion

The numerous studies performed up to now demonstrated increased MT (MT-1/2) expression in several tumours, including tumours of lungs, ovary, urinary bladder, large intestine and kidneys [26–30]. In most of the cases high level of the protein was linked to poor prognosis. Several authors showed an evident relation-

Table 2. Evaluation of HER-2 receptor expression [25].

Intensity of membranous colour reaction	Points
No colour reaction or weak membranous colour reaction in <10% cancer cells	0
Weak membranous colour reaction in >10% cancer cells, stained fragments of cells membranes	1
Weak or moderate staining of the entire cell membrane, present in >10% cancer cells	2
Intense, evident staining of the entire cell membrane, present in >10% cancer cells	3

ship between intensity of MT expression and clinical advancement of the tumours, frequency of metastasis development, as well as shortening of survival rate and disease free survival [17,28,31–34]. In some tumours also a significant correlation between high levels of MT and unfavourable results of treatment was observed [30,35,36].

An increased MT (MT-1/2) expression was demonstrated also in breast cancers, but clinical significance of the proteins has not been yet unequivocally determined. In the normal mammary gland MT expression is noted mainly in myoepithelial cells, while in epithelial cells of ducts MT expression is found very rare [15]. A similar pattern of MT expression was observed in the examined benign lesions of mammary gland, including adenomas, adenofibromas and papillomas [37]. In lobular breast cancers as well as in other, less frequent histological types of the tumour, including mucinous carcinoma and papillary carcinoma, in most cases no MT expression or weak cytoplasmic reactions was shown [13,15]. On the other hand, a definitely increased MT expression was noted in ductal breast cancer, both in *in situ* and invasive forms. According to authors of the till now published reports 26% to 100% of all ductal breast cancer cases manifest overexpression of MT [15].

It is suggested that the role of MT in carcinogenesis and tumour progression could be linked to the involvement of MT in processes of cell proliferation and differentiation [10,12,13]. Using flow cytometry, the highest concentration of MT was found in cells at the end of G1 phase and at the threshold of G1/S phases [38]. Moreover, a significant MT translocation was immunocytochemically demonstrated to take place from cytoplasm to cell nucleus during S phase of cell cycle [3,15]. In *in vitro* studies conducted on human breast cancer cell line MCF-7, the inhibition of MT expression by anti-sense oligonucleotides was observed to inhibit cell growth and to activate apoptosis [39]. Disturbances in cell cycle linked to uncontrolled cell proliferation and inhibition of apoptotic process are widely known to represent a significant component of neoplastic progression. The increased

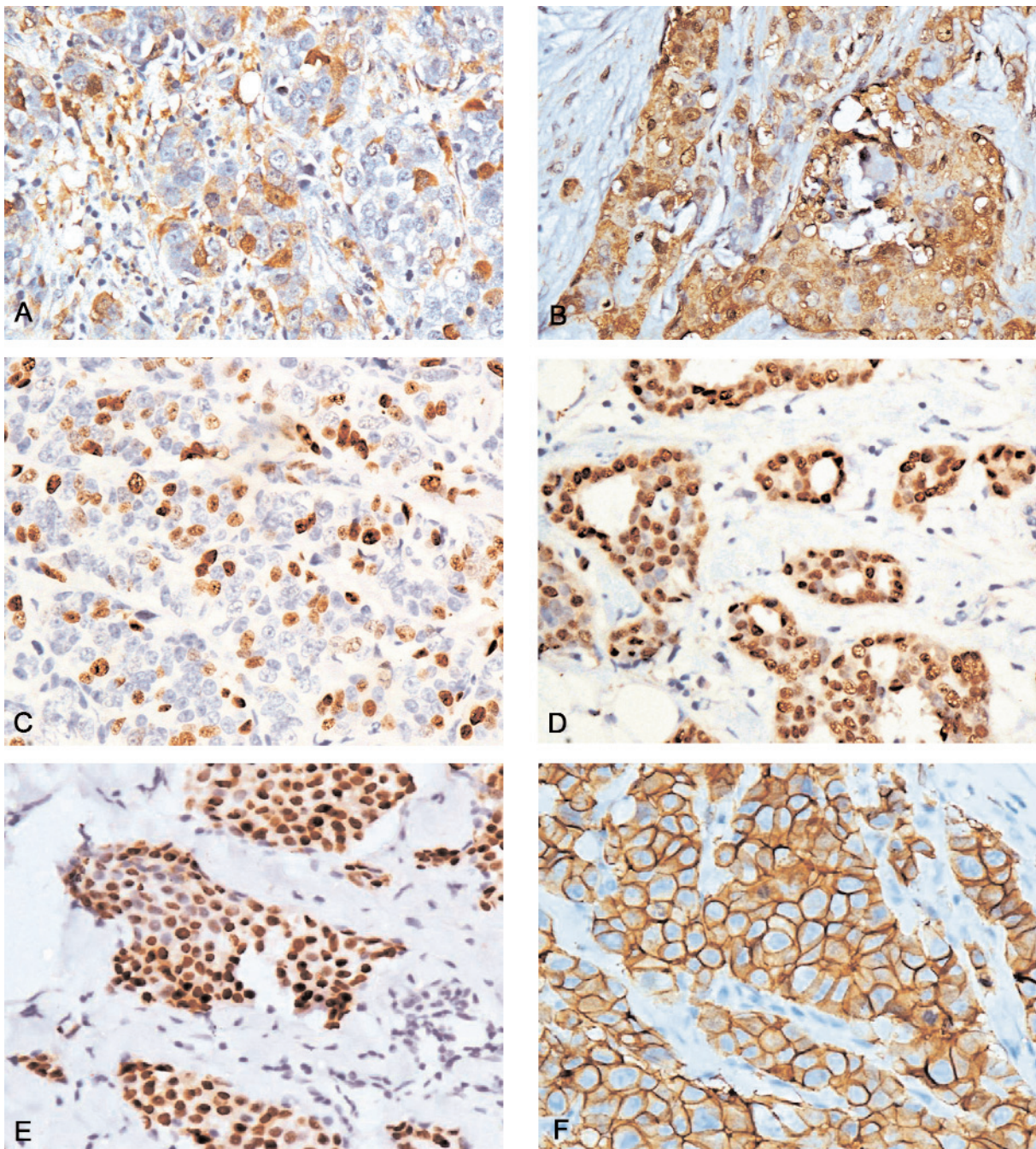


Fig. 1. Expression of studied proteins in cells of ductal breast cancers. MT, a weak nuclear-cytoplasmic reaction (A). MT, moderate nuclear-cytoplasmic reaction (B). Ki-67, moderate nuclear reaction (C). ER, strong nuclear reaction (D). PgR, strong nuclear reaction (E). HER-2, strong membranous reaction (F). Immunohistochemical technique, counterstained with hematoxylin (original magnification $\times 200$).

proliferative activity results in a more pronounced expansion of the tumour, in accelerated course of the disease and, thus, in worse prognosis.

In our investigations we have evaluated expression of MT (MT-1/2) in cells of ductal breast cancer and its relationship with an increased proliferative potential of neoplastic cells, manifested by expression of Ki-67 antigen. Ki-67 antigen is thought to represent one of the most sensitive indices of cell proliferative activity and numerous till now conducted studies have corrob-

orated prognostic value of its expression in various tumours [40]. In the examined cases of invasive ductal breast cancer we have demonstrated a positive correlation between MT and Ki-67 antigen expression, corroborating the earlier results of Jin *et al.*, obtained in the same type of tumour [41]. A similar relationship has been documented, i.e., in squamocellular oesophageal carcinoma, nasopharyngeal carcinoma, as well as in cancers of ovary, large intestine and kidneys [27,29,30,32,42]. Positive correlation between MT and

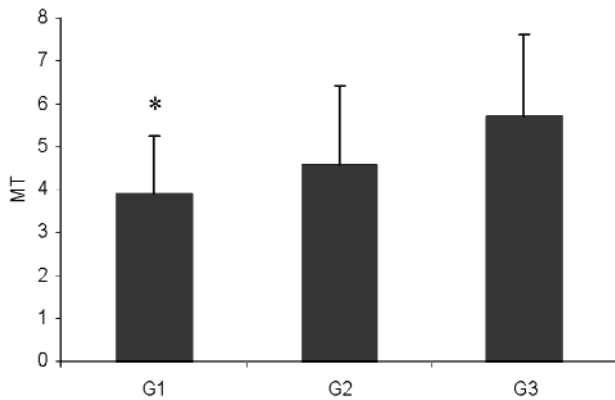


Fig. 2. Intensity of MT expression (IRS) in ductal breast cancers as related to their grade of histological differentiation, G (* $p=0.020$ G1 vs G3).

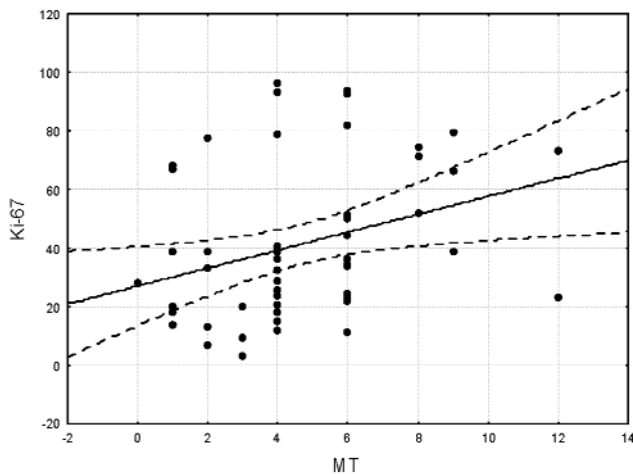


Fig. 3. Correlation between expression of MT (IRS) and expression of Ki-67 antigen (% cells) in the studied group of ductal breast cancers ($r=0.32$, $p=0.019$).

Ki-67 expression has been confirmed also in tumours of mesodermal origin, i.e. in histiocytic fibrosarcoma, adiposarcoma and synovial sarcoma [43]. Results of our studies as well as reports of other authors corroborate the hypothesis on involvement of MT in processes linked to cell proliferation.

The involvement of MT in processes of tumour differentiation, proliferation and progression is also indicated by the relationship between expression levels of the proteins and tumour grade of histological differentiation (G). The grade of histological differentiation or, in other words, grade of malignancy is a resultant of three tumour characteristics, including the ability to form glandular structures, polymorphism of cells which form tumour structures and mitotic activity. Jin *et al.* found that in breast cancer cases of the highest malignancy G3, expression of MT was significantly

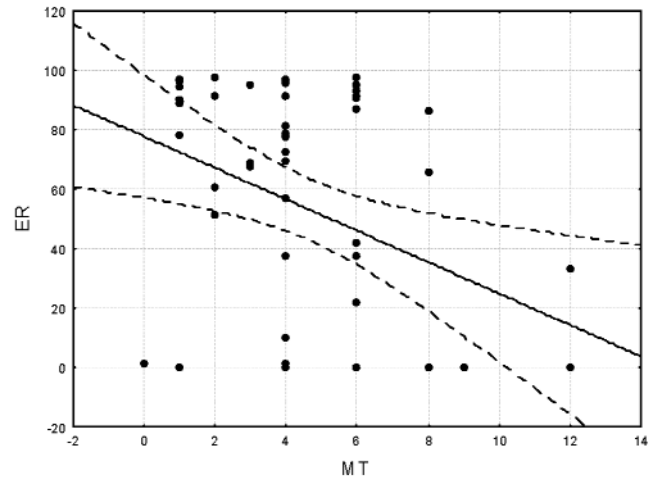


Fig. 4. Correlation between expression of MT (IRS) and expression of ER receptor (% cells) in the studied group of ductal breast cancers ($r=-0.35$, $p=0.008$).

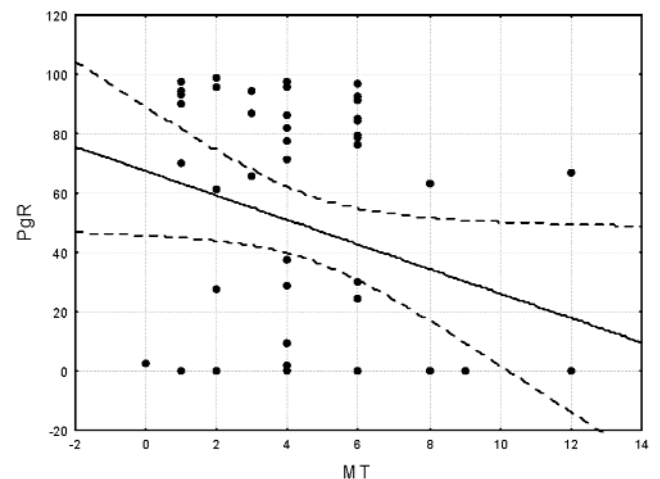


Fig. 5. Correlation between expression of MT (IRS) and expression of PgR receptor (% cells) in the studied group of ductal breast cancers ($r=-0.27$, $p=0.046$).

higher than in better differentiated cases, G1 and G2 [41,44]. Similar observations were made recently by Yap *et al.* [36]. In turn, Gallicchio *et al.* in the studied group of 110 cases of ductal breast cancer demonstrated a strong positive correlation between intensity of MT expression and grade of malignancy [45]. Our results have corroborated results of other authors: we have noted a clearly lower expression of MT in cases of a lower malignancy grade G1, and the expression has increased with increasing grade of cancer malignancy.

Analysis of the obtained results demonstrated also negative correlation between MT expression on one hand and expression of estrogen (ER) and progesterone (PgR) receptors on the other. Presence of ER and PgR in cells of breast cancer and other hormone-dependent tumours, including ovarian and uterine can-

cers, is linked to a more favourable prognosis. The estrogen-positive cases of mammary cancers have been shown to manifest lower proliferative potential and lower expression of receptors for epidermal growth factors, such as EGFR and HER-2 [46,47]. The increased expression of MT in cases of breast cancers devoid of estrogen and progesterone receptors confirms the unfavourable prognostic significance of the proteins. Results of our studies corroborate reports of other authors. Ioachim *et al.* observed that in ductal breast cancers MT expression is significantly higher in cases characterized by absence of ER and PgR [48,49]. The same authors in endometrial carcinoma showed an evident negative correlation between intensity of MT expression and contents of ER and PgR [50]. In turn, in ovarian cancers no relationship between expression of MT and status of receptors for steroid hormones was demonstrated [27].

On the other hand, in the examined group of ductal breast cancers no relationships could have been detected between expression of MT and HER-2 receptor, belonging to the family of membranous epidermal growth factor receptors (EGFR). Cases of breast cancer with over-expression of HER-2 receptor are characterized by a more aggressive course and less favourable prognosis [25]. In the available references no data could have been noted on relationships between MT and HER-2, which would allow to verify the result obtained in this study.

The conducted studies indicate that pronounced expression of MT protein in ductal breast cancers may represent an unfavourable prognostic index. Results of this report as well as data of other authors suggest a need for continuing studies which will permit to better understand role of MT in the neoplastic process and to define clinical significance of their expression in various types of tumours, including breast cancer.

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