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Elevated metallothionein (MT) expression in invasive ductal breast cancers predicts tamoxifen resistance

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Summary. Elevated expression of the low molecular weight metallothionein (MT) proteins can be found typically in breast cancer cases with less favourable prognosis. The MT gene has been described to be potentially down-regulated by estrogen receptor alpha. The present study is aimed at examining the predictive value of MT expression for results of tamoxifen treatment in breast cancer in relation to steroid receptor status. Sixty patients with primary invasive ductal breast cancers with post-operative tamoxifen treatment were enrolled in the study. In paraffin sections of the studied tumours immmunohistochemical reactions were performed using antibodies directed against MT, estrogen receptors (ER) and progesterone receptors (PgR). Results of the immunohistochemical reactions and of clinical observations were analysed using multivariate progression analysis based on the Cox proportional hazard model. Elevated MT expression was demonstrated to be typical for cases with documented relapse of the disease ($P<0.001$) or terminated by death ($P=0.03$). Decreased ER expression was found to be typical for cases of a higher grade ($P=0.02$) and cases terminated by death ($P=0.006$). The multivariate analysis showed that elevated MT expression was characteristic for cases with shorter overall survival time ($P=0.04$). The data showed that MT carried an independent, and also independent from ER status, unfavourable predictive value as far as results of tamoxifen treatment were concerned.

Key words: Breast cancer, Metallothionein, Tamoxifen

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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. It is of general importance to predict the biology of the tumour and, thus, the course of the disease in the individual patient to ensure adequate therapy and patient surveillance (Fitzgibbons et al., 2000).

In the treatment of breast cancer, tamoxifen represents at present the most frequently employed agent (Plouffe, 2000). It exerts its curative action by blocking the function of estrogen receptor alpha (ER). Clinical trials on tamoxifen in post-menopausal females with breast cancer demonstrated remissions in 15-35% of the patients. In ER positive cases the remission developed in over 60% of the patients. On the other hand, in the ER negative cases the remissions occurred in only less than 10% of the patients (EBCTCG, 1998). A more accurate definition of sensitivity to tamoxifen in individual cases would permit the employment of, in the tamoxifen-resistant cases, some other therapeutic approaches, thus improving efficiency of breast cancer treatment.

Metallothioneins are low molecular weight proteins (6 to 7 kDa), the chains of which are composed of 61 or 62 amino acids. Typically, they contain multiple cystein residues and few aromatic amino acids. MT are thought to mediate several functions, including the control of indispensable trace element levels (e.g., zinc, copper), alleviation of toxic effects of cadmium and mercury or protection of cells from oxidative stress (Hamer, 1986; Kagi, 1991).

Numerous reports documented the presence of MT in various tumour cells, including breast cancer (Dziegiel et al., 2003; Hishikawa et al., 1999; Zhang et al., 2000). Augmented expression of MT has been reported in less differentiated tumours (Ioachim et al.,

1999). MT expression used to be linked to higher proliferative activity of tumour cells and to shorter survival of the patients (Oyama et al., 1996; Dziegiel et al., 2003). A negative correlation was also described between expressions of MT and of ER, which suggests involvement of ER in the control of MT gene (Jin et al., 2000).

Opinions on prognostic value of MT expression in breast cancer are divergent. Oyama et al. (1996) failed to demonstrate links between MT expression and survival time. On the other hand, Zhang et al. (2000), Goulding et al. (1995), Haerslev et al. (1995), Vazquez-Ramirez et al. (2000) and Fresno et al. (1993) demonstrated that MT expression represented an unfavourable prognostic factor in breast cancers. Ioachim et al. (2003) showed in a mixed group of 134 patients with breast cancer a limited prognostic value of immunohistochemical MT expression.

Negative correlation between expression of MT on one hand and of ER on the other as well as unfavourable prognostic significance of MT expression in breast cancer suggests that elevated expression of MT may be typical for tamoxifen-resistant cases of the cancer. In our present study we aimed to evaluate the predictive value of MT expression examined by immunohistochemistry in relation to estrogen and progesterone receptors status in primary invasive ductal breast cancers post-operatively treated with tamoxifen.

Table 1. Patient and tumor characteristics. Univariate survival analysis (Kaplan-Meier) of relationships between age, grade and stage and overall survival time.

CHARACTERISTICS	No. (%)[a]	LOG RANK *P* value
All patients	60 (100)	
Age (mean 60.2±9.57 SD)		0.43
≤ 50	5 (8)	
>50-60	14 (23)	
>60	41 (69)	
Grade		0.02
2	48 (80)	
3	12 (20)	
pTNM		0.006
II a	19 (32)	
II b	41 (68)	
Histology		
Invasive ductal breast cancer	60 (100)	
Therapy (in total)		
Pateys mastectomy	60 (100)	
Tamoxifen 20mg / day	60 (100)	
MT expression		0.0096
Lower MT expression (IRS 0-4)	24 (40)	
Higher MT expression (IRS 5-12)	36 (60)	
ER expression		0.09
ER positive (IRS 3-12)	42 (70)	
ER negative (IRS 0-2)	18 (30)	
PgR expression		
PgR positive (IRS 3-12)	38 (63)	0.43
PgR negative (IRS 0-2)	22 (37)	

[a]: Differences in the sum to 100 % in groups are due to rounding. MT: metallothionein, ER: estrogen receptor, PgR: progesterone receptor.

Material and methods

Patients

Immunohistochemical analysis was performed retrospectively on tissue samples which were taken for routine diagnostic purposes. The cases were selected based on availability of tissue and were not stratified for known preoperative or pathological prognostic factors. The study was approved by an Institutional Review Board (IRB) and the patients gave their informed consent before their inclusion in the study. Sixty post-menopausal patients with primary invasive breast cancer who were diagnosed in the years 1993 to 1994 in Lower Silesian Centre of Oncology in Wroclaw, Poland were included in the study. All the patients were subjected to mastectomy according to Patey. Following the surgery all the patients were subjected to monotherapy, applying tamoxifen at 20 mg daily dose. The compliance was monitored by the doctors in charge. The patients were monitored by periodic medical check-ups, ultra-sonographic and radiological examinations (Table 1). Fragments sampled from studied tumours were fixed in 10% buffered formalin and then embedded in paraffin. In every case, hematoxylin and eosin stained preparations were subjected to histopathological evaluation by two pathologists (P.D. and A.W.). Tumour histology was determined according to the criteria of the WHO. The stage of tumours was assessed according to Unio Internationale Contra Cancrum. Tumour grade was estimated according to Bloom-Richardson in the modification of Elston and Ellis. During the follow-up period, 28 patients (47%) had a recurrent disease and 20 patients (33%) died of the disease. The mean (median) progression-free survival time was 48.9 months (range 8 to 90 months), while the mean (median) overall-free survival time was 59.6 months (range 13 to 90 months).

Immunohistochemistry

Formalin-fixed paraffin embedded tissue was freshly cut (4 µm). The 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. Immunohistochemical reactions were performed using the following antibodies: monoclonal (clone E9) mouse antibodies detecting isoforms 1 and 2 of metallothionein (DakoCytomation, Denmark) at dilution 1:100 in the Antibody Diluent, Background Reducing (DakoCytomation, Denmark), monoclonal mouse antibodies (clone 1 D5) to ER (DakoCytomation, Denmark)(optimaly prediluted) and

monoclonal mouse antibodies (clone 1 A6) to PgR (Dako, Denmark) (optimaly prediluted). The sections were incubated with antibodies for one hour at room temperature. Subsequently, incubations were performed with biotinylated antibodies (15 min, room temperature) and with streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB2, 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 every case controls were included in which the specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation, Denmark).

Evaluation of reaction intensity

Intensity of immunohistochemical reactions was estimated independently by two pathologists (P.D. and A.W.). In doubtful cases a re-evaluation was performed using a double-headed microscope and staining was discussed until a consensus was achieved. In order to evaluate the expression of studied antigens a semi-quantitative scale of ImmunoReactive Score (IRS) was applied, in which intensity of colour reaction and percentage of positive cells were taken into account. The score represented a product of points given for the evaluated characters and it ranged from 0 to 12 (Remmele and Stegner, 1987) (Table 2). In analyses of ER and PgR, cases with expression of 0 to 2 in IRS scale (Remmele and Stenger, 1987; Fitzgibbons et al., 2000; Ogawa et al., 2004) were treated as negative cases.

Statistical analysis

Statistical analysis of the results was performed using Statistica 98 PL software (Statsoft, Poland). Kaplan-Meier statistics and log-rank tests were performed to estimate the significance of differences in survival times. To examine relationships between individual variables ANOVA rank test of Kruskal-Wallis and Spearmanns rank correlation were used. *P* values <0.05 were considered to indicate significant differences. A multivariate progression analysis, based on the Cox proportional hazard model, was performed to test the independent value of each parameter predicting overall survival.

Table 2. Evaluation criteria of MT expression using IRS (ImmunoReactive Score) score (by Remmele and Stegner, 1987).

PERCENTAGE OF POSITIVE CELLS	POINTS	INTENSITY OF REACTION	POINTS
No positive cells	0	No reaction	0
<10% positive cells	1	Weak colour reaction	1
10-50% positive cells	2	Moderate intensity	2
51-80% positive cells	3	Intense reaction	3
>80% positive cells	4		

Results

In the case of MT, performed immunohistochemical reactions yielded a colour reaction localized in the cytoplasm as well as in cell nuclei of cancer cells. The reaction varied in individual cases (Fig. 1A,B). The mean MT immunoreactivity score was 4.68±3.17 SD. The non-invasive components of studied invasive cancers demonstrated strong cytoplasmic and nuclear reactions in myoepithelial cells while non-invasive cancerous cells manifested no colour reactions (Fig. 1C). In the case of ER alpha the obtained colour reaction was localized in cell nuclei (Fig. 2A). The intensity of the reaction varied in individual cases. The mean immunoreactivity score amounted to 4.06±4.23 SD. In the case of PgR the colour reaction was localized in cell nuclei (Fig 2B). The intensity of the reaction varied in individual cases. The mean immunoreactivity score was 4.35±4.11 SD.

Using the ANOVA rank test of Kruskal-Wallis, relationships were examined between the overall immunoreactivity scores of MT, ER and PgR and relapses, patient deaths, grade, stage and patients age. The cases with relapse of the neoplastic disease demonstrated a higher MT immunoreactivity score, as compared to the cases with no relapse ($P<0.0001$) (Fig. 3A). In the cases terminated by death of the patient the immunoreactivity score of MT was significantly higher ($P=0.03$) than that noted in surviving patients (Fig. 3B). No significant relationships were detected between the MT immunoreactivity score and grade, stage and patients' age ($P=0.43$, $P=0.09$, $P=0.78$, respectively). In the cases terminated by death of the patient the overall ER immunoreactivity score was also significantly lower ($P=0.006$) than that noted in surviving patients (Fig. 4A). In the G2 grade ER immunoreactivity score was significantly ($P=0.02$) higher than in G3 grade (Fig. 4B). No significant relationships were found between the overall immunoreactivity score of ER and relapses, stage and patients' age ($P=0.09$, $P=0.38$, $P=0.76$) and between the overall immunoreactivity score of PgR and studied clinico-pathological variables (P>0.05).

Spearmann's rank correlation was applied in order to examine relationships between the overall immunoreactivity score of MT on one hand and the overall immunoreactivity score of ER and PgR. We have found negative correlations between MT and ER expression ($P=0.003$) (Fig. 5A) and also between MT and PgR expression ($P=0.003$) (Fig. 5B).

Using log-rank the overall survival time and the progression-free time were examined for groups manifesting an overall MT immunoreactivity score extent of 0-4 (lower MT expression, 24 cases) and those manifesting an overall immunoreactivity score of 6-12 (higher MT expression, 36 cases). The calculations demonstrated that cases of lower MT expression showed a significantly longer overall survival time (log-rank

test: P=0.0096). No significant relationships could be documented between MT expression and a disease-free time (log-rank test: P=0.12). It should be added that 12 (20%) cases manifesting higher MT expression demonstrated a parallel expression of ER. Both in the case of ER and in the case of PgR differences in overall survival time and in a disease-free time were examined between groups which clinically demonstrated no expression of a given receptor (IRS 0-2, 19 cases in the case of ER and 24 in the case of PgR) (Remmele and Stenger, 1987; Fitzgibbons et al., 2000, Ogawa et al., 2004) and groups manifesting such an expression (IRS 3-12, 41 cases in the case of ER and 36 in the case of PgR). The calculations demonstrated no relationship between the expression of ER or that of PgR and overall survival time (log-rank test: P=0.09 and P=0.43, respectively) or disease-free time (log-rank test: P=0.14 and P=0.68, respectively).

MT expression as well as other clinico-pathological parameters that were significant in univariate analysis (grade and stage) (Table 1) and the immunoreactivity score of ER expression were included in multivariate analysis. This showed a significant relationship between MT expression and overall survival time (F Cox test: P=0.03) (Fig. 6). Out of the other parameters, only stage was demonstrated to represent an independent prognostic factor (P=0.009) for overall survival.

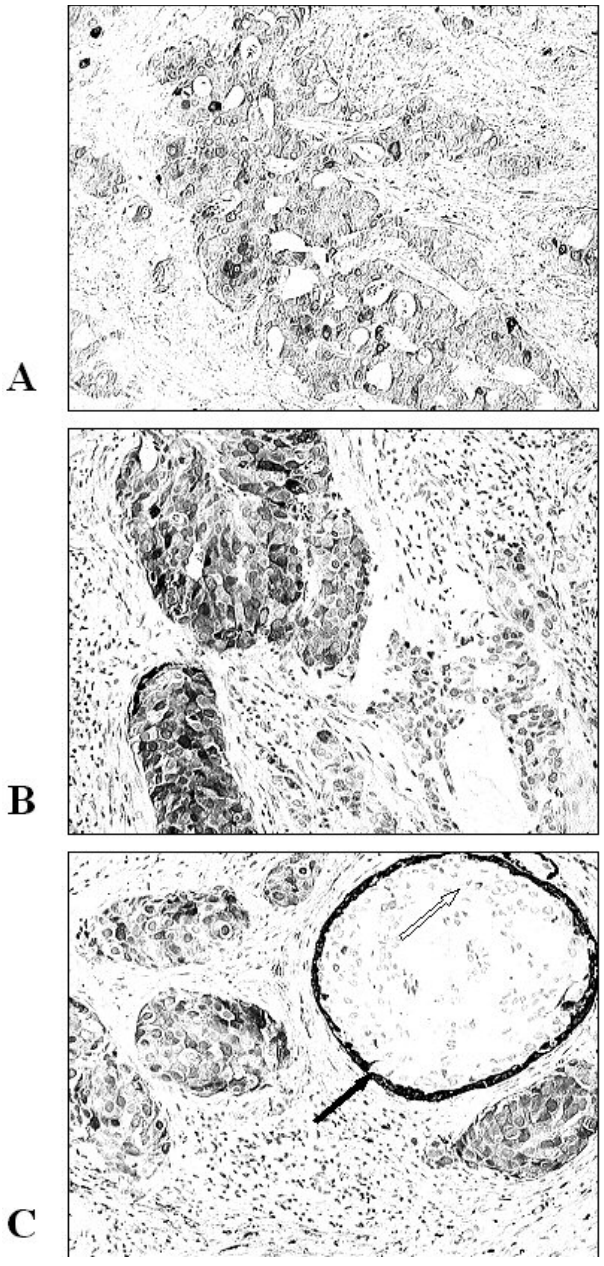

Fig. 1. A, B, C. Immunohistochemical localization of metallothionein expression in primary invasive ductal breast cancers. **C.** Note strong reaction in myoepithelial cells (black arrow) and absence of reaction in non-invasive cancer cells (white arrow). A, x 200; B, C, x 400

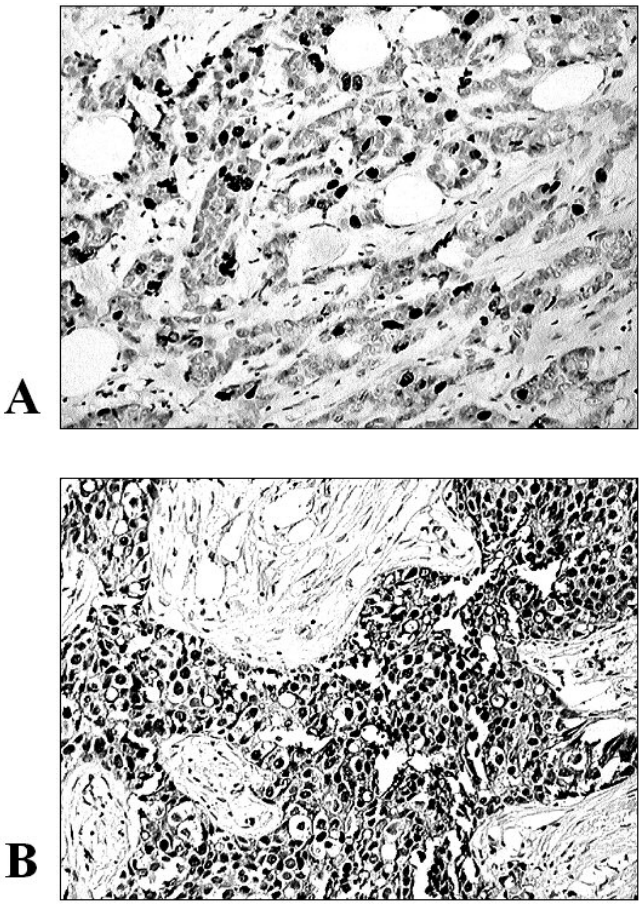

Fig. 2. A. Immunohistochemical localization of estrogen receptor expression in primary invasive ductal breast cancers. **B.** Immunohistochemical localization of progesterone receptor expression in primary invasive ductal breast cancers. A, x 400; B, x 200

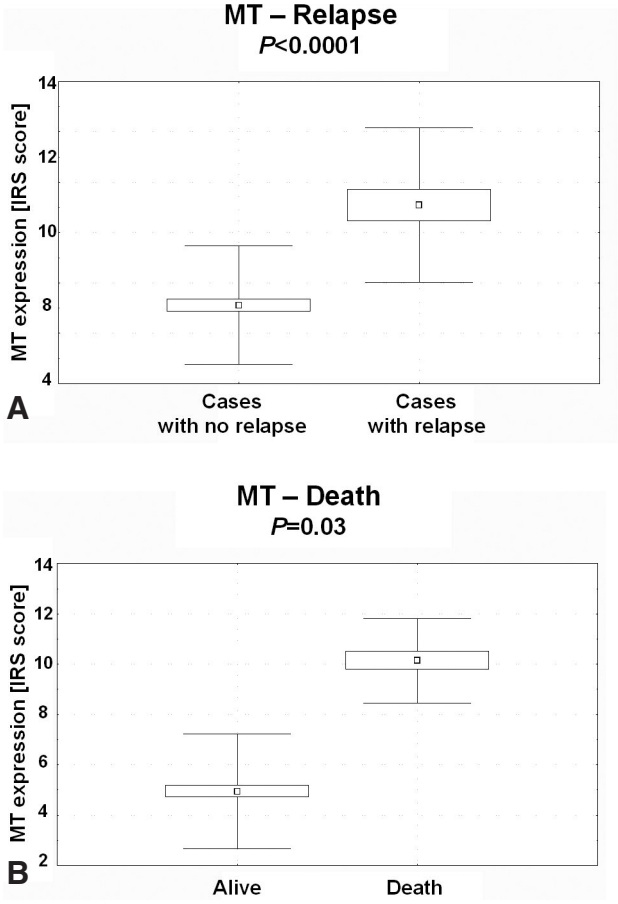

Fig. 3. Relationships between MT expression and relapse of the neoplastic disease and patients death. Cases with relapse **(A)** or of death **(B)** manifested a significantly higher overall MT immunoreactivity score.

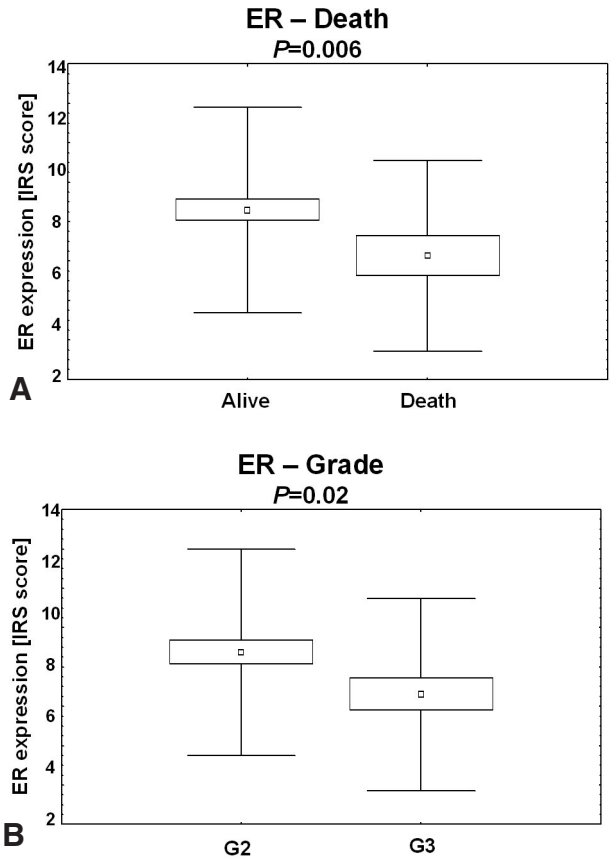

Fig. 4 Relationships between ER expression and patient death and grade. Cases of death **(A)** or of higher grade **(B)** manifested a significantly lower overall ER immunoreactivity score.

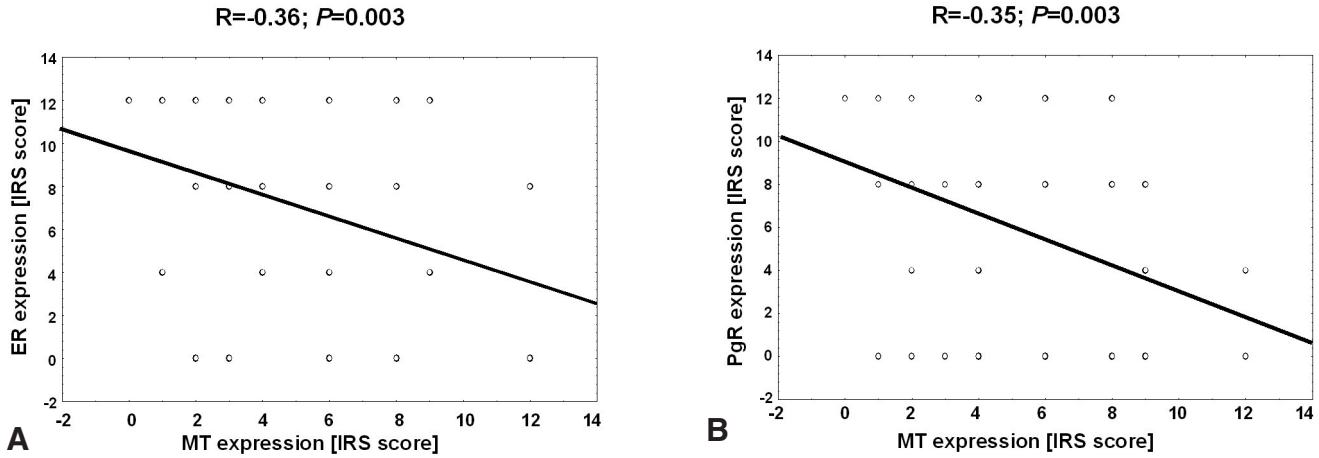

Fig. 5. Negative correlation between the overall MT immunoreactivity score and **(A)** overall ER immunoreactivity score, **(B)** overall PgR immunoreactivity score.

Discussion

In this work we have described the expression of MT as detected by immunohistochemistry in primary invasive ductal breast carcinomas. We have confirmed that MT protein is expressed in a subset of breast cancers, as described by other authors (Fresno et al., 1993; Goulding et al., 1995). The significance of MT expression has been studied in a group of 60 cases of primary invasive ductal breast cancers. All patients were postoperatively treated with tamoxifen. Those patients who died during the observation period manifested a higher overall immunoreactivity score of MT as compared to surviving patients. Our calculations have demonstrated also that cases of lower overall immunoreactivity score of MT have shown a significantly longer overall survival time. Cases with relapse of the tumour have shown a significantly higher MT expression. MT expression has shown no relationship with a disease-free survival. Also, no relationship has been uncovered between intensity of MT expression on one hand and grade, stage and age of the patients on the other.

Using immunohistochemistry we have demonstrated at present that 70% cases of invasive ductal mammary carcinoma exhibited also expression of ER and 63% of such cases exhibited a parallel expression of PgR. The data are consistent with those published by other authors (Fitzgibbons et al., 2000; Ogawa et al., 2004) and with earlier reports of our own (Surowiak et al., 2001, 2004). Similarly to our earlier investigations (Surowiak et al., 2004), we have shown that the overall ER immunoreactivity score is lower in the cases of higher grade. In the present study on the group of 60 patients with primary invasive ductal mammary carcinoma we have demonstrated that fatal cases manifested a lower overall ER immunoreactivity score as compared to surviving patients. No relationship could have been disclosed between the expression of ER and PgR on one hand and overall survival time and a disease-free time on the other, as well as between the expression and the other clinico-pathological variables studied.

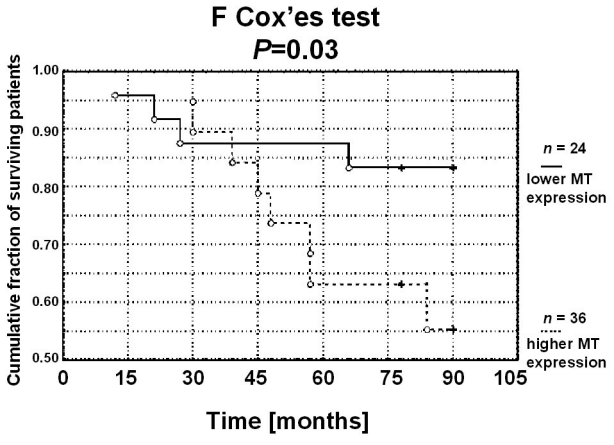

Fig. 6. A Cox regression plot of cumulative survival of patients with higher (IRS 6-12) and lower (IRS 0-4) MT expression in ductal breast cancer. All the patients were treated with tamoxifen. In the group with lower MT expression 83% of patients and in the group with higher MT expression only 55% of patients survived until the 90th month following surgery.

MT expression in breast cancers and in breast cancer cell lines has been noted by several authors (Haerslev et al., 1995; Friedline et al., 1998; Ioachim et al., 2003). High overall MT expression has been consistently associated with increased tumour grade and more severe nuclear pleomorfism (Fresno et al., 1993; Zhang et al., 2000; Jin et al., 2002), as compared to the low MT expressing counterparts. Most studies have shown no significant associations of MT expression with tumour size and with presence of lymph node metastases at diagnosis (Jin et al., 2000, 2002). Tai et al. (2003) in *in vitro* investigations have demonstrated that MT-2A isoform was up-regulated in breast cancer cells of a higher invasive potential. Jin et al. (2001a,b) have found that increased MT-1F and MT-2A mRNA were separately associated with higher histological grade, but not with patients' age and lymph node status. Fresno et al. (1993) and Jin et al. (2000) have shown a negative correlation between expressions of MT and ER in breast cancer samples. Haerslev et al. (1995) have documented a negative correlation between MT expression and another classical exponent of sensitivity to tamoxifen treatment, the progesterone receptor (PgR). In this work we have found negative correlation between the overall MT immunoreactivity score and the overall immunoreactivity score of ER and PgR. Friedline et al. (Friedline et al., 1998) has found that in some cell lines MT-1E gene might be down-regulated by estrogen receptor alpha (ER alpha). Harris et al. (2001) has noted the estradiol-induced up-regulation of MT expression in cells which carried estrogen receptor beta. The above described relationships suggest that MT gene may be down-regulated by ER alpha and, thus, that MT expression may increase when ER alpha is inactive. In a few reports an unfavourable prognostic significance of MT expression in breast cancer cases has been noted (Fresno et al., 1993; Goulding et al., 1995; Haerslev et al., 1995; Vazquez-Ramirez et al., 2000; Zhang et al., 2000; Ioachim et al., 2003). In a single study only (Oyama et al., 1996) MT expression has been found to bear no relation to duration of patient's survival. To our knowledge, this study demonstrates for the first time an independent prognostic value of MT expression in human breast cancers treated with tamoxifen. It should be mentioned that 12 (20%) cases with higher MT expression demonstrated a parallel expression of ER. In our investigations we have not been able to document any relationship between the the overall ER immunoreactivity score and overall survival time of studied patients. The data have indicated that the negative predictive value of MT expression is not linked

to negative correlation between MT and ER.

In this study we have confirmed also the phenomenon described by Jin et al. (2001b): in the components of *in situ* examined invasive cancers myoepithelial cells manifest a strong colour reaction while cancer cells remain negative. Hence, MT may also provide an interesting marker which may facilitate differentiation between pre-invasive and invasive cancers.

The studies have shown that MT expression is an unfavourable predictive factor for results of tamoxifen treatment. Taking into account the fact that *MT* gene is down-regulated by ER, the group of cancers with expression of both MT and ER should provide an interesting material for studies. No literature data are available which would indicate that MT *per se* may cause resistance to tamoxifen. Expression of MT in ER(+) cases may be explained by ER damage or by its malfunction. Hence, MT most probably represents a phenotypic marker of resistance to tamoxifen. A group of ER(+) and MT(+) cases is important from the clinical point of view since as many as around 40% cases with ER alpha expression fail to respond to tamoxifen treatment (EBCTCG, 1998). Parallel estimation of MT and of other exponents of sensitivity to tamoxifen, such as ER or PgR may improve predictive power and, thus, efficiency of breast cancer treatment. Further studies on other patients with breast cancer are needed to validate this finding. Considering the fact that MT is also operative in phenomena of resistance to cytostatic drugs and to radiotherapy (Theocharis et al., 2004), the protein should be targeted by investigations on the novel therapeutic approaches.

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