Correlation of Intensity of MT-I/II Expression with Ki-67 and MCM-2 Proteins in Invasive Ductal Breast Carcinoma

ANDRZEJ WOJNAR[1], BARTOSZ PULA[2], ALEKSANDRA PIOTROWSKA[2], ALEKSANDRA JETHON[2], KRZYSZTOF KUJAWA[3], CHRISTOPHER KOBIERZYCKI[2], JANUSZ RYS[4], MARZENA PODHORSKA-OKOLOW[2] and PIOTR DZIEGIEL[1,2,5]

[1]*Lower Silesian Oncology Centre, Wroclaw, Poland;*
[2]*Department of Histology and Embryology, Medical University, Wroclaw, Poland;*
[3]*Institute for Agricultural and Forest Environment, Polish Academy of Sciences, Poznan, Poland;*
[4]*Department of Tumour Pathology, Centre of Oncology Maria Sklodowska-Curie Memorial Institute, Krakow, Poland;*
[5]*Department of Histology and Embryology, Medical University, Poznan, Poland*

Abstract. *The purpose of the study was to evaluate the clinical significance of the intensity of metallothionein (MT-I/II) expression and its relationship to two different proliferation markers, Ki-67 antigen and minichromosome maintaince 2 protein (MCM-2) in 117 patients with invasive ductal breast carcinoma (IDC). A significantly higher MT-I/II expression was noted in the grade 3 (G3) carcinomas as compared to those of G1 and G2. A positive correlation was observed between the MT-I/II expression and both proliferation markers, Ki-67 (r=0.20, p=0.0343) and MCM-2 (r=0.25, p=0.0065). Also a strong positive correlation was noted between Ki-67 and MCM-2 expression (r=0.52, p<0.0001). No significant correlations were found between the analyzed markers and tumour size, lymph node metastasis, oestrogen expression (ER), progesterone receptor (PR) or human epidermal growth-factor receptor (HER-2) expression. Out of the three studied markers only the high expression of Ki-67 exhibited a negative impact on patient overall and event free survival and was an independent prognostic factor. MT-I/II and MCM-2 protein expression was not correlated with poor patient outcome, although MCM-2 expression correlated (Fisher's exact test) positively with grade of malignancy (p=0.0018) and negatively with ER (p=0.0002) and PR (p=0.0056) expression. To conclude, MT-I/II may play a key role in IDC proliferation, but is not a useful prognostic factor of this disease.*

Breast cancer poses a great health problem worldwide and is the most frequent malignant tumour with more than 600,000 new cases diagnosed in Europe and the United States annually (1). In the USA, it is the most frequent cause of death of women aged 20 to 59 years (2). In view of the above, an early diagnosis and effective treatment are immensely important, therefore reliable prognostic and predictive factors are required. Currently, the predictive factors for breast cancer are oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth-factor receptor (HER-2) status. The proliferation marker Ki-67 has been proven to be a valuable prognostic marker in many studies, but due to some concerns, is not used in diagnostic routine (3).

Metallothioneins (MT) belong to a family of low molecular weight proteins which are expressed in many species and various tissues. Until now four main isoforms have been discovered (4, 5). The physiological role of MT involves binding and transport of zinc and copper ions and detoxification by binding heavy metals, *e.g.* cadmium, mercury and lead (4, 5). It has been shown that metallothionein isoforms I and II (MT-I/II) may stimulate cell proliferation by supplying zinc ions to enzymes participating in DNA replication (6). Moreover, MT protect cells from apoptosis, and reactive oxygen species and mediate resistance to certain chemotherapeutic agents (by inactivating free radicals formed due to metabolism of certain cytostatic drugs, *e.g.* anthracyclins and binding to other agents, *e.g.* to cisplatin) (6-9). MT-I/II may protect cell's DNA from damage caused by radiation or drugs intercalating with its strands *e.g.* cisplatin (7, 8, 10). Increased MT-I/II expression has been demonstrated in lung, ovary, urinary bladder, larynx, oral cavity and breast tumours (11-15, 30-32). In the majority of this studies, MT-I/II expression was associated with poor clinical outcome and predicted chemoresistance to cisplatin chemotherapy (10). In comparison to IDC, MT-I/II is rarely found in lobular breast carcinonoma and other rare histological types including

Correspondence to: Piotr Dziegiel, MD, Ph.D., Department of Histology and Embryology, Medical University, Chalubinskiego 6a, 50-368 Wroclaw, Poland. Tel: +48 717841354, Fax: +48 71 7840082, e-mail: piotr@hist.am.wroc.pl

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mucinous and papillary carcinoma (16, 17). An association between chemoresistance and MT-I/II overexpression has also been noted in breast cancer (18).

MCM-2 belongs to the family of MCM (minichromosome maintenance proteins), which consists of six homogenous proteins (MCM-2 to 7) (19). At the early G_1 phase of the cell cycle, MCM proteins participate in the formation of the pre-replication complex, what differs them from the Ki-67 antigen, which is mostly present in late G_1, S, G_2 stages of the cell cycle (20-25). MCM-2 may be useful as a new prognostic marker, because of its elevated expression in rapidly dividing cells, including those of breast cancer (19, 26-28). Compared to Ki-67, which is commonly used in various tumour types (including soft tissue tumours, breast, lung, gastric or larynx carcinomas), examination of MCM-2 protein still requires further research to establish its prognostic and predictive value (13, 19, 29-31).

This study aimed to examine the relationship between MT-I/II expression and proliferation markers: MCM-2 protein and Ki-67 proliferation antigen and to investigate the impact of these three proteins on patients outcome.

Patients and Methods

Patients. The studies were performed on 117 IDC cases, sampled in the years 1999-2002 during resection and biopsy procedures in the Lower Silesian Oncology Centre in Wroclaw. The ages of the patients ranged from 30 to 83 years (mean 56.9±11.1 years), all the patients were female. In total 95 patients had undergone Patey's mammectomy and 22 quadrantectomy accompanied by lymph node resection. The tumour size ranged from 1 to 80 mm (mean 23.3±17.0 mm). All tissue specimens used in this study had been collected before the beginning of the treatment. Each patient was treated with suitable adjuvant therapy, according to the stage of the disease. In cases of stage 3 and higher, neoadjuvant therapy had been administered. The histological malignancy grade (G) was determined using guidelines suggested by WHO (32). The clinical and pathological data were obtained from the archives of the Lower Silesian Oncology Centre in Wroclaw (Table I). The patients had been followed up for 56.3±38.7 months (range 1-125 months).

Immunohistochemistry (IHC). Tissue samples had been fixed in 10% buffered formalin, dehydrated and embedded in paraffin. In each case, haematoxylin and eosin-stained preparations were subjected to histopathological evaluation by two pathologists to confirm the diagnosis and estimate the grade of malignancy. MT-I/II IHC staining was performed on 4-μm-thick paraffin sections mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany), deparaffinized in xylene and rehydrated. Activity of endogenous peroxidase was blocked by 5 min incubation with 3% H_2O_2. The expression of MT-I/II was demonstrated using mouse primary monoclonal antibody (E9) (DakoCytomation, Glostrup, Denmark). The MT-I/II primary antibody was visualized using biotinylated secondary antibodies and streptavidin conjugated with horseradish peroxidase (DakoCytomation LSAB+ System-HRP). Diaminobenzidine (DAB, DakoCytomation) was used as the substrate. All the sections were counterstained with Meyer's haematoxylin (20 second).

For Ki-67 (MIB-1, 1:100, DakoCytomation), MCM-2 (CRCT2.1, 1:50, Novocastra/Leica, Newcastle, UK), ER (1D5, 1:100, DakoCytomation) and PR (636, 1:100, DakoCytomation) staining 4-μm-thick paraffin sections were cut. Deparaffinization and antigen retrieval were performed in Target Retrieval Solution (pH 6 for Ki-67, ER, PR; pH 9 for MCM-2; 97˚C, 20 min; DakoCytomation) and a Pre-Treatment Link Rinse Station (DakoCytomation). The sections were then washed in TBS and incubated (RT, 20 min) with primary antibodies in Link48 Autostainer (DakoCytomation). EnVision FLEX (DakoCytomation) was used for the visualization of antibodies, in accordance with the manufacturer's instructions. All the slides were counterstained with Mayer's haematoxylin and conducted with negative controls. HER-2 expression was examined using a HercepTest™ kit (DakoCytomation), following the procedure recommended by the manufacturer.

Evaluation of the intensity of IHC reaction. The evaluation of MT-I/II reaction intensities was conducted using the semiquantitative immunoreactive (IRS) method of Remmele and Stegner (33), which is based on the intensity of the colour reaction and the percentage of positive cancer cells in the slide. The scale ranges from 0-12 points, where 0 denotes absence of reaction, 1-2 corresponds to poor reaction, 3-4 to moderate reaction and 6-12 to a pronounced reaction. The intensity of Ki-67 antigen and MCM-2 protein expression in the cancer cells was evaluated according to the percentage of positive cells among all the cancer cells, where 0 corresponds to the absence of the reaction, 1 denote 1-10% positive cells, 2 to 11-25% positive cells, 3 26-50% and 4 >50% positive cells. All the slides were scored using a BX-42 light microscope (Olympus, Tokyo, Japan). The examining pathologists knew no clinical details related to the respective patients (AW, PD).

Statistical analysis. The results were subjected to statistical analysis using The Prism 5.0 (GraphPad, La Jolla, CA, USA) and STATISTICA 8.0 (StatSoft, Krakow, Poland) software. The relationship between the expression of MT-I/II, Ki-67 and MCM-2 protein was examined using Spearman's rank correlation. The relationship between expression intensity of the two proteins and histological malignancy grade (G) was examined using ANOVA rank test of the Kruskal-Wallis and Mann-Whitney tests. Spearman's rank correlation test and Fisher's exact test was applied to analyze the relationship of the studied markers with the clinicopathological data. Overall survival (OS) and event free survival (EFS) were determined by the Kaplan-Meier method and the significance of the differences was determined by a log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. The odds ratio (OR) and 95% confidence interval (95% CI) were estimated for each variable. The differences were accepted as significant at $p<0.05$.

Results

A cytoplasmic-nuclear pattern of MT-I/II expression was noted in 86 (73.5%) of the examined cases (Figure 1A). As many as 73 (62.4%) cases showed low MT-I/II expression (IRS 0-2), whereas 44 (37.6%) cases were classified as moderate or high expression (IRS 3-12). Nuclear Ki-67 antigen expression was noted in all the study cases (Figure 1B). MCM-2 nuclear expression was observed in 112

Table I. *Clinical and pathological characteristics of 117 studied patients*.

Parameter	No.	%
Age		
≤50	35	29.9
>50	82	70.1
Menopausal status		
Pre	38	32.5
Post	79	67.5
Tumour size		
T1	67	57.3
T2	37	31.6
T3	10	8.5
T4	3	2.6
Lymph nodes		
N0	62	53.0
N1, N2, N3	55	47.0
pTNM		
I	42	35.9
IIA	38	32.5
IIB	17	14.5
III	19	16.2
IV	1	0.9
Grade		
G1	12	10.3
G2	69	59.0
G3	36	30.7
ER		
Positive	90	76.9
Negative	27	23.1
PR		
Positive	80	68.4
Negative	37	31.6
HER2 by IHC		
Positive	18	15.4
Negative	99	84.6

IHC: Immunohistochemistry.

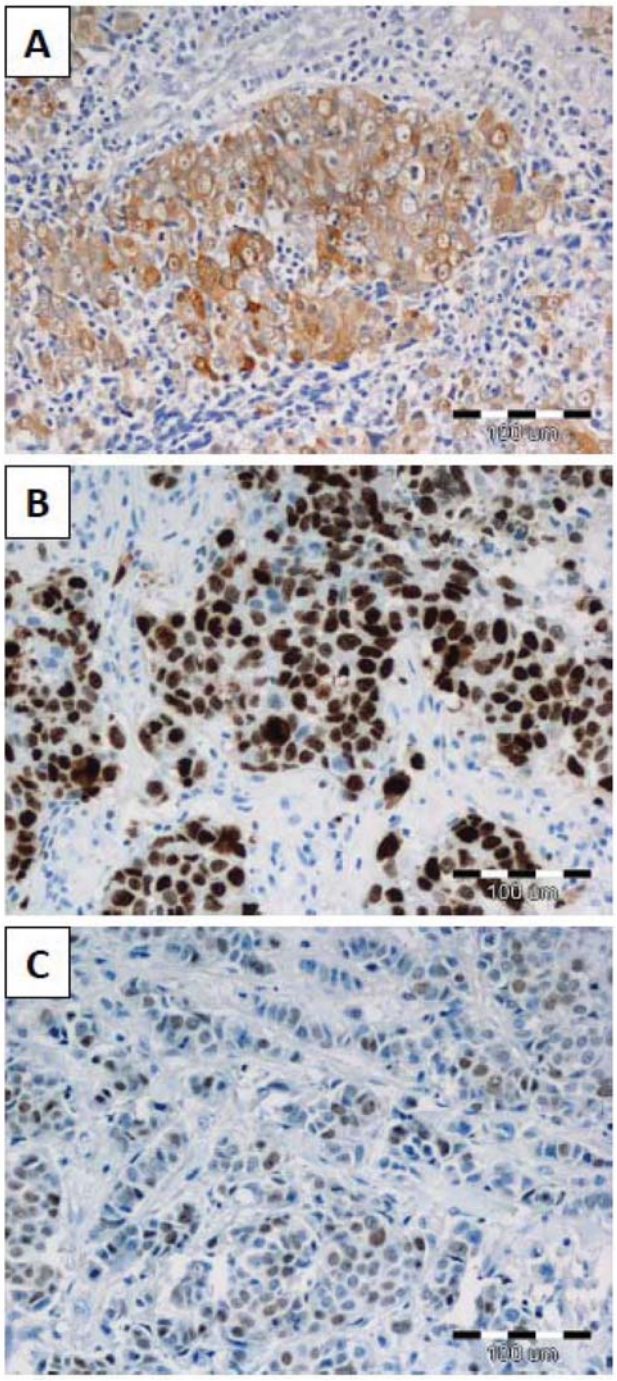

Figure 1. *Cytoplasmic-nuclear expression of MT-I/II (A) and nuclear expression of Ki-67 (B) and MCM-2 protein (C) in invasive ductal breast cancer. Magnification ×200.*

(95.7%) cases (Figure 1C). Repeated staining of the negative MCM-2 sections did not changed the results. The distribution of the studied markers is summarized in Table II. A positive correlation was observed between MT-I/II expression and both proliferation proteins: Ki-67 (r=0.20, p=0.0343) and MCM-2 (r=0.25, p=0.0065). Also a strong positive correlation was noted between Ki-67 and MCM-2 expression (r=0.52, p<0.0001).

Significant differences were found for MT-I/II (p=0.0473), Ki-67 (p<0.0001) and MCM-2 (p=0.0018) regarding the malignancy grade (Figure 2). MT-I/II expression was significantly lower in the G1 cases when compared to the G3 cases (Figure 2A). Significantly higher Ki-67 expression was noted in the G3 cases as compared to G2 (p=0.0001) and G1 cases (p=0.007) (Figure 2B). The same was also true for the expression of MCM-2 protein (G1 *vs*. G3 p=0.0041; G2 *vs*. G3 p=0.0055) (Figure 2C). From the other analyzed clinicopathological factors, tumour size correlated positively with Ki-67 antigen (r=0.32, p=0.0005) and MCM-2 protein (r=0.21, p=0.0255) expression. MT-I/II expression was associated with negative PR status (p=0.0424), but no relationship was found between MT-I/II expression and the

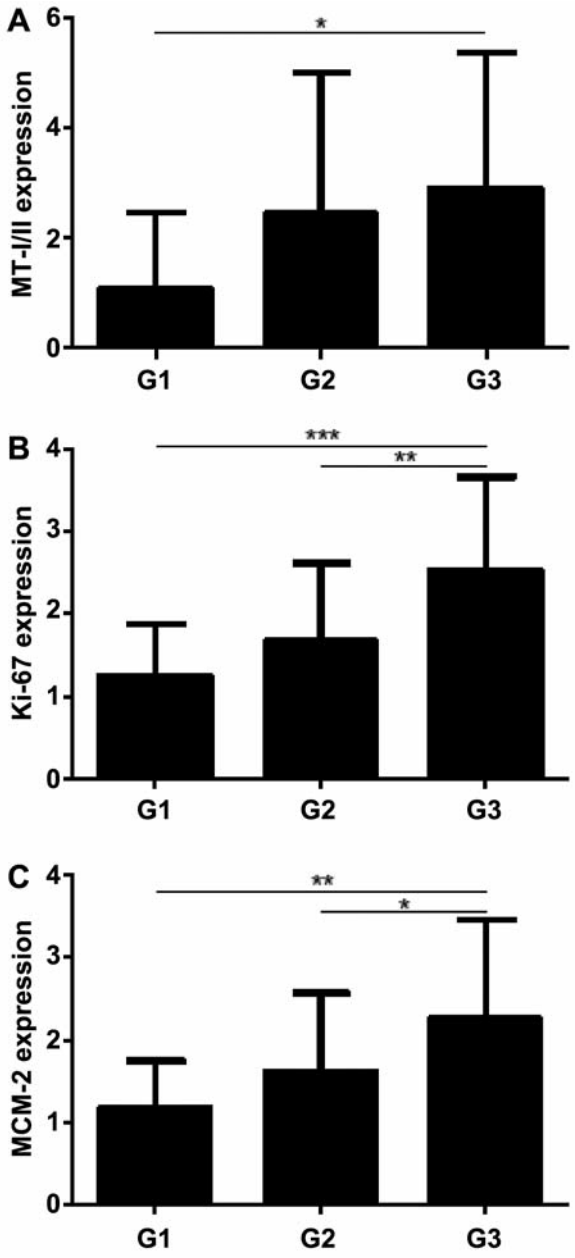

Figure 2. *MT-I/II (p=0.0473) (A), Ki-67 (p<0.0001) (B) and MCM-2 (p=0.0018) (C) expression in different grades of malignancy of invasive ductal breast cancer. *p<0.05; **p<0.005, ***p<0.0005; Kruskall-Wallis test.*

expression of ER or HER-2 (Table III). On the other hand high Ki-67 expression was associated with the absence of ER ($p<0.0001$) and PR ($p<0.0001$) expression. Also, tumours with high MCM-2 expression were associated with a lack of ER ($p=0.0002$) and PR ($p=0.0056$) expression. No associations between the studied markers and patient age, menopausal status or presence of lymph node metastasis were found (Table III).

Table II. *Distribution of MT-I/II, Ki-67 and MCM-2 staining.*

Marker	No.	%
MT-I/II IRS		
0	31	26.5
1-2	42	35.9
3-12	44	37.6
Ki-67 Score		
0	0	0.0
1	59	50.4
2	27	23.1
3	16	13.7
4	15	12.8
MCM-2 Score		
0	5	4.3
1	58	49.6
2	24	20.5
3	20	17.1
4	10	8.5

IRS: Immunoreactive scale.

Univariate analysis revealed that patients with tumours expressing high levels of Ki-67 had a significantly shorter OS ($p=0.0313$) and EFS ($p=0.0027$) than those expressing low levels (Table IV). For the multivariate analysis, also, tumour size (pT) and lymph node involvement (pN) were taken for the analysis, as both these parameters were shown to be associated with poor patient outcome in the univariate analysis. Multivariate analysis showed that Ki-67 ($p=0.042$), pT ($p=0.0003$) and pN ($p=0.0006$) were independent prognostic factors in terms of OS. No differences in OS and EFS were noted regarding MT-I/II and MCM-2 expression. Of note, analysis using the Cox regression model for survival of combined MT-I/II and Ki-67 or MT-I/II and MCM-2 results, did not improve the statistical significance of the results of patients OS ($p=0.835$ and $p=0.34$, respectively).

Discussion

This study confirmed the role of MT-I/II in cancer cell proliferation and the results obtained by our laboratory and Jin *et al*. (16, 34). In particular MT-I/II expression have been positively correlated with proliferation markers Ki-67 antigen and MCM-2 protein, which have been shown to be up-regulated in breast cancer cells (3, 28, 34). The involvement of MT-I/II in cancer progression was confirmed by its relationship with tumour grade of malignancy. Its expression was significantly higher in undifferentiated G3 carcinomas as compared to the well-differentiated G1 carcinomas. Surprisingly, although strong correlations with tumour cell proliferation and grade of malignancy were observed, the MT-I/II expression did not affect the size of

Table III. *Relationship of MT-I/II, Ki-67 and MCM-2 expression with selected clinicopathological parameters. Significant p values are marked bold.*

Parameters	No. (%)	MT-I/II – No. (%)		*P*-value	Ki-67 – No. (%)		*P*-value	MCM-2 – No. (%)		*P*-value
		IRS 0-2	IRS 3-12		≤25%	>25%		≤25%	>25%	
Age										
≤50	35 (29.9)	22 (62.9)	13 (37.1)	1.0000	28 (80.0)	7 (20.0)	0.3645	27 (77.1)	8 (22.9)	0.8167
>50	82 (70.1)	51 (62.2)	31 (38.8)		58 (70.7)	24 (29.3)		60 (73.2)	22 (26.8)	
Menopause										
Pre	38 (32.4)	22 (57.9)	16 (42.1)	0.5434	29 (76.3)	9 (23.7)	0.8233	29 (76.3)	9 (23.7)	0.8235
Post	79 (67.6)	51 (64.6)	28 (35.4)		57 (72.2)	22 (27.8)		58 (73.4)	21 (26.6)	
pN										
Positive	55 (47.0)	34 (61.8)	21 (38.2)	1.0000	40 (72.7)	15 (27.3)	1.0000	42 (76.4)	13 (23.6)	0.6765
Negative	62 (53.0)	39 (62.9)	23 (37.1)		46 (74.2)	16 (25.8)		45 (72.6)	17 (27.4)	
ER										
Positive	90 (76.9)	58 (64.4)	32 (35.6)	0.4976	75 (83.4)	15 (16.6)	**<0.0001**	75 (83.4)	15 (16.6)	**0.0002**
Negative	27 (23.1)	15 (55.6)	12 (44.4)		11 (40.7)	16 (59.3)		12 (44.4)	15 (55.6)	
PR										
Positive	80 (68.4)	55 (68.8)	25 (31.2)	**0.0424**	68 (85.0)	12 (15.0)	**<0.0001**	66 (82.5)	14 (17.5)	**0.0056**
Negative	37 (31.6)	18 (48.6)	19 (52.6)		18 (48.6)	19 (51.4)		21 (56.8)	16 (43.2)	
HER-2										
Positive	18 (15.4)	10 (55.6)	8 (44.4)	0.5994	10 (55.6)	8 (44.4)	0.0812	11 (61.1)	7 (38.9)	0.2381
Negative	99 (84.6)	63 (63.6)	36 (36.7)		76 (76.8)	23 (23.2)		76 (76.8)	23 (23.2)	

Table IV. *Univariate Cox proportional hazards analysis of 117 studied patients. HR: Hazard-ratio, CI: confidence interval.*

Clinicopathological parameter	Overall survival			Event free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (≤50 *vs*. >50)	0.5726	0.2587-1.2670	0.1689	0.5208	0.2708-0.9336	**0.0294**
Tumour size (≤2 cm *vs*. >2 cm)	0.3006	0.1341-0.6738	**0.0035**	0.4508	0.2385-0.8518	**0.0141**
Lymph node involvement (N0 *vs*. N+)	0.2758	0.1288-0.5903	**0.0009**	0.4281	0.2336-0.7846	**0.0061**
MT-I/II (0-2 *vs*. 3-12)	1.2610	0.5803-2.7410	0.5580	0.9199	0.4950-1.7100	0.7919
MCM-2 (<25% *vs*. ≥25%)	0.8672	0.3548-2.1190	0.7547	0.8984	0.4429-1.8220	0.7666
Ki-67 (<25% *vs*. ≥25%)	0.3732	0.1522-0.9154	**0.0313**	0.3216	0.1534-0.6740	**0.0027**

the primary tumour or metastases to lymph nodes. The MT-I/II expression did not differ in regard to the ER or HER-2 expression status, but was associated with negative PR expression status. These results did confirmed partially the data obtained by Iochim *et al*., which showed higher intensity of MT-I/II expression in breast carcinomas devoid of oestrogen and progesterone receptors (36, 37).

Although, the intensity of MT-I/II expression correlated with factors, which were shown to be associated with poor patient outcome, *e.g*. grade of malignancy, Ki-67 and MCM-2 expression, no impact on the overall survival and event free survival regarding MT-I/II expression was noted. Out of the three studied IHC markers, only Ki-67 was proven to have an impact on OS and EFS, as has also been shown by numerous studies conducted with this marker in large subsets of breast cancer (3).

Interestingly, MCM-2 expression did not affect patient survival, although such a relationship was noted for Ki-67 expression and both these proliferation markers correlated strongly in the studied cases. The present data did not confirm the results obtained by Gonzalez *et al*. which showed that MCM-2 was an independent prognostic factor for overall survival of breast cancer patients (28). The present study was the first to show that, MCM-2, in spite of its advantages over Ki-67, could not predict patient outcome in contrast to other reports (19, 29). Of note, the combined analysis of the MT-I/II and Ki-67 or MT-I/II and MCM-2 results, did not improve the statistical significance of the OS or EFS results in multivariate analysis, which confirmed that MT-I/II and MCM-2 may not be used as reliable prognostic markers of IDC.

The present IHC study confirmed the results of studies conducted on breast cancer cell lines, which showed MT-I/II

involvement in breast cancer differentiation and progression (39-42). However, the analysis of MT-I/II expression in breast cancer showed no impact on patient outcome, which points to a limited use of this protein as a prognostic factor in IDC.

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