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Prognostic significance of augmented metallothionein (MT) expression correlated with Ki-67 antigen expression in selected soft tissue sarcomas

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Summary. In soft tissue sarcomas, the most important prognostic criteria include extent of malignancy (G), size of the tumour and intensity of Ki-67 antigen expression. In recent times expression of metallothionein (MT) in cells of some malignant processes of epithelial origin was found to correlate with intensity of Ki-67 antigen expression and to carry a possible prognostic significance. The present study aimed at a demonstration of prognostic value of MT expression and at comparing it with Ki-67 antigen expression and G grade in selected soft tissue sarcomas. Immunohistochemical studies were performed on paraffin sections in 54 cases of malignant fibrous histiocytoma (MFH), 18 cases of liposarcoma and 20 cases of synovial sarcoma. The extent of MT and Ki-67 antigen expression was evaluated and an attempt was made to correlate the results with each other and with grade of the tumour. Expression of MT was evident both in the cytoplasm and in cell nuclei of all studied sarcomas. The most pronounced MT expression was noted in MFH-type tumours. The extent of Ki-67 antigen expression was similar in MFH and liposarcoma and was the lowest in synovial sarcoma. In MFH, liposarcoma and synovial sarcoma a pronounced positive correlation was documented between expression of MT and Ki-67 antigen ($r=0.85$; $p<0.001$; $r=0.93$, $p<0.0001$; $r=0.79$, $p<0.0001$). In all types of the tumours a positive relation was detected between MT expression, expression of Ki-67 and G grade of malignancy in the tumour. Moreover, patients with higher MT expression in the studied tumours demonstrated a shorter survival. MT expression in soft tissue tumours of MFH, liposarcoma and synovial sarcoma type strongly correlated with intensity of proliferation (Ki-67) and G grade and could be useful in defining the extent of malignancy and in prognostic appraisal in the tumours.

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Introduction

Metallothioneins (MT) represent low molecular weight proteins of around 7 kDa in size. They consist of a polypeptide chain of 61 to 68 amino acids, including around 30% cystein residues (Coyle et al., 2002). Studies on structure and function of MT permitted the distinguishing of four main types of the proteins, including MT-I, MT-II, MT-III and MT-IV. In human cells, MT-II, MT-III and MT-IV are coded by 15 genes, and MT-I by a group of 12 genes. All the genes for MT are localised in chromosome 16. Expression of genes for MT-I and MT-II was noted in several tissues, including tumour cells (Boon-Huat et al., 2001; Romero-Isart and Vašák, 2002). Immunohistochemistry is one of the most frequently applied techniques to detect MT in cells of various tissues and in tumour cells. Under a light microscope it is possible to pinpoint the site of the protein expression, its distribution in the cell (cell nucleus, cytoplasm) and the intensity of the colour reaction. MT expression is noted in cell nucleus and/or in cytoplasm and it varies depending upon the type of studied normal or neoplastic tissue (Cherian and Apostolova 2000; Boon-Huat et al., 2001).

The principal and the earliest described function of MT is their effect on metal homeostasis in cells. MT function as the main protective mechanism of cells exposed to toxic metal ions (Cd, Pb, Hg, Cu) binding the metals which results in formation of inactive complexes (Vašák and Hasler, 2000). Apart from the detoxicative activity, MT bind zinc and control zinc-dependent enzymes, which are involved in DNA replication, transcription, translation and in cellular metabolism. They affect cell growth and proliferation (Beyersmann and Haase, 2001). In addition, augmented expression of MT in tumour cells is linked to resistance of the cells to

Table 1. Clinical variables of studied soft tissue sarcomas.

HISTOLOGICAL TYPE	N	MEAN AGE	LOWER EXTREMITY	UPPER EXTREMITY	RETROPERITONEAL	MALE:FEMALE
MFH	54	58.5 (43 – 72)	23 (42.6%)	21 (38.9%)	10 (18.5%)	3:2
liposarcoma	18	49.3 (26 – 69)	11 (61.1%)	4 (22.3%)	3 (16.6%)	3:2
synovial sarcoma	20	32.2 (16 – 58)	12 (60%)	8 (40%)	-	3:2

		TNM STAGING					
HISTOLOGICAL TYPE	N	T1	T2	N0	N1	M0	M1
MFH	54	42	12	54	0	54	0
liposarcoma	18	11	7	18	0	18	0
synovial sarcoma	20	17	3	20	0	20	0

MFH: malignant fibrous histiocytoma.

Table 2. Histopathological data on studied soft tissue sarcomas.

HISTOLOGICAL TYPE	N	G1	G2	G3
MFH	54	-	22	32
pleomorphic	41	-	13	28
inflammatiory	3	-	-	3
myxoid	10	-	9	1
Liposarcoma	18	14	-	4
well differentiated	7	7	-	-
myxoid	7	7	-	-
round cell	2	-	-	2
pleomorphic	2	-	-	2
Synovial sarcoma	20	-	12	-
biphasic	9	-	7	2
monophasic	6	-	5	1
poorly differentiated	5	-	-	5

MFH: malignant fibrous histiocytoma; G: grade of malignancy.

cytostatic drugs and radiotherapy (Kotoh et al., 1994; Cai et al., 1999; Nakano et al., 2003).

In recent years, MT expression was demonstrated in cells of various tumours, both of epithelial and mesenchymatic origin [Boon-Huat et al., 2001; Dziegiel et al., 2002; Surowiak et al., 2002; Cherian et al., 2003; Gaumann et al., 2003). In numerous studies on epithelial tumours positive correlation was detected between MT expression and expression of Ki-67 antigen (Jin et al., 2002; Dziegiel et al., 2003). The data confirm the role played by MT in cell proliferation. In some types of tumours of epithelial origin other reports demonstrated a strict relation between MT expression and the grade of histological malignancy (G) (Shukla et al., 1998; Paraskevakou et al., 1999; Jin et al., 2001). In turn, the grade represents one of the most important prognostic parameters in any tumour. Till now, MT expression in various sarcomas has provided the topic of only a few studies (Trieb and Kotz, 2001; Dziegiel et al., 2002; Gaumann et al., 2003). MT manifestation has not been examined in liposarcoma and malignant fibrous histiocytoma (MFH) - type tumours and only one study has dealt with expression of the protein in synovial sarcoma (Dziegiel et al., 2002). All the above mentioned types of neoplasms are burdened with a difficult to predict clinical course, and prognosis based on recognised variables (grade, number of mitoses, extent of tumour cell necrosis) remains unsatisfactory (Oliveira and Nascimento, 2001).

Examination of cell proliferative activity by appraisal of Ki-67 antigen expression (Jensen et al., 1998) represents one of the most significant aspects in estimation of tumour (including sarcoma) aggressiveness. Correlation of expression intensity in the case of the antigen and MT in cells of soft tissue sarcomas may provide additional data useful in the evaluation of the disease course and of the efficiency of appropriate therapeutic techniques.

In our study we aimed at the demonstration of prognostic significance of MT (MT-I and MT-II isoforms) expression in cells of selected soft tissue sarcomas (malignant fibrous histiocytoma – MFH, liposarcoma, synovial sarcoma) and at attempts to correlate its intensity with expression of Ki-67 antigen and grade of histological malignancy (G).

Materials and methods

Material for the studies was obtained from 43 patients subjected to surgery due to soft tissue tumours in the Lower Silesia Centre of Oncology of Wroclaw in 1998 – 2003 and from 49 archival blocks kept in the Department of Pathological Anatomy, University of Medical Sciences in Poznan. The studied tumours included 54 cases of MFH, 18 cases of liposarcoma and 20 cases of synovial sarcoma. All tumours were excised with the margin of healthy tissue and no additional therapy was administered. The recurrence in the place of the tumour resection was observed in 11 cases of MFH, 6 of liposarcoma and 4 of synovial sarcoma. The clinico-

pathological data are presented in Tables 1 and 2.

The tumour samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. All immunohistochemical reactions were performed in paraffin sections. Expression of MT (isoforms MT-I and MT-II) and of Ki-67 antigen was demonstrated using mouse monoclonal antibodies (clone E9; dilution: 1:100 and clone MIB-1; dilution: 1:50, respectively). In cases of paraffin sections of MFH-type tumours the diagnosis was confirmed by performing immunohistochemical reactions with polyclonal anti-alpha-1-antichymotrypsin antibodies. All the reactions were accompanied by negative controls in which specific antibodies were substituted by the Primary Negative Control reagent. In the cases of anti-Ki-67 antigen reactions the studied paraffin sections were boiled in the Antigen Retrieval Solution in a microwave oven in order to unblock antigenic determinants. The investigated antigens were visualised by using biotinylated antibodies and streptavidin-biotinylated peroxidase (LSAB2 kit) and diaminobenzidine (DAB). All the employed antibodies and reagents were produced by DAKO (Denmark).

Intensity of the immunohistochemical reactions was blindly evaluated by two independent pathologists. For evaluation of MT expression the semi-quantitative IRS scale according to Remmele (Remmele and Stegner, 1987) was used, taking into account intensity of the colour reaction and proportion of positive cells. Results represented a product of scores given for individual parameters and fitted the range of 0-12 points: weak reaction 1-2 points, moderate reaction 3-5 points, strong reaction 6-12 points. Ki-67 antigen expression was appraised in the scale reflecting the proportion of cells showing nuclear colour reaction: no reaction, 0 points; 1-10%, 1 point; 11-25%, 2 points; 26-50%, 3 points; and over 50%, 4 points. In studied sarcomas, grade of histological malignancy (G1, G2, G3) was defined considering the following variables: differentiation of tumour cells, number of mitoses per ten high magnification fields, and percentage of necrotically altered tumour cells (Weiss and Goldblum, 2001).

The results were subjected to statistical analysis using STATISTICA PL (StatSoft, Poland) software, tests of Mann-Whitney, F Cox, Spearman's correlation and Kaplan-Meier's survival analysis.

Results

MT expression (in cell nucleus and in cytoplasm) was detected in all 54 examined cases of MFH tumours (Fig. 1a). In 7 cases its intensity was low, in 8 cases moderate and in 39 cases high. High intensity of MT expression dominated in pleomorphic MFH tumours of G3 grade. Intensity of Ki-67 antigen expression (Fig. 1d) in MFH tumours depended on histological type and grade of the tumour and resembled intensity of MT expression. This was reflected by statistically significant positive correlation between expressions of MT and of Ki-67 antigen ($r=0.85$; $p<0.001$) (Fig. 2a). Increasing grade of the tumour was associated with significantly higher values of MT and Ki-67 antigen expression ($U=3.75$, $p<0.001$; $U=3.49$, $p<0.005$, respectively).

In liposarcoma-type tumours nuclear-cytoplasmic expression of MT could be noted in all the 18 examined cases (Fig 1b). Its peak intensity could be noted in the pleomorphic type of the tumour and in round cell tumours. The lowest intensity of MT expression was observed in the myxoid type of liposarcoma. Similar results were obtained in studies on Ki-67 antigen expression (most pronounced expression in the pleomorphic and round cell types of the tumour) (Fig. 1e). This resulted in the high, positive correlation between expression of the markers ($r=0.93$; $p<0.0001$) (Fig. 2b). In liposarcoma, MT expression and Ki-67 antigen expression coincided also with G grades: the higher grades were accompanied by higher expression of MT and Ki-67 antigen ($U=0$, $p<0.005$; $U=2$, $p<0.01$, respectively).

In 20 examined cases of synovial sarcoma no clearly different MT expression could be noted (Fig. 1c) in various histological types of the tumour. In the tumours, however, positive correlation was detected between MT expression and expression of Ki-67 antigen (Fig. 1f) ($r=0.79$, $p<0.0001$) (Fig. 2c). Moreover, augmented malignancy of the tumour (G) was accompanied by increased expression of both MT and Ki-67 antigen ($U=6$, $p<0.001$; $U=8$, $p<0.01$, respectively).

The positive and significant correlations between intensities of MT and Ki-67 antigen expressions on the one hand and grade of the tumour on the other prompted us to conduct further analyses. We compared expressions of the two antigens (MT and Ki-67) between groups representing various grades of malignancy. In all the studied types of sarcoma both MT expression and Ki-67 antigen expression proved to be more intense in groups of higher grade (G) (Fig. 3).

In addition, the highest expression of MT was detected in MFH-type tumours, Ki-67 antigen expression level was similar in MFH and in liposarcoma tumours and it was the lowest in synovial sarcoma (Fig. 4).No correlation between MT expression and other clinical parameters (TNM) was observed.

In 18 cases of sarcomas (T1N0M0) with restricted intensity of MT expression (1-2 points) and in 21 (T1N0M0) cases with high intensity of MT expression (6-12 points) survival analysis of the patients was conducted. Longer survival was demonstrated for patients with low intensity of MT expression as compared to patients with highly intense MT expression ($p<0.05$) (Fig. 5).

Discussion

Soft tissue sarcomas account for around 1% of all malignant tumours. However, their obscure etiopathogenesis and unsatisfactory results of treatment cause that the tumours pose a relatively serious problems in oncology (Weiss and Goldblum, 2001). Due to late

diagnosis of soft tissue sarcomas and the resulting marked advancement of the oncological process, evaluation of histological malignancy grade (G) (Kempson et al., 2001) represents a very important element of histopathological diagnosis of the tumours. The grade allows the determination of aggressiveness of the disease and, thus, defines the prognosis. The till now accepted criteria for determining grade, including

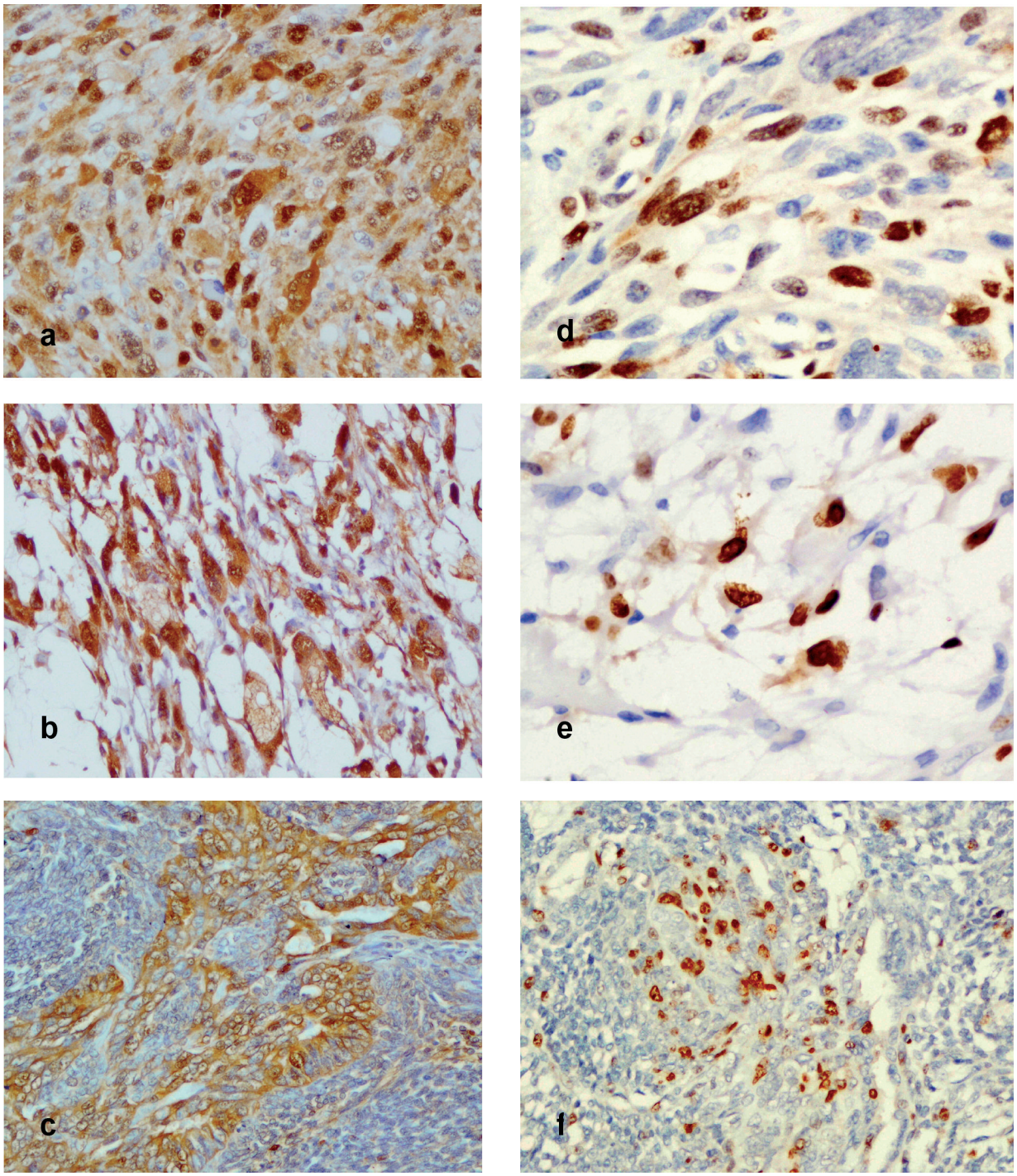

Fig. 1. Expression of MT in cells (cell nucleus and/or cytoplasm): **a,** MFH; **b,** liposarcoma; **c,** synovial sarcoma. Expression of Ki-67 antigen in cells (cell nuclei): **d,** MFH; **e,** liposarcoma; **f,** synovial sarcoma. Background staining with hematoxylin. a, b, x 200; d, e, x 400; c, f , x 100

cellular pleomorphism, mitotic activity and frequency of tumour cell necrosis, remain, to a large extent, subjective and insufficient. Moreover, the soft tissue tumours frequently exhibit an unpredictably rapid growth and metastases, which find no correspondence in histopathological evaluation including determination of the grade (Daugaard et al., 1997; Mann et al., 1999). This has prompted us to examine the new exponent (MT

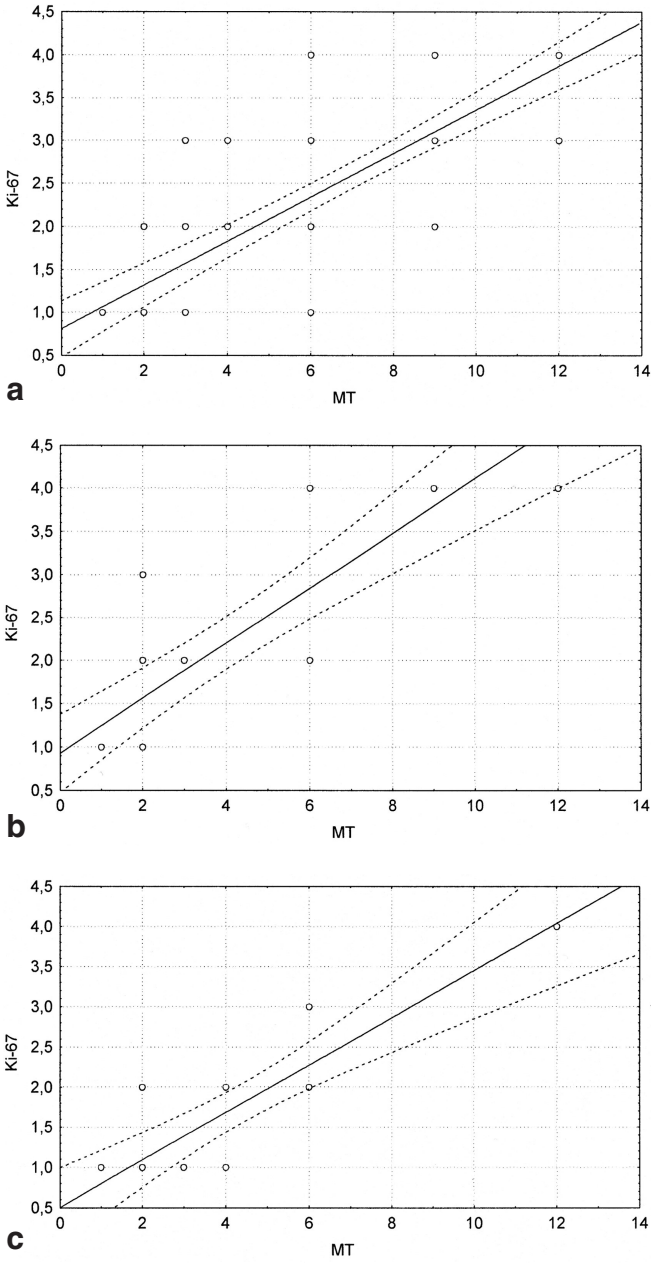

Fig. 2. Correlation between intensities of MT expression and Ki-67 antigen expression in cells of sarcomas **(a)**: MFH, r=0.85; p<0.001 **(b)**: liposarcoma, r=0.93; p<0.0001 **(c)**: synovial sarcoma, r=0.79, p<0.0001.

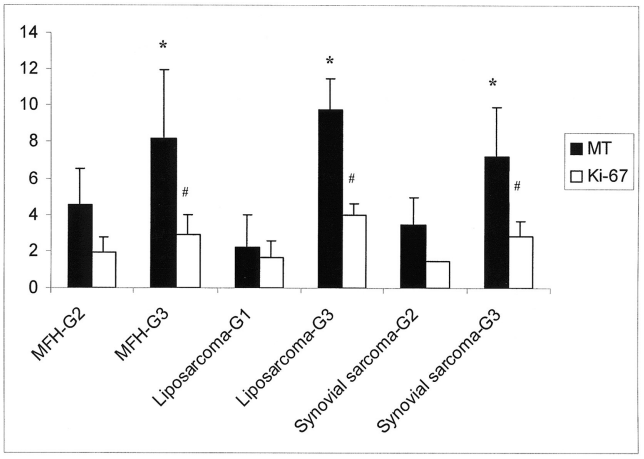

Fig. 3. Intensity of MT expression and Ki-67 antigen expression in sarcomas of MFH, liposarcoma and synovial sarcoma types as related to grade of malignancy, G, *p<0.01; #p<0.05.

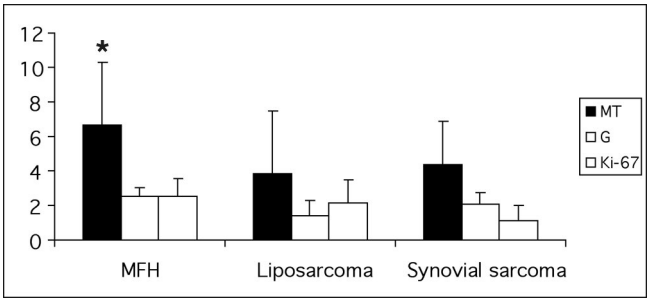

Fig. 4. Intensity of MT expression and of Ki-67 antigen expression in individual types of studied sarcomas: MFH, liposarcoma, synovial sarcoma, *p<0.05.

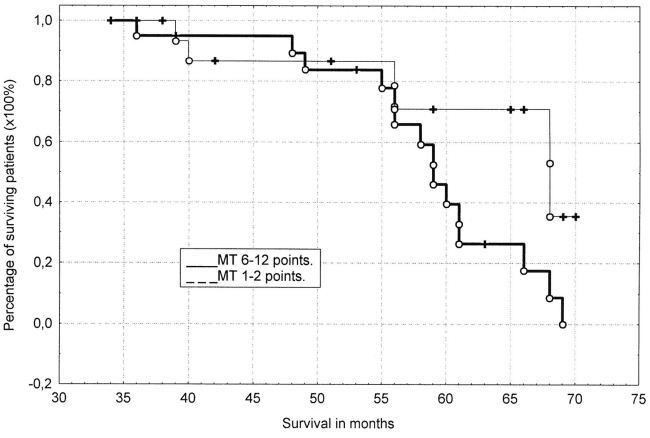

Fig. 5. Analysis of survival for patients with low (18 cases; MT 1-2 points) and high (21 cases; MT 6-12 points) intensity of MT expression in cells of MFH, liposarcoma and synovial sarcoma), p<0.05.

expression) of maturity and proliferative activity of soft tissue sarcomas as compared to the recognised and corroborated marker such as Ki-67 antigen. Significance of MT in cell proliferation has been confirmed in cases of several epithelial tumours (Jin et al., 2001, 2002; Dziegiel et al., 2003). Its role was also noted in the mechanism of multi-drug resistance (including resistance to cytostatic drugs) (Kotoh et al., 1994; Nakano et al., 2003). In addition, in several cases augmented expression of MT in cells of various malignant tumours correlated with shorter survival of the patients, suggesting prognostic importance of the protein (Yamamoto et al., 1999; Dziegiel et al., 2003; Weinlich et al., 2003).

Until now, MT expression has not been examined in tumours of mesodermal origin, except for two recently published reports (Dziegiel et al., 2002; Gaumann et al., 2003). Similarly to tumours of epithelial origin, we have demonstrated strong correlation between expressions of MT and of Ki-67 antigen in all studied types of sarcoma, including MFH, liposarcoma and synovial sarcoma. The significant correlation between augmented MT expression and grade of studied sarcomas has also been observed in mammary and colonic tumours (Jin et al., 2001; Dziegiel et al., 2003). Evaluating intensity of MT expression in the studied sarcomas we have also performed analysis of survival of patients of high versus low level of expression of the protein. Similarly to authors who tested other types of tumours, we have noted shorter survival of patients with tumours manifesting high level of MT expression (Yamamoto et al., 1999; Dziegiel et al., 2003; Weinlich et al., 2003). The results point to the potential of applying MT expression as another prognostic marker in soft tissue sarcomas.

Numerous studies documented augmented MT expression in tumour cells exposed to action of various cytostatic drugs and radiotherapy (Kotoh et al., 1994; Cai et al., 1999; Yamamoto et al., 1999; Nakano et al., 2003). In such therapeutic methods their mechanism of action involved generation of high amounts of free oxygen radicals which damage tumour cells. MT protects cells from the damage induced by reactive oxygen forms, causing their inactivation (Bouzourene et al., 2002; Tcheocharis et al., 2003). Evaluation of MT expression intensity in sarcoma cells may be useful in planning chemo- and radiotherapy of the tumour types.

Summing up our results we may note that MT provides another index of malignancy and a prognostic parameter in MFH, liposarcoma and synovial sarcoma-type tumours.

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