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Prognostic significance of metallothionein expression in correlation with Ki-67 expression in adenocarcinomas of large intestine

P. Dziegiel[1], J. Forgacz[2], E. Suder[3], P. Surowiak[1], J. Kornafel[4] and M. Zabel[1,5]

[1]Chair and Department of Histology and Embryology, Wroclaw Medical University, Wroclaw , Poland, [2]Lower Silesia Centre of Oncology, Wroclaw, Poland, [3]Chair and Department of Anatomy, Wroclaw Medical University, Wroclaw, Poland, [4]Chair of Oncology and Department of Gynaecologic Oncology, Wroclaw, Medical University, Wroclaw, Poland, [5]Chair of Histology and Embryology, Poznan Medical University, Poznan, Poland

Summary. The study aimed at determining levels of metallothionein (MT) and Ki-67 antigen expression in adenocarcinomas of large intestine and examining relation of the expression levels with various clinical and pathological variables. The studies were performed on 81 cases of large intestine adenocarcinoma. Using immunocytochemistry, expressions of MT (positive reaction in 73 cases) and of Ki-67 (positive reaction in 79 cases) antigen were examined and the obtained results were compared with, i.a., grade (G) of the tumour and depth to which intestinal wall was infiltrated by individual tumours. Patient survival analysis was also performed, as correlated to expression levels of the two antigens. The obtained results permitted to disclose that the lower was grade of histological differentiation (G2, G3), the more pronounced was expression of MT and Ki-67. Also, the deeper was neoplastic infiltration of intestinal wall, the more pronounced was MT and Ki-67 expression. Despite the relatively strong correlation between MT expression and Ki-67 expression ($r=0.536$; $p<0.05$), only Ki-67 antigen expression in large intestine adenocarcinomas was inversely correlated to survival of the patients. Ki-67 proved to be a better prognostic marker, as compared to MT, in large intestine adenocarcinomas.

Key words: Metallothionein, Ki-67, Large intestine adenocarcinoma

Introduction

Metallothionein (MT) is a low molecular weight protein of high cysteine content and a low content of aromatic amino acids. Among others, it participates in the control of heavy metal levels (Zn, Cu, Pb, Cd). Till now, four isoforms of the protein have been recognised, including MT-I, MT-II, MT-III and MT-IV. Apart from heavy metal levels, expression of the metallothionein gene is controlled by various agents, including glucocorticoids, interferon, interleukin-1, vitamin D and ionising radiation. MT is suggested to participate in anti-oxidative protection as a strong oxygen radical scavenger (Moffat and Denizean, 1997). Results of recent years documented augmented expression of intracellular MT in various tumours, including ovarian cancer, thyroid carcinoma, cancer of urinary bladder, breast cancer, malignant melanoma, synovial sarcoma and in tumours of salivary glands (Nartey et al., 1987; Bahnson et al., 1991; Murphy et al., 1991; Fresno et al., 1993; Zelger et al., 1993; Sunardhi-Widyaputra et al., 1995; Dziegiel et al., 2002). In other experiments a significant role of MT was demonstrated in multi-drug resistance (MDR) toward cytostatic drugs in various types of malignant tumours, in the cells of which expression of the protein was demonstrated using immunocytochemical techniques (Kelley et al., 1988; Hishikawa et al., 1997; Monden et al., 1997). The data obtained up to now indicate that the role and functions played by MT in neoplastic diseases have not been fully clarified. In studies on MT expression in cells of mammary cancer, MT expression level correlated with a less favourable prognosis and a rapid progress of the disease (Fresno et al., 1993; Oyama et al., 1996). In cancers of urinary bladder and oesophagus, augmented intracellular levels of MT co-existed with a pronounced resistance to cytostatic drugs (Kotoh et al., 1994; Hishikawa et al., 1997).

Till now, few studies have dealt with MT expression in malignant tumours of alimentary tract and in large intestine in particular. The obtained results were equivocal and failed to correlate with MT expression or specific clinical or pathological variables. While some of

Offprint requests to: Piotr Dziegiel, Chair and Department of Histology and Embryology, Wroclaw Medical University, ul. Chalubińskiego 6a, 50-368 Wroclaw, Poland. Fax: (4871)784-00-82. e-mail: piotr@hist.am.wroc.pl

the results suggested that MT expression level correlated with advancement of the tumour, they did not clarify whether expression of the protein in tumour cells was related to other prognostic factors (Öfner et al., 1994; Giuffré et al., 1996; Ioachim et al., 1999; Sutoh et al., 2000).

Considering the above and the fact that MT may play a certain role in the cell cycle control (Nagel and Vallee, 1995; Cherian and Apostolova, 2000) it seemed purposeful to compare expression of the protein and of Ki-67 antigen (an index of cell proliferation) and various clinical and pathological variables in an attempt to estimate their prognostic value in large intestine cancers.

Material and methods

Material for the studies was obtained from 81 patients of 35 to 88 years of age, including 37 women and 44 men, treated in the Lower Silesia Centre of Oncology in Wroclaw in 1993-94. The follow up data and disease status were obtained from the Medical Archive, Lower Silesia Centre of Oncology. In each case the diagnosis of primary adenocarcinoma of large intestine was confirmed by histopathology. The latter defined also histological differentiation of studied tumours (G), distinguishing highly differentiated tumours (G1) – 11 cases, moderately differentiated tumours (G2) – 43 cases and poorly differentiated tumours (G3) – 27 cases. Like in adenocarcinomas of other organs, the cathegories of highly, moderately or poorly differentiated adenocarcinomas (G1, G2, G3, respectively) reflects evaluation of histological structure elements, including the extent of glandular structure formation, polymorphism of cells and cell nuclei, mitotic activity. Indirectly, the tumour grading allows to appraise how malignant is the tumour (Jass et al., 1986). Material for immunocytochemical studies was obtained from paraffin blocks, which contained tumour tissue, sampled during the surgery. Histological appraisal of the depth to which the tissue was penetrated by the tumour was conducted according to Dukes (Dukes and Bussey, 1958) and Astler-Coller classification (Astler and Coller, 1954), in our own modification. The cases were grouped as containing: a) tumour infiltrate restricted to mucosa and submucosa; b) tumour infiltrate involving mucosa, submucosa and muscle layer; and c) tumour infiltrate involving the above -mentioned layers and the peri-intestinal adipose tissue. Tumour samples were fixed in 10% buffered formaline, dehydrated and embedded in paraffin blocks. In the studies on MT and Ki-67 expression two series of the immunocytochemical reactions were performed in parallel, using paraffin sections from the same blocks. In studies on MT expression (isoforms I and II) monoclonal E9 antibodies (dilution 1:100), (Dako, Denmark) were used and in studies on Ki-67 expression Ki-S5 antibodies (dilution 1:50), (Dako, Denmark) were employed, taking advantage of the EnVisionTM system and incubation with diaminobenzidine solution (DAB), (Dako, Denmark). In the case of reactions with anti-MT antibodies, the positive control involved paraffin sections of mammary ductal carcinoma while the negative control employed Primary Negative Control antibodies (DAKO, Denmark). In the case of reactions with anti-Ki-67 antibodies, the sections were incubated in Antigen Retrieval Solution (DAKO, Denmark) in a microwave oven. The slides with paraffin sections, overlaid with primary antibodies, were incubated at 5 ºC overnight.

Intensity of the immunocytochemical reactions for MT was evaluated in a blind fashion independently by two pathologists, employing the semiquantitative IRS scale, according to Remmele (Remmele and Stegner, 1987), taking into account intensity of the colour reaction and the number of positive cells. The final score represented a product of scores representing the two variables and ranged from 0 to 12 points (low reaction: 1 to 2 points, average reaction: 3 to 4 points, intense reaction: 6 to 12 points) (Table 1). Expression of Ki-67 was quantified taking into account the percentage of positive cells (no positive cells: score 0, up to 10% positive cells: score 1 to 2, 10 to 30% positive cells: score 3 to 5, over 30% positive cells: score 6 to 12).

The obtained results were subjected to statistical analysis, employing the Statistica Pl software and the Kruskall-Wallis test, Spearman rank test, Kaplan-Meier test, F test of Cox and χ^2 test.

Results

Upon evaluation of intensity of MT expression (in cell nucleus and in cytoplasm) in 81 cases of studied adenocarcinomas, in 8 cases no reaction was noted, in 31 cases the reaction was low (Fig. 1A), in 22 cases the reaction was of an average intensity (Fig. 1B) and in 20 cases the reaction was intense (Fig. 1C). Intensity of

Table 1. Evaluation of MT antigen expression in studied preparations. The final score represents a product of the positive cell score **(A)** and the score reflecting intensity of the colour reaction **(B)**.

A	B
0: no cells with positive reaction	0 : no colour reaction
1: to 10% cells with positive reaction	1: low intensity of colour reaction
2: 11 to 50% cells with positive reaction	2: average intensity of colour reaction
3: 51 to 80% cells with positive reaction	3: intense colour reaction
4: >80% cells with positive reaction	

expression of Ki-67 antigen in individual tumours was as follows: in 2 cases no reaction was detected, in 17 cases the reaction was low (Fig. 1D), in 35 cases it had an average intensity (Fig. 1E) and in 27 cases the reaction was intense (Fig. 1F). Mean scores in the IRS scale for all the tumours are given in Table 2.

The other aim of the study was to examine the relation between expression of MT and Ki-67 on the one hand and selected parameters of histopathological appraisal of the large intestine adenocarcinomas on the other. In the case of the histological differentiation of the tumour (grade - G), mean levels of MT and Ki-67 expression were calculated for individual grade groups. The most intense reaction for MT was detected in the grade 3 (G3) group, i.e. in the least differentiated tumours. Similar results were obtained upon analysis of Ki-67 expression: the lower the differentiation of the tumours, the higher the Ki-67 antigen expression in the tumour (Fig. 2).

In tumour fragments, sampled in the same and standardised way, when MT and Ki-67 antigen expression was compared with the depth of intestinal

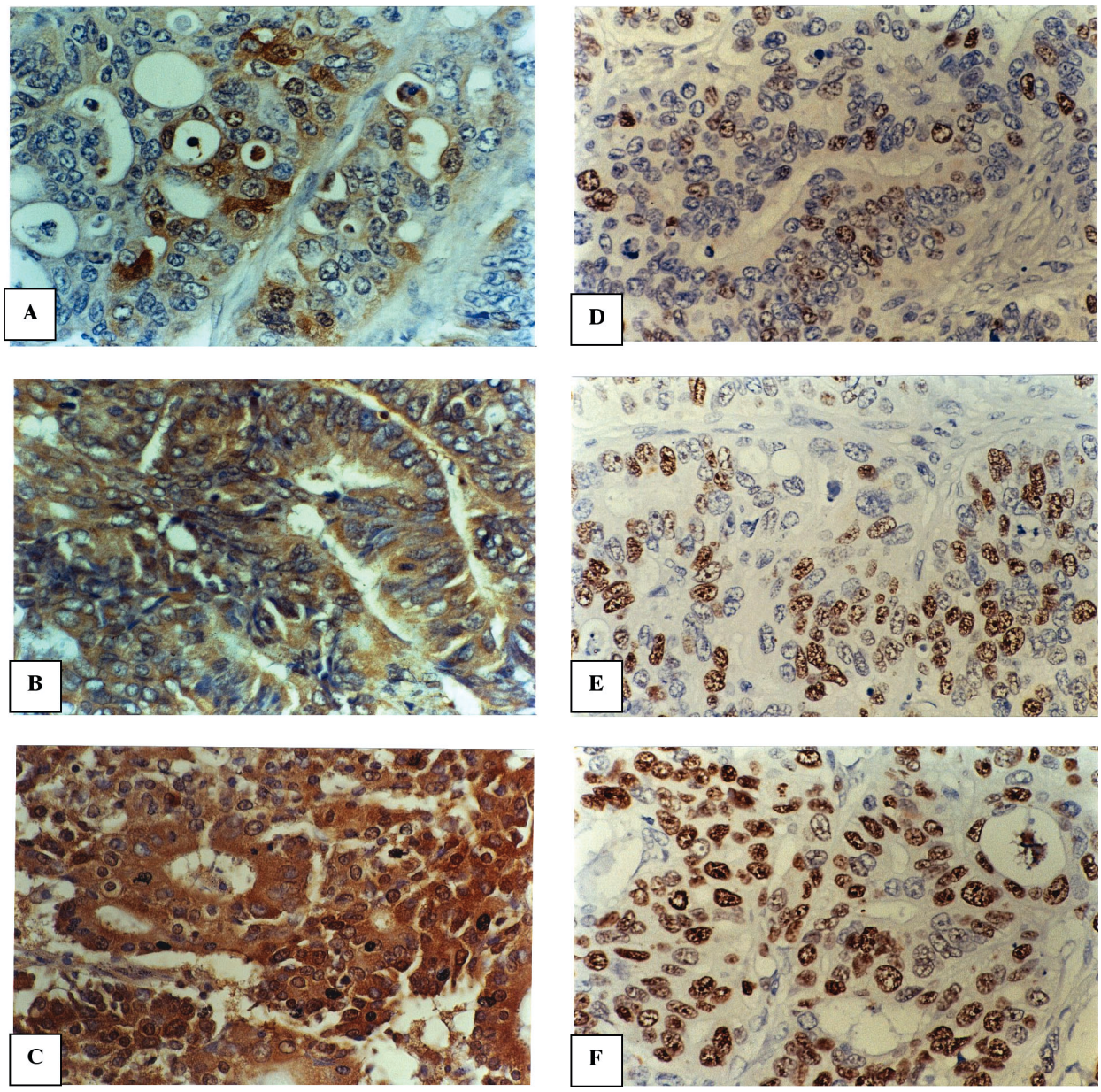

Fig. 1. Intensity of MT expression in adenocarcinomas of large intestine: **A,** low; **B,** average; **C,** intense. Intensity Ki-67 expression: **D,** low; **E,** average; **F,** intense. x 400

wall infiltration by cancerous cells, the increasing depth of the penetration was found to be associated with increasing expression of MT and Ki-67 (Fig. 3).

Also, the remaining clinical and pathological variables were evaluated, which according to Jass (2000) affect prognosis in the large intestine carcinoma, i.e. type of tumour growth, inflammatory reaction, metastases to lymph nodes, in relation to MT and Ki-67 antigen expression, but no significant relationships between the variables were demonstrated.

Also, the reciprocal relationship of MT and Ki-67 expression was examined in the 81 cases of large intestine adenocarcinoma. Taking advantage of the rank correlation coefficient according to Spearman, which was r=0.536 (p<0.05), a relatively strong linkage of expression of the two antigens was detected (Fig. 4). Subsequently, an analysis was performed of the MT and Ki-67 antigen expression as related to survival of the patients (percentages of surviving patients in individual years following the surgery). The evaluated cases were grouped with respect to MT and Ki-67 expression (Table 2). The Kaplan-Meier statistical analysis of patient survival was performed and significance of detected

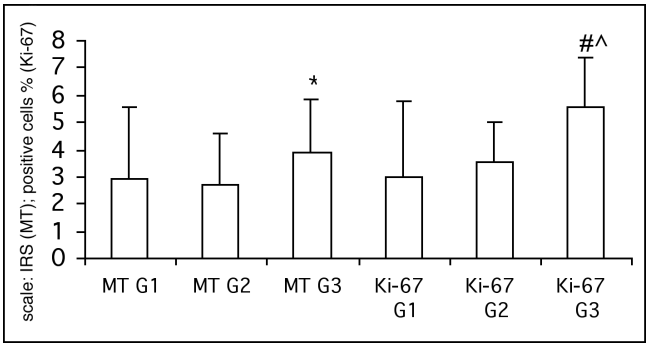

Fig. 2. Extent of expression of MT (IRS scale) and Ki-67 (score 0 to 2: up to 10 % positive cells, score 3 to 5: 10-30 % positive cells, score 6 to 12: over 30 % positive cells) in large intestine adenocarcinomas of individual grades of histological differentiation (G). *: p<0.01 (MT G3 vs MT G2); #: p<0.05 (Ki-67 G3 vs Ki-67 G1); ^: p<0.001 (Ki-67 G3 vs Ki-67 G2). Kruskall-Wallis test.

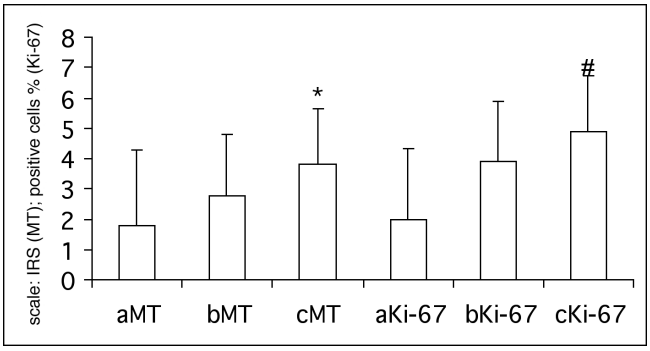

Fig. 3. Extent of expression of MT and Ki-67 antigen as related to the depth of large intestine wall infiltration by adenocarcinoma. **a.** Mucosa and submucosa. **b.** Mucosa, submucosa and muscle layer. **c.** Mucosa, submucosa, muscle layer and adipose tissue. *: p<0.05 (cMT vs bMT) and (cMT vs aMT); #: p<0.05 (cKi-67 vs bKi-67) and (cKi-67 vs aKi-67). Kruskall-Wallis test.

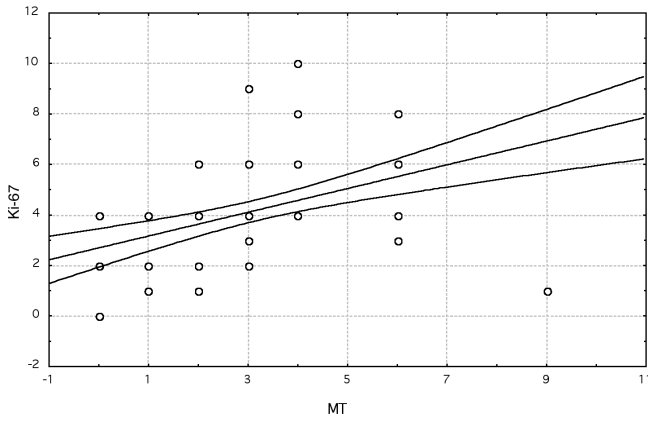

Fig. 4. Correlation between expression of MT and Ki-67 antigen in large intestine adenocarcinoma; r=0.536; p<0.05

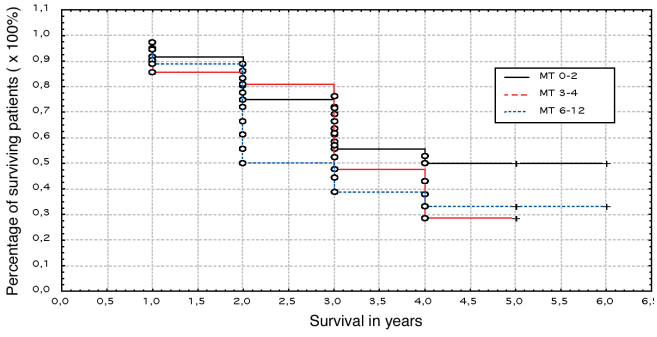

Fig. 5 Survival of patients with large intestine adenocarcinoma as related to extent of expression of MT (IRS scale: 0 to 2 – low expression, 3 to 4 – average expression, 6 to 12 – pronounced expression)

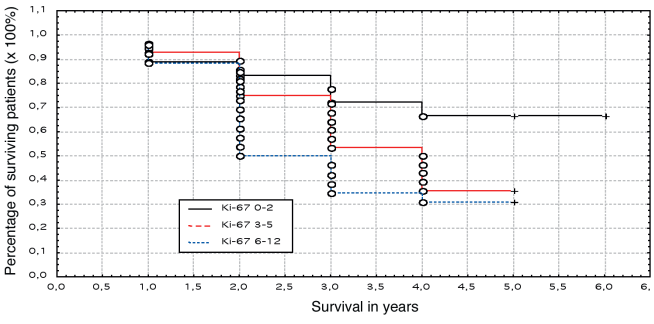

Fig. 6. Survival of patients with large intestine adenocarcinoma as related to the extent of expression of Ki-67 antigen (score 0 to 2: up to 10 % positive cells; low expression), score 3 to 5: 10-30 % positive cells; moderate expression), score 6 to 12: over 30 % positive cells; pronounced expression). Significant differences: low vs average expression: p<0.05; low vs pronounced expression: p<0.01.

differences was estimated using the $\chi 2$ test and the F test of Cox. In the case of MT expression, the detected differences in survival between the groups proved to be insignificant (Fig. 5). In the case of Ki-67 expression, it was found to be inversely related to survival of the patients (Fig. 6).

Discussion

Frequency of large intestine carcinoma is increasing worldwide. This reflects civilisation progress and an increasing living standard in society. At present, large intestine carcinoma is the most frequent tumour of the alimentary tract. Despite intense efforts, results of treatment of the disease remain unsatisfactory. Thus, early diagnosis and determination of reliable prognostic factors linked to various therapeutic measures are of utmost importance. Rapid progress in adjunct therapy (chemotherapy, radiotherapy) has stressed the need for better diagnostic and prognostic evaluation of the patients in order to select patient groups who may benefit from new strategies of therapy (Jass, 2000).

MT represents a widely distributed and only recently characterised protein of living organisms. Interest in the protein has increased in particular when it has been found to be present in cells of various tumours. However, multiple studies have failed till now to clarify the role of MT in the cells (Moffat and Denizean, 1997). Numerous studies have shown that increased expression of MT is related to worse prognosis in various types of tumours. This has been most clearly shown in invasive ductal mammary carcinoma and in malignant melanoma (Zelger et al., 1993; Zhang et al., 2000). Similar results have been expected in studies on other types of tumours of various organs and tissues, including large intestine adenocarcinoma. Till now, few studies on the topic have appeared and their results have proven contradictory in certain respects. Moreover, the studies have not taken advantage of the potential offered by comparing various markers and prognostic factors, related to MT expression (Giuffré et al., 1996; Ioachim et al., 1999; Öfner et al., 1994; Sutoh et al., 2000).

In our studies we have evaluated MT expression in cells of large intestine adenocarcinoma, as related to Ki-67 expression and to various clinical and pathological variables. Ki-67 antigen is known to represent one of the most sensitive indicators of cell proliferation and to serve as a prognostic factor, capable of reflecting the activity of neoplastic process (Gerdes et al., 1991). In turn, augmented expression of MT has been detected by immunocytochemical techniques in cells of human colon carcinoma (HT-29) at various stages of cell cycle. MT expression has been reaching maximum in the late G1 and at the G1/S threshold of interphase. Two- to threefold increase in MT in rapidly growing cells and variability in the protein level during mitosis suggest that the protein may play a role in cell proliferation (Nagel and Vallee, 1995).

Analysis of the obtained results has shown a relatively strong correlation between MT expression and Ki-67 expression in cells of large intestine carcinoma. It seems that MT might be used as a certain index of proliferative activity of the tumour cells. We have also examined the correlation between MT and Ki-67 expression and histological differentiation (grade, G) of the examined tumours. A significantly ($p<0.01$) higher MT expression has been noted in the poorly differentiated tumours (G3), associated with the worst prognosis. In the case of Ki-67 the relation has been similar. The results suggest certain prognostic significance of MT in primary adenocarcinoma of large intestine. The results on the correlation between grade of the tumour (G) and MT expression have not been compatible with those of other authors (Öfner et al., 1994; Giuffré et al., 1996; Ioachim et al., 1999; Sutoh et al., 2000). However, in our studies we have employed a distinct technique for evaluating expression level of the (MT) we have employed the IRS scale, according to Remmele (Remmele and Stegner, 1987). The modified extent of infiltration of large intestinal walls by the tumour, compared with expression of MT and Ki-67, has represented another prognostic element. The analysis was simplified by distinguishing three infiltration levels (mucosa and submucosa, muscle layer, adipose tissue). Both MT expression and Ki-67 expression have proven to be significantly ($p<0.05$) higher in tumour cells infiltrating adipose tissue, as compared to tumour cells infiltrating mucosa, submucosa and muscle layer only. The above data justify the conclusion that the deeper is intestinal wall infiltration by the tumour the higher is the expression of both antigens (MT, Ki-67). The latter is associated with the more aggressive process and worse prognosis. Also in this case, the literature data, in which Dukes' classification (including the depth of intestinal wall infiltration by the tumour) was used to evaluate advancement of the disease, yield distinct results as

Table 2. Expression of MT and Ki-67 in 81 cases of primary large intestine adenocarcinoma (IRS scale).

EXPRESSION INTENSITY		NUMBER OF CASES	
MT (IRS scale)	Ki-67	MT	Ki-67
No reaction: score 0	No positive cells: score 0	8	2
Weak reaction: score 1 to 2	<10 % positive cells: score 1-2	31	17
Average intensity of the reaction: score 3 to 4 points	10-30% positive cells: score 3-5	22	35
Intense reaction: score 6 to 12	>30 % positive cells: score 6-12	20	27

compared to those presented here. In the quoted studies no relation could have been detected between grade of the tumour (G) and depth of neoplastic infiltration and augmented MT expression (Dukes and Bussey, 1958; Öfner et al., 1994; Giuffré et al., 1996; Ioachim et al., 1999; Sutoh et al., 2000). Due to the relatively strong correlation of MT and Ki-67 expression in the studied material we performed Kaplan-Meier analysis of patient survival. Individual groups of patients with a various extent of MT expression have not differed in survival time. However, the patients with low MT expression tended to survive 5 years after the therapy more frequently than patients with strong MT expression (those tended to die already beginning in the second year after the therapy). The absence of statistically significant differences may reflect low numerical force of the studied groups. On the other hand, other authors in more advanced cases, with metastases to lymph nodes and to liver, demonstrated negative correlation between MT expression in primary tumours and survival of the patients. Lack of such a correlation in the present study might have resulted from the earlier stages of the disease in our patients (Hishikawa et al., 2001). On the other hand, in the case of Ki-67 antigen expression the identical analysis has demonstrated significant differences in survival and has confirmed clinical usefulness of the marker (Pietiläinen et al., 1996). The lower extent of Ki-67 antigen expression has correlated with longer survival of the patients.

The results we have presented have in part been confirmed in the literature. The latter points to a weak relation of MT expression and survival of patients with primary large intestine adenocarcinoma and confirms the relation for Ki-67 antigen expression (Öfner et al., 1994; Giuffré et al., 1996; Pietiläinen et al., 1996; Ioachim et al., 1999; Sutoh et al., 2000).

Summing up, we can note that expression of MT is relatively strongly correlated with Ki-67 antigen expression in primary large intestine adenocarcinomas and may, to a certain extent, constitute an index of tumour cell proliferation. Moreover, we have demonstrated a significantly higher expression of the two studied immunocytochemical markers in tumours of a poor histological differentiation (G3) and in tumours which infiltrate deeper intestinal walls.

The results presented by us contradict in part the earlier published results of other authors and substantiate the conclusion that the MT role in neoplastic processes and in large intestine carcinoma in particular remains far from being clarified.

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