Original paper

Diagnostic significance of TIMP-1 level in serum and its immunohistochemical expression in colorectal cancer patients

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Tissue inhibitor of metalloproteinase-1 (TIMP-1) inhibits the ability of cancer cells to metastasize, but it can also stimulate cancer development. The aim of this study was to assess the level of TIMP-1 in serum and its expression in patients with colorectal cancer (CRC).

The study group consisted of 43 patients diagnosed with colorectal cancer and 24 healthy volunteers. The level of TIMP-1 was assessed by the ELISA method while the expression of this protein was performed immunohistochemically.

The concentration of TIMP-1 in the sera of colorectal cancer patients was significantly higher than in the healthy control group ($p = 0.004$). Higher level of TIMP-1 in the sera correlated with female gender ($p = 0.045$), tumor location in colon ($p = 0.016$), poorly differentiated tumor ($p = 0.034$) and higher platelet count in whole blood ($p < 0.004$). A positive reaction of the protein in cancer cells was observed in 31 cases and was found to correlate negatively with its reaction in peritumoral stroma ($p < 0.001$).

According to this study, TIMP-1 protein may play an important role in cancer development. The assessment of this molecule in serum and tissue can be useful at the time of diagnosis and can help us to understand the nature of colorectal pathogenesis.

Key words: TIMP-1, serum, colorectal cancer, immunohistochemistry.

Introduction

Extracellular matrix degradation results from an imbalance between the activity of matrix metalloproteinases and their tissue inhibitors (TIMPs) in both physiological and pathological conditions. The following types of tissue inhibitors of metalloproteinase have been recognized so far: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The presence of TIMPs allows for elimination of matrix metalloproteinases (MMPs) or inhibition of the proteolytic activity of their active as well as lethal forms. TIMPs and MMPs form non-covalent bonds at a 1 : 1 ratio due to the ability to disconnect the *N*-terminal fragment of metalloproteinase blocks [1, 2]. The main tissue inhibitor of metalloproteinases is the tissue inhibitor of metalloproteinase-1 (TIMP-1), which is a soluble protein released by fibroblasts, endometrial cells, and cancer

cells, and which is present in the intercellular matrix, plasma, and other body fluids. TIMP-1 expresses strong affinity to MMP-9, and their interplay is the exponent of the tissue microenvironment during homeostasis [1, 2].

It has been shown that excessive secretion of MMPs frequently contributes to the increase in TIMPs. However, its inhibiting activity is not effective. Moreover, crossing the extracellular matrix (ECM) barrier determines the ability of cancer cells to give rise to metastases. It has been proven that the overexpression of TIMP-1 inhibits the ability of cancer cells to metastasize. Overexpression of the molecule inhibits both tumor growth of melanoma and its ability to metastasize as well as impeding the development of stomach cancer and preventing the progression of oral squamous cell carcinoma [3-5]. However, TIMP-1 participates in the protection of cells from apoptosis. It has been demonstrated that the protein inhibits apoptosis in T lymphocytes, human breast cancer epithelial cells and in melanomas [6, 7]. It has been confirmed that TIMP-1 is regulated by Bcl-2, which participates in the cellular apoptotic pathway [8]. However, recent reports indicate that TIMP-1 can stimulate cancer development, activate cell growth, promote migration and invasion of cancer cells, and increase the risk of metastasis formation [8-10].

Colorectal cancer (CRC) is one of the most common cancers in the developed countries, as it takes the second place among the causes of malignant cancer related deaths in Poland [11]. As early as in the 1960s it was established that serous carcinoembryonic antigen (CEA) is a biological prognostic marker in patients with colorectal cancer, as recommended by the American Society of Clinical Oncology (ASCO) and the European Group on Tumour Markers (EGTM). Monitoring the antigen level allows for detection and monitoring of early stage metastases, including metastases to lungs and liver [12]. However, EGTM is still seeking a new independent CRC biomarker [13]. The latest numerous reports have confirmed the significance of determining the TIMP-1 level in colorectal cancer patients, but the confirmation of its unequivocal role arouses much controversy. Thus, the main aim of our study was to assess the concentration of TIMP-1 in the serum and its immunohistochemical expression in patients diagnosed with CRC compared to healthy control volunteers.

Material and methods

Materials

The study group consisted of 43 patients, including 16 women and 27 men, treated surgically in the 2nd Department of General and Gastroenterological Surgery in the Medical University of Bialystok. The patients' age range was 34-86 years old (mean 67.11 ±1.89). The pathological diagnosis confirmed colorectal cancer and its stage (TNM) according to the WHO classification. The adenocarcinoma type was diagnosed in 38 individuals, whereas adenocarcinoma with a mucous component was identified in 5 individuals. A pT1 tumor was observed in 1 case, pT2 in 4 patients and pT3 in 38 patients. The investigated tumors were classified as moderately differentiated (G2) in 40 patients, and poorly differentiated (G3) in 3 patients. At the time of the diagnosis, metastases to local lymph nodes were observed in 23 out of 43 cases, whereas the presence of metastases to distant organs was noted in 11 out of 43 of the studied cases.

The control group consisted of 24 healthy volunteers (13 males and 11 females aged 45-75, mean 55.7 ±7.3, median 54.5). The healthy controls neither had any acute/chronic inflammatory conditions nor were being treated with any temporary medication. There were no significant differences in age or sex between patients and controls.

The study material consisted of serum samples obtained from both the blood of the patients with colorectal carcinoma collected prior to the surgery and the healthy controls. Blood serum was stored at –80°C immediately after centrifugation until the assay was performed.

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and received the approval of the local Bioethics Committee. All the participants signed informed consent forms prior to the examination.

Methods

Enzyme-linked immunosorbent assays (ELISA)

TIMP-1 concentration was determined by means of the enzyme-linked immunosorbent assay (ELISA) method. Serum samples were prepared according to the manufacturer's instructions. Prior to the assay, the samples were 100-fold diluted with Calibrator Diluents. A monoclonal antibody specific for TIMP-1 had been pre-coated onto a microplate and incubated with serum samples. After the first washing, an enzyme-linked polyclonal antibody specific for TIMP-1 was added to the wells. Following the second wash, a substrate solution was added. Next, the color development was stopped. The reaction measurement was based on the intensity of the sample color. All the specimens were assayed twice. No statistically significant differences between the measurements were found. The TIMP-1 Quantikine ELISA kit recognized both the recombinant and natural human TIMP-1. The minimum detectable dose (MDD) of TIMP-1 ranged from 87 to 524 ng/ml. Mean MDD was 190 ng/ml. The sensitivity was 0.08 ng/ml. The serum concentration of TIMP-1 was expressed in ng/ml.

Table I. Level of TIMP-1 protein in serum of patients with colorectal cancer and healthy controls

	TIMP-1 (ng/ml)					
	n	Mean	Median	SD	Range	p
Normal	24	529.16	527.05	54.78	427.5-630.5	0.004
Tumor	43	580.96	572.80	66.42	455.1-704.1	

SD – standard deviation

Immunohistochemistry

The immunohistochemistry method was carried out in 38 of 43 patients with CRC. Formalin-fixed and paraffin-embedded tissue specimens cut on a microtome into 4 μm sections were deparaffinized in xylenes and hydrated in alcohols. To visualize the antigens of TIMP-1 protein, the sections were heated in a microwave oven for 20 min in a citrate buffer (pH = 6.0). Then, they were treated with 3% hydrogen peroxide solution for 5 min and incubated with polyclonal rabbit antibody against human TIMP-1 (Novocastra, dilution 1 : 150) at room temperature overnight. The reaction was carried out using Novocastra Novolink Polymer Detection System (NCL – Novocastra, Leica Biosystems) and developed with chromogen DAB. Positive and negative controls were performed according to the producer's protocol (Novocastra, UK). Counterstaining was performed with hematoxylin.

Immunohistochemical staining was evaluated by two independent pathologists blinded to the clinical information. The expression of proteins was found in cytoplasm of the tumor cells and stroma and graded separately in the same manner. Expression was determined using the semiquantitative method and defined in relation to the intensity of staining (0 – absent, 1 – weak, 2 – moderate, 3 – strong) and the percentage of positive tumor cells. H-score was derived by summing percentages of cell staining at each intensity and then multiplied by the weighted intensity of staining. Score values ranged from 0 to 300. The study group was divided into negative cases (H-score < 150) and positive cases (H-score > 150).

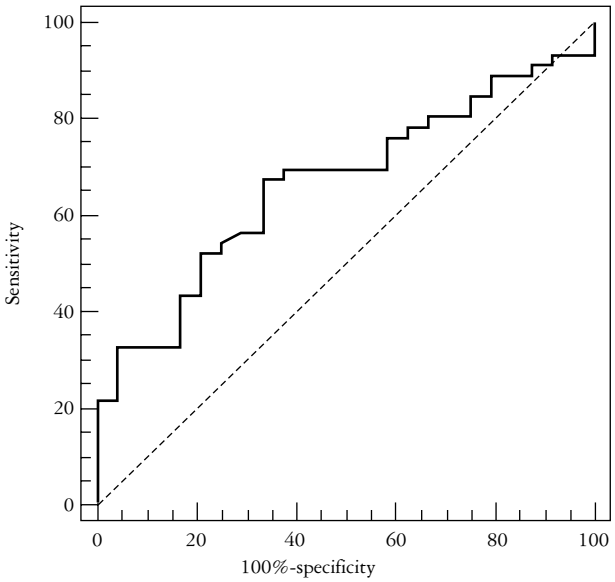

Fig. 1. ROC curve analysis for TIMP-1 levels in serum of patients with colorectal cancer

Statistical analysis

Statistical analysis was conducted based on the STATISTICA 8.0 program (StatSoft, Cracow, Poland). In order to compare the two groups, the Mann-Whitney *U* test was used. Correlations between the serum level and the expression of protein and other parameters were calculated by Spearman's correlation coefficient tests. The level of significance was < 0.05. The missing data were removed in pairs. The analysis of the receiver-operating characteristics (ROC) curve was performed using MedCalc statistical software (MedCalc Software, Belgium).

Results

Preoperative level of TIMP-1 protein in sera of patients with CRC and healthy controls

The mean value of TIMP-1 concentration in the sera of patients with colorectal cancer was 580.96 ng/ml (range 455.1-704.1 ng/ml) and was significantly higher than in the healthy control group, in which it was 529.16 ng/ml with a 427.5-630.5 ng/ml range (p = 0.004). The level of TIMP-1 protein in the sera of patients with colorectal cancer and the control group is shown in Table I.

The analysis of the ROC curve was performed to determine the diagnostic value and the cutoff point of serum TIMP-1 level. The sensitivity and specificity of serum TIMP-1 level were 67.4% and 66.7%, respectively, at a cutoff value of 537.8 ng/ml. The area under the ROC curve for TIMP-1 showed that the protein exhibits moderate diagnostic power (AUC = 0.666) (Fig. 1).

Correlations between preoperative level of TIMP-1 protein in sera and clinicopathological parameters in CRC patients

The level of TIMP-1 protein in patients with colorectal cancer correlated with gender, tumor location and the degree of histological malignancy (G)

Table II. Correlations between TIMP-1 levels and clinicopathological parameters in patients with colorectal cancer

PARAMETER		TIMP-1 (NG/ML)				
		N	MEDIAN	RANGE	COEFFICIENT	P VALUE
Age	≤ 60	14	574.5	491.3-688.6	0.075	NS
	> 60	29	584.0	455.1-704.1		
Gender	Male	27	564.4	455.1-688.6	**–0.306**	**0.045**
	Female	16	608.9	496.2-704.1		
Localization	Colon	24	601.3	491.3-704.1	**–0.362**	**0.016**
	Rectum	19	555.3	455.1-701.0		
Adenocarcinoma type	Nonmucinous	38	579.1	455.1-704.1	0.076	NS
	Mucinous	5	595.5	461.8-701.0		
Grade of malignancies	2	40	575.5	455.1-704.1	**0.323**	**0.034**
	3	3	652.6	643.9-657.9		
pT stage	1	1	641.7	–	0.151	NS
	2	4	524.5	455.1-583.4		
	3	38	585.3	461.8-704.1		
Lymph node metastasis	Absent	20	569.6	461.8-701.0	0.154	NS
	Present	23	590.8	455.1-704.1		
Distant metastasis	Absent	32	584.1	455.1-704.1	–0.096	NS
	Present	11	571.7	461.8-688.6		

Spearman's correlation coefficient tests. Data missing in pairs. NS – not significant

(Table II). A significantly lower level of the protein was observed in the sera of male patients compared to female patients with CRC ($p = 0.045$). The relation between the increase in TIMP-1 in patients with CRC and the colon tumor occurrence ($p = 0.016$) was statistically significant. Higher level of TIMP-1 was associated with colon localization of tumor rather than in the rectum ($p = 0.016$). Moreover, the statistical analysis showed that the level of TIMP-1 was significantly lower in patients with moderately differentiated colon cancer (G2) in comparison with patients with G3 (poorly differentiated) ($p = 0.034$). No statistically significant differences between the concentration of TIMP-1 protein in the sera of patients with CRC and the remaining clinicopathological parameters such as age, histological type, stage and local lymph node involvement and distant organ metastases were observed.

Preoperative level of TIMP-1 protein and morphological blood parameters

The level of TIMP-1 protein in the sera of patients with CRC correlated positively with the platelet count ($p = 0.004$) (Fig. 2). The platelet count in patients with CRC increased with the rise of TIMP-1 concentration in the serum. No other correlations of TIMP-1 level in the serum of the study group with particular morphological blood parameters were noted (Table III).

Immunohistochemical expression of TIMP-1 protein in CRC tissue

The expression of TIMP-1 protein was observed in the cytoplasm of tumor cells and peritumoral stroma (Fig. 3). The positive reaction of protein in cancer cells was observed in 31 cases, whereas no reaction in this localization was observed in 7 cases. Positive TIMP-1 immunoreactivity of pericancer stromal cells was detected in only one case. The TIMP-1 expression localized in peritumoral stroma was negative in most cases (37 subjects). Moreover, the expression of TIMP-1 in tumor cells was found to correlate negatively with its reaction in peritumoral stroma ($p < 0.001$) (Table IV). However, the protein expression of cancer cells did not correlate with any clinicopathological features (Table V).

Discussion

The presence of TIMP-1 was confirmed in plasma, serum, tissue homogenates and in tissue samples of patients with CRC. The overexpression of the

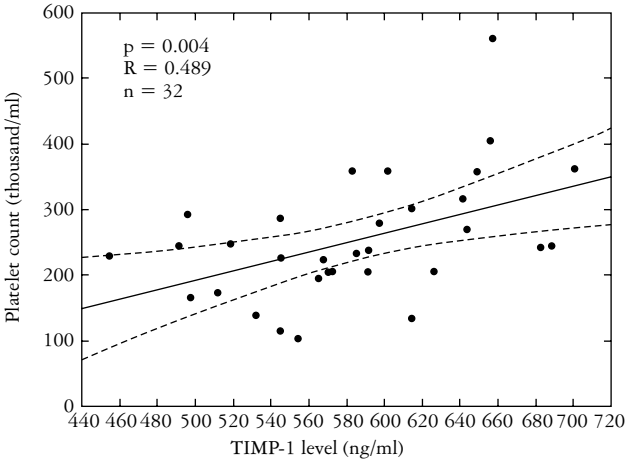

Fig. 2. Correlation between TIMP-1 in serum of patients with colorectal cancer and platelet count

TIMP-1 protein in cancer cells has also been confirmed in molecular studies [14, 15]. However, the analyses of tissue homogenates obtained from the patients diagnosed with CRC with the ELISA method indicated that the level of TIMP-1 protein increased in comparison to its concentration in normal colon tissue in biological material of this type [16-18]. Furthermore, Baker *et al*. [17] noted that the growth of TIMP-1 in tumor homogenates was closely connected with Duke's cancer stage, lymph node involvement and MMP-1 level. The protein level in tissue homogenates of the tumor in all the reports mentioned above was significantly lower than its plasma concentration [19]. Based on the findings of the tissue analysis, the researchers conducted the experiments on other material as well. In the preoperative plasma, the increase in TIMP-1 protein level in patients with CRC was related to the patient's age. The level of the protein increased in the elderly [20-24]. Moreover, the increase in TIMP-1 level also correlated with Dukes' D stage of cancer [20, 25, 26]. Yukawa *et al.* [25] observed that the plasma level of TIMP-1 in patients with advanced disease was significantly higher in comparison with the patients with an early stage of CRC. According to the author,

Table III. Correlations between level of TIMP-1 protein in serum and morphological blood parameters

Parameter	TIMP-1		
	n	Coefficient	p value
Red blood cell count	32	0.191	NS
White blood cell count	32	0.139	NS
PLT	32	**0.489**	**0.004**
Hematocrit	32	–0.089	NS
Hemoglobin	32	–0.137	NS
Sodium	32	–0.230	NS
Potassium	32	–0.068	NS
Prothrombin time	31	0.023	NS
Prothrombin ratio	31	–0.066	NS
Total proteins	21	0.104	NS
Aspartate transaminase	22	0.111	NS
Alanine transaminase	22	–0.325	NS
Glucose	16	–0.305	NS
Urea	28	–0.273	NS
Creatinine	25	–0.172	NS

Spearman's correlation coefficient tests. Data missing in pairs.
NS – not significant

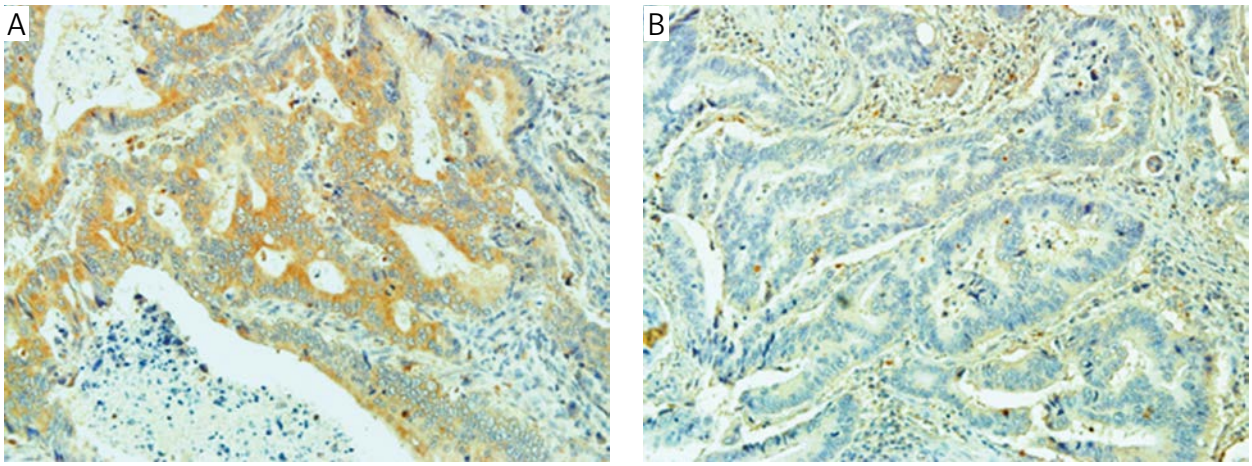

Fig. 3. Immunohistochemical expression of TIMP-1 in colorectal cancer. A strong reaction of TIMP-1 protein in cytoplasm of tumor cells and negative expression of this protein in stroma were seen in most cases (**A**). In only one case we observed positive TIMP-1 immunoreactivity in peritumoral stromal cells without color reaction in colorectal cancer cells (**B**). Total original magnification 200×

the five-year survival rate was observed in TIMP-1 positive patients [27]. Other researchers have also observed the relation between the level of TIMP-1 protein in plasma and other parameters such as primary tumor location in colon, the presence of metastases to lymph nodes and distant organs, the attachment of blood and lymph vessels, and the growth of serum CEA level [20, 24, 25]. Holten-Andersen *et al.* [21] noted the increase in the level of TIMP-1 protein in postoperative plasma of patients with CRC compared to the control group. However, the concentration of the protein did not differ notably from its level in preoperative plasma of patients with CRC [21].

According to this study, which was based on the ELISA method, a statistically significantly higher level of TIMP-1 protein in the sera of patients with CRC compared to the healthy control group was observed. Similar findings were noted by Mroczko *et al.* [28] who observed an increase in the serum protein of patients with CRC in relation to healthy controls and patients with diagnosed colon adenoma. Moreover, other researchers also noted an increase in the concentration of TIMP-1 in the serum of patients with colorectal cancer [29, 30]. However, the lack of healthy control group excludes the possibility of a direct comparison of the studies to our results. Oberg *et al.* [31] reported the lack of statistical significance of the serum protein level of patients with colorectal cancer and the control group, yet a wide range of the obtained values was the same in both groups. The differences in all the mentioned reports on the deter-

Table IV. Characteristics of TIMP-1 expression in colorectal cancer tissue

	TIMP-1 EXPRESSION		P VALUE
	NEGATIVE N (%)	POSITIVE N (%)	
Cancer cells	7 (22.5)	31 (77.5)	< 0.001
Peritumoral stroma	37 (97.4)	1 (2.6)	

Missing data are removed in pairs.

Table V. Correlations between TIMP-1 expression and clinical-pathological parameters in patients with colorectal cancer

PARAMETER		TIMP-1 EXPRESSION			
		CANCER CELLS		COEFFICIENT	P VALUE
		NEGATIVE N (%)	POSITIVE N (%)		
Age	≤ 60	1 (2.6)	12 (31.5)	–0.199	NS
	> 60	6 (15.7)	19 (50.2)		
Gender	Male	4 (10.5)	10 (26.3)	0.200	NS
	Female	3 (7.8)	21 (55.4)		
Localization	Colon	6 (15.7)	17 (44.7)	0.245	NS
	Rectum	1(2.6)	14 (37)		
Adenocarcinoma type	Nonmucinous	7 (18.4)	28 (73.8)	0.139	NS
	Mucinous	0 (0)	3 (7.8)		
Grade of malignancies	2	6 (15.7)	29 (79.1)	0.105	NS
	3	0 (0)	2 (5.2)		
Tumor size	< 5 cm	4 (10.5)	17 (44.7)	0.075	NS
	> 5 cm	2 (5.2)	13 (39.6)		
pT stage	1	0 (0)	1 (2.6)	–0.108	NS
	2	0 (0)	3 (7.8)		
	3	6 (15.7)	25 (71.3)		
	4	0 (0)	1 (2.6)		
Lymph node metastasis	Absent	4 (10.5)	12 (31.5)	0.135	NS
	Present	3 (7.8)	18 (47.3)		
Distant metastasis	Absent	4 (10.5)	19 (50.2)	–0.032	NS
	Present	3 (7.8)	12 (31.5)		

Spearman's correlation coefficient tests. Data missing in pairs. NS – not significant

mination of TIMP-1 protein in the serum of patients with CRC result from using particular ELISA tests or the number of patients in the study group.

The statistical analysis of this study showed that the level of TIMP-1 protein in the sera of patients with CRC correlated with gender, tumor location and the degree of cancer malignancy. The serum of male patients diagnosed with CRC compared with its concentration in female counterparts was characterized by a significantly lower level of the protein. We found no other data confirming the relation between the serous level of TIMP-1 in patients with CRC and gender. The relation between the increase in TIMP-1 concentration in the sera of patients with CRC and occurrence of the tumor in the colon appeared to be statistically significant. Holten-Andersen *et al.* [20] also proved that the level of TIMP-1 protein in the plasma of patients with primary tumor located in the colon was higher compared to the patients with rectal tumor. What is more, statistical analysis of this study indicated that the level of TIMP-1 protein was statistically significantly higher in patients with a poorly differentiated CRC. The degree of differentiation determines the malignancy of the cancer. Therefore, the serum TIMP-1 increase in those patients may result from considerable degradation of tissue stroma and the expansion of the cancer. The above observations confirm the results of the research conducted by Giaginis *et al.* [30] who claimed that the level of the TIMP-1 protein in the serum was higher in patients with a poorly differentiated histological type of CRC. The authors drew attention to the fact that the level of TIMP-1 protein was connected with the degree of malignancy [30]. This observation is confirmed by numerous studies showing that TIMP-1 level was related to Duke's classification and the highest protein concentration was found in the samples of Duke's D stage cancer patients [28, 31, 32]. According to these observations, there is a connection between the increase in the TIMP-1 concentration in the sera of patients with CRC and the following factors: the occurrence of metastases to lymph nodes and distant organs including the liver [28, 32], tumor size [32], and shorter time of survival [28, 30]. However, we observed no such correlations.

The following observations were connected with the relation between the level of TIMP-1 protein in the serum of patients with CRC and morphological blood parameters obtained preoperatively. We noted that the level of TIMP-1 in the serum correlated positively with platelet count (Fig. 2). The concentration of TIMP-1 in the sera of patients with CRC rose with the increase in platelet count. Therefore, the degree of tumor malignancy did not indicate any statistically significant relation with the platelet count (data not shown). The results of our studies are in accordance with the observations presented below. The studies by other authors indicated that the TIMP-1 protein occurs in α granules of platelets [33, 34], which indicates that these cells can determine the protein level in plasma and serum [35]. Possible participation of the platelet count in TIMP-1 protein secretion was observed in patients with CRC [36, 37]. Holten-Andersen *et al.* [36] proved the existence of a weak yet significant correlation between the concentration of TIMP-1 in the plasma of patients diagnosed with CRC and the platelet count, which indicates that they are not the only source of the protein. The authors suggest that the weak relation between the two parameters resulted not from the platelet count but from the degree of their activation or failure. In the following years Frederiksen *et al.* [37] made a detailed analysis of the blood obtained from tumor veins and arteries of patients with rectal cancer. The researchers observed a weak yet statistically significant correlation between the preoperative concentration of TIMP-1 in plasma and platelet count from peripheral blood as well as a significant correlation between the level of the protein and the counts of platelets from tumor veins and peripheral blood obtained intraoperatively. According to the studies conducted by the author, activated or damaged platelets are the source of the TIMP-1 protein, which means that the protein may also be derived from other cellular elements. Sorensen *et al.* [19] analyzed the level of TIMP-1 protein in plasma, serum and tissue extracts collected from patients with primary CRC. The authors of this study observed an increase in the level of TIMP-1 in the serum compared to the plasma of patients with CRC. Also, no correlation between the concentration of the protein in plasma and tissue tumor extracts was observed.

According to our immunohistochemical study, we found a positive reaction of TIMP-1 in cancer cells in most cases and in peritumoral stromal cells only in 1 subject. We also noted significantly higher expression of TIMP-1 protein in cancer cells compared to peritumoral stroma. Our findings are in line with those of Jensen *et al.* [38] and Roca *et al.* [39]. The authors demonstrated that the TIMP-1 protein was overexpressed in colorectal cancer cells. However, Holten-Andersen *et al.* [14] found a lack of TIMP-1 expression in benign and malignant colorectal cells and weakly distributed in normal mucosa, while strong TIMP-1 immunoreactivity was seen in peritumoral stromal cells such as myofibroblasts in the invasive front of the tumor. Also Joo *et al.* [40] found the positive reaction of TIMP-1 in peritumoral stromal cells while the normal mucosa and CRC cells did not express this protein. The increased expression of TIMP-1 was connected with the degree of malignancy and the five-year survival rate of those patients [14, 39, 40]. However, our investigation did not confirm such correlations.

In conclusion, it is highly indicated that both the positive TIMP-1 immunoreactivity in CRC cells and the increased level in sera of those patients may suggest that those types of cancer cells can produce and release this protein. Due to this fact, CRC cells may acquire protective properties against various degradation mechanisms and increase their longevity.

The authors declare no conflict of interest.

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