Clinical usefulness of MMP-2 and TIMP-2 concentrations in the preoperative serum of patients with colorectal cancer

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ABSTRACT

Purpose: To evaluate the clinical significance of MMP-2 and TIMP-2 concentrations in the sera of patients diagnosed with colorectal cancer.

Methods: The study group comprised 48 patients with colorectal carcinoma and 24 healthy controls. The serum concentration of MMP-2 and TIMP-2 proteins was evaluated by the ELISA method.

Results: The mean level of MMP-2 in the sera of patients with colorectal cancer was 39.4 ng/ml and was significantly lower compared with the control ($p<0.001$). A decrease in TIMP-2 protein in the sera of patients with colorectal cancer was also observed where its mean level was 132.3 ng/ml. The concentration of MMP-2 and TIMP-2 did not correlated with any clinicopathological parameters, except for the patients' age. In addition, the concentration of MMP-2 in the sera of patients with CRC correlated negatively with the number of white blood cells in the blood and the prothrombin index. The concentration of TIMP-2 correlated positively with potassium and urea concentration in the blood.

Conclusions: The results of our study indicate lack of clinical usefulness of determining the levels of MMP-2 and TIMP-2 in the sera of patients with colorectal cancer. However, these proteins play an important role in the carcinogenesis of colorectal cancer.

Key words: Colorectal cancer, matrix metalloproteinases, MMP-2, TIMP-2, serum marker

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INTRODUCTION

The extracellular matrix (ECM) is a specific tissue part which participates in cell migration, adhesion, differentiation and intercellular interactions. It also ensures the bi-directional transport of nutrients out of arterial cells into cells and the transport of residual products from cells to veins and lymph. The components of EMC include structural proteins such as collagen, elastin, proteoglycans, and other proteins produced by fibroblasts. Remodelling of the extracellular matrix constitutes a very important process in the creation of the tumor microenvironment. Initially, the extracellular matrix is involved in tumor initiation, proliferation, local tumor development and neovascularization, and subsequently impacts on the migration of cancer cells leading to the formation of metastases. It is achieved through the process of intratumoral transformation of fibroblasts to myo-fibroblasts, which have the ability to overproduce proteolytic enzymes called metalloproteinases (MMPs)[1]. Metalloproteinases can in turn degrade and remodel extracellular matrix proteins. Numerous studies have demonstrated the overexpression of MMPs in many types of cancer [2].

Metalloproteinase 2 (MMP-2, gelatinase A) is one of the members of the MMP family. The characteristic feature of MMP-2 structure is the presence of fibronectin type II modules in the catalytic domain, resulting in the high substrate specificity for gelatin and collagen type I, II, III, IV, VII and X [3]. Thanks to this MMP-2 plays a very important role in extracellular matrix remodelling as well as in other physiological processes. The activity of metalloproteinase 2 is controlled at three levels: at the gene transcription, proenzyme activation and the regulation by tissue inhibitors of metalloproteinases (TIMPs). The proteolytic activation of pro-MMP-2 takes place on the cell surface with the participation of one of the six MT-MMP (membrane metallo-proteinases), especially MT1-MMP. MT1-MMP forms dimmers and connects with TIMP-2, then the complex binds pro-MMP-2 and facilitates its proteolytic activation. This is a unique process since TIMP-2 belongs to a group of endogenous protein inhibitors of metalloproteinases and shows a high affinity for gelatinases. Therefore, it also has the ability to bind to both, the active and inactive forms of MMP-2 [2,4].

The participation of MMP-2 and TIMP-2 proteins in colorectal cancer development (CRC) has been fairly well established by numerous research on the expression of these proteins in tissues. Therefore, the aim of this study was to evaluate the concentration of MMP-2 and TIMP-2 in the sera of patients with colorectal carcinoma.

MATERIALS AND METHODS

Patients

The study group consisted of 48 patients diagnosed with metastatic colorectal cancer (30 men and 18 women aged 34-86 years) treated surgically at the Department of General Surgery and Gastroenterology, Medical University of Bialystok in the years 2007-2009. Colorectal cancers were confirmed histopathologically. Adenocarcinoma was found in 42 out of 48 patients and the mucous type was discovered in 6 out of 48 patients. The malignancy grade of the investigated tumors was moderately (45 out of 48 patients) or poorly differentiated (3 out of 48 patients). According to the TNM classification, only in one case did the tumor infiltrate the submucosa (pT1), in 4 cases- the muscular layer and in 43 cases reached the subserous tissue. In 27 out of 48 patients it had metastasized to lymph nodes and in 14 out of 48 patients it had distant metastases mainly to the liver but also to the pancreas, lungs, kidneys, ovaries and the body of the uterus.

The blood of colorectal cancer patients was drawn for clot prior to surgery. Blood serum was stored frozen at -80^0C immediately after separation by centrifugation until the assay was performed. The control group consisted of 24 healthy subjects (13 men and 11 women aged 45-75 years).

The study protocol was approved by the Bioethics Committee and written informed consent was obtained from all the participants.

Enzyme-linked immunoabsorbent Assays (ELISA)

MMP-2 and TIMP-2 concentrations were measured with a quantitative sandwich enzyme immunoassay (R & D Systems, Abingdon, UK). Serum samples were incubated on microtiter plates precoated with a murine Monoclonal antibody against MMP-2/TIMP-2. The platelets were washed and an enzyme-linked polyclonal antibody specific for MMP-2/TIMP-2 was added to the wells. After the second washing procedure, a substrate solution was added. This coloured substrate was in proportion to the amount of MMP-2 or TIMP-2, which were bound to the microtiter plate. All the specimens were assayed twice and there were no statistically significant differences between the measurements. The MMP-2 Quantikine ELISA kit recognized only both the free recombinant (pro-and mature) and natural human MMP-2. The human TIMP-2 Quantikine ELISA kit recognized recombinant and natural human TIMP-2 and detected approximately 50% of recombinant human TIMP-2 when complexed with recombinant human

active MMP-9 in a 1:1 molar ratio. The results are presented in nanograms per milliliter.

Statistics

The statistical analysis was performed using the STATISTICA 8.0 program. The Mann-Whitney U test was used in order to compare the two groups. Correlations between the serum level of proteins, the clinicopathological features and morphological blood parameters was calculated by Spearman's correlation coefficient test. The value $p<0.05$ was considered statically significant. Missing data were removed in pairs. Additionally, the area under the ROC curve (AUC) was analysed using MedCalc Statistical Software.

RESULTS

Serum MMP-2 and TIMP-2 levels in colorectal cancer and control tissue.

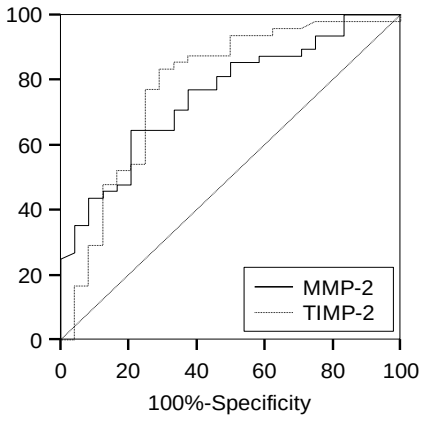
The mean serum levels of MMP-2 in patients with colorectal cancer were 39.4 ng/ml of 14.8-54.4 ng/ml and were significantly lower than those in the control group (47.0 ng/ml in a range of 36.0-67.0 ng/ml, $p<0.001$). A decrease in TIMP-2 protein in the sera of patients with colorectal cancer, where the mean level was 132.3 ng/ml (range 111.8-185.6 ng/ml), was also observed. A similar range of serum TIMP-2 levels in the control group was reported (111.6-183.7 ng/ml), but the mean value was significantly higher than that in colorectal cancer patients and it amounted to 146.3 ng/ml ($p<0.001$) (Table 1).

Table 1. Serum MMP-2 and TIMP-2 levels in colorectal cancer and control tissue

		MMP-2 (ng/ml)				
	N	**Mean**	**Median**	**SD**	**Range**	**P- value**
Normal	24	47.0	45.3	8.86	36.0-67.0	**<0.001**
Tumor	48	39.4	39.4	7.13	14.8-54.4	
		TIMP-2 (ng/ml)				
	N	**Mean**	**Median**	**SD**	**Range**	**P- value**
Normal	24	146.3	145.5	16.98	111.6-183.7	**<0.001**
Tumor	48	132.3	131.0	12.00	111.8-185.6	

Mann-Whitney U-test

On the basis of the designed ROC curves we showed that the cutoff point for MMP-2 concentration was 42.5 ng/ml (sensitivity and specificity was respectively 74.1% and 62.5%), while the cutoff point for TIMP-2 concentration was 139.7 ng/ml (sensitivity here and specificity was respectively 83.3% and 70.8%). The area under the ROC curve designed for MMP-2 and TIMP-2 was 0.754 and 0.777 respectively, which indicates the average differential properties of these tests in patients with colorectal cancer (Figure 1).



Test Result Variables		Area under the ROC curve (AUC)	Standard Error[a]
MMP-2		0.754	0.058
TIMP-2		0.777	0.064

Figure 1. Areas under the ROC curves for MMP-2 and TIMP-2 in differentiation between colorectal cancer patients and health subjects

Correlations between serum MMP-2 and TIMP-2 levels and clinicopathological parameters in colorectal cancer patients.

The serum concentration of MMP-2 protein correlated with the patients' age. The level of this protein was lower in the sera of subjects aged 60 and above ($p<0.01$). A similar relationship was shown for TIMP-2 protein ($p<0.01$). No correlations were found between the levels of these proteins and age in controls (data not shown). There were no significant differences between the levels of MMP-2 and TIMP-2 in the sera of patients with colon carcinoma and any other clinicopathological parameters (Tables 2, 3).

Table 2. Correlations between serum MMP-2 levels and clinicopathological parameters in colorectal cancer patients

Parameter		**MMP-2 (ng/ml)**				
		N	**Mean**	**Range**	**Coefficient**	**P- value**
Age	≤60	16	36.9	30.9 – 49.4	**0.363**	**<0.01**
	>60	32	40.6	14.8 – 54.4		
Gender	Male	30	39.5	30.3 – 54.4	–0.079	NS
	Female	18	39.1	14.8 – 50.0		
Localization	Colon	28	38.9	14.8 – 54.4	–0.001	NS
	Rectum	20	40.1	32.0 – 54.4		
Adenocarcinoma type	Nonmucinous	42	39.4	14.8 – 54.4	–0.063	NS
	Mucinous	6	39.5	30.3 – 54.4		
Grade of malignancies	2	45	39.4	14.8 – 54.4	–0.065	NS
	3	3	38.5	34.8 – 45.3		
pT stage	1	1	45.4	45.4	–0.097	NS
	2	4	40.1	33.2 – 49.4		
	3	43	39.2	14.8 – 54.4		
Lymph node metastasis	Absent	21	40.5	30.3 – 54.4	–0.057	NS
	Present	27	38.5	14.8 – 50.0		
Distant metastasis	Absent	34	38.9	14.8 – 54.4	0.079	NS
	Present	14	40.6	30.6 – 54.4		

Spearman's correlation coefficient test. Missing data were removed in pairs. NS- no significant

Table 3. Correlations between serum TIMP-2 levels and clinicopathological parameters in colorectal cancer patients

Parameter		**TIMP-2 (ng/ml)**				
		N	**Mean**	**Range**	**Coefficient**	**P- value**
Age	≤60	16	126.8	111.8 – 144.7	**0.358**	**<0.01**
	>60	32	135.1	115.6 – 185.6		
Gender	Male	30	131.8	111.8 – 185.6	–0.155	NS
	Female	18	133.2	120.9 – 153.2		
Localization	Colon	28	132.8	111.8 – 185.6	–0.010	NS
	Rectum	20	131.6	117.4 – 148.8		
Adenocarcinoma type	Nonmucinous	42	132.0	111.8 – 153.2	–0.145	NS
	Mucinous	6	134.4	115.6 – 185.6		
Grade of malignancies	2	45	132.8	111.8 – 185.6	–0.180	NS
	3	3	125.4	120.9 – 130.8		
pT stage	1	1	135.0	135.0	–0.097	NS
	2	4	133.2	132.5 – 139.7		
	3	43	132.2	111.8 – 185.6		
Lymph node metastasis	Absent	21	132.9	115.6 – 185.6	0.036	NS
	Present	27	131.9	111.8 – 153.2		
Distant metastasis	Absent	34	131.2	111.8 – 185.6	0.261	NS
	Present	14	135.0	120.9 – 148.8		

Spearman's correlation coefficient test. Missing data were removed in pairs. NS- no significant

However, a statistically significant positive correlation between the levels of MMP-2 and TIMP-2 in the sera of patients with colon carcinoma was shown (Figure 2).

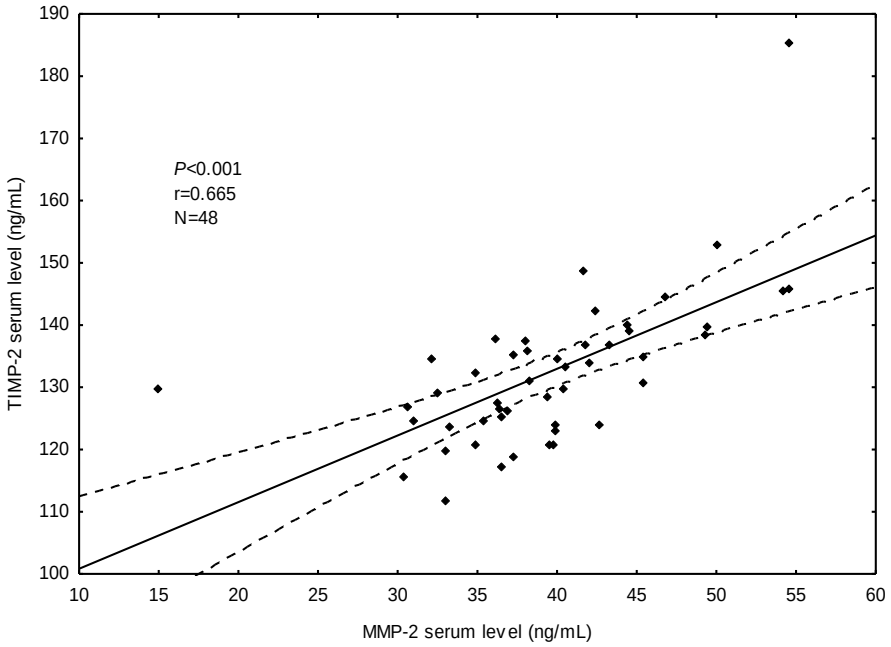


Figure 2. Correlation between the levels of MMP-2 and TIMP-2 in serum of patients with colorectal cancer

Correlations between serum MMP-2 and TIMP-2 levels and blood morphology parameters in colorectal cancer patients

The serum level of MMP-2 correlated negatively with the number of white blood cells ($p<0.05$). The higher the number of white blood cells in the patients' blood, the less MMP-2 protein was found in the serum. In addition, a negative correlation between MMP-2 levels and the prothrombin index was observed ($p<0.05$).

Statistical analysis showed a significant correlation of blood potassium level and serum TIMP-2 level in patients with colorectal cancer ($p<0.01$). Concurrently with a decrease in TIMP-2, the level of potassium in the blood increased. A similar relationship existed in the case of MMP-2 protein, but it was on the border of statistical significance ($p=0.056$). In addition, the levels of serum TIMP-2 were found to correlate positively with urea concentration in the blood of patients with colorectal cancer. (Tables 4, 5).

Table 4. Correlation between serum MMP-2 levels and blood morphology parameters

Parameter	**MMP-2**		
	N	**Coefficient**	**P- value**
Red Blood Cell Count	35	–0.173	NS
White Blood Cell Count	35	**–0.338**	**<0.05**
PLT	35	–0.173	NS
Hematocrit	35	–0.106	NS
Hemoglobin	35	–0.033	NS
Sodium	35	0.026	NS
Potassium	35	–0.325	**0.056**
Prothrombin time	34	0.174	NS
Prothrombin index	33	**–0.349**	**<0.05**
Total proteins	23	–0.074	NS
Aspartate Transaminase	25	0.259	NS
Alanine Transaminase	25	–0.032	NS
Glucose	17	–0.267	NS
Urea	31	0.292	NS
Creatinine	28	0.018	NS

Spearman's correlation coefficient test. Missing data were removed in pairs. NS- no significant.

Table 5. Correlation between serum TIMP-2 level and blood morphology parameters

Parameter	TIMP-2		
	N	Coefficient	P- value
Red Blood Cell Count	35	–0.014	NS
White Blood Cell Count	35	–0.249	NS
PLT	35	–0.230	NS
Hematocrit	35	0.049	NS
Hemoglobin	35	0.130	NS
Sodium	35	0.010	NS
Potassium	35	**–0.431**	**<0.01**
Prothrombin time	34	–0.045	NS
Prothrombin index	33	–0.107	NS
Total proteins	23	–0.001	NS
Aspartate Transaminase	25	0.118	NS
Alanine Transaminase	25	–0.058	NS
Glucose	17	–0.345	NS
Urea	31	**0.370**	**<0.05**
Creatinine	28	–0.134	NS

Spearman's correlation coefficient test. Missing data were removed in pairs. NS- no significant

DISCUSSION

A key mechanism of the extracellular matrix degradation is the disturbance in the ratio of the matrix metalloproteinase to their inhibitors. Numerous studies have shown that the overexpression of MMP-2, which occurs in colon cancer, results in an increased expression of TIMP-2 inhibitor. Even at the molecular level an increase in MMP-2 transcripts and a decrease in the expression of TIMP-2 mRNA in colon cancer can be observed [5,6]. Similar changes are observed when the expression of these proteins is measured in tumor tissues by immunohistochemistry [7,8]. However, some researchers report results of an increased expression of TIMP-2 mRNA and TIMP-2 protein in colon cancer cells as well as in the stroma [9,10]. More significant differences are observed in the concentrations of these proteins in tumor tissue homogenates, the serum or plasma of patients with colorectal cancer [11-13]. Oberg et al. [11] observed an increased concentration of free MMP-2 and total TIMP-2 in the preoperative serum of patients compared to the healthy controls group and a statistically significantly lower level of the MMP-2/TIMP-2 complex. Other studies have shown an increased concentrations of native forms of TIMP-2 in the plasma of patients with colorectal carcinoma but only with Dukes A; its level was significantly lower in patients with Dukes B, C, D or in healthy controls [12]. However, Gershtein et al. [13] demonstrated no significant changes in the concentrations of total MMP-2 and TIMP-2 in the plasma of patients with colorectal cancer and control groups. Nonetheless, they documented an increase in the total MMP-2 level in tumor homogenates compared to normal intestinal mucosa. In the case of TIMP-2 protein they did not show such differences [13]. Our results differed from those reported above. We evaluated free MMP-2 and total TIMP-2 in the sera of patients with colorectal cancer by ELISA. The protein concentration ranges in colorectal cancer patients were found to overlap as compared to the control group, which excluded their use as diagnostic markers of the neoplastic process. The serum levels of MMP-2 and TIMP-2 were statistically significantly lower in cancer patients compared to healthy subjects. Our results are similar to the observations noted by Groblewska et al. [14,15] who demonstrated that the level of MMP-2 and TIMP-2 decreased in CRC. Furthermore, the MMP-2 concentration was lower in colorectal cancer compared to colorectal adenoma patients. In addition, Waas et al. [16,17] found a decrease in inactive pro-MMP-2 in plasma. To our knowledge, no data referring to a decrease in plasma TIMP-2 levels in patients with colorectal cancer have been reported. Nevertheless, a decrease in the TIMP-2 concentration in tumor tissue homogenates tested with ELISA was documented by Baker et al. [18].

Plasma MMP-2 concentrations were also studied by Hanke et al. [19] who focused on patients with CRC before cytoreduction treatment and after chemotherapy. However, there are no results of plasma MMP-2 levels from a control group of healthy individuals to allow a comparison with our findings. Despite this, the authors showed that MMP-2 levels were lower in pre-treated patients compared to patients after chemotherapy and those without progression. In addition, the plasma concentration of MMP-2 in chemotherapy treated patients was lower in subjects with cancer progression than in those without progression. The higher plasma levels of MMP-2 in patients after chemotherapy may indicate that the protein can be released by the damaged tissues.

Our study did not demonstrate any statistically significant differences between the concentration of free MMP-2 and total TIMP-2, and histological parameters such as tumor location, histological type, malignancy grade, stage and the presence of metastases to local lymph nodes and distant organs. It was solely the patients' age (above 60 years of age) which correlated with decreased levels of these proteins. However, other researchers have demonstrated a correlation between changes in MMP-2 and TIMP-2 levels and the Dukes's classification [12,18], a decrease in pro-MMP-2 and the presence of lymph node

metastases [17], an increased level of free MMP-2 and a shorter survival time, or increased total TIMP-2 and a poorer prognosis for the patient [11].

The relationship of MMP-2 and TIMP-2 concentrations in blood serum and total morphological parameters determined in routine preoperative diagnostics was of great interest. A negative correlation between the concentration of free MMP-2 and the white blood cell count and the prothrombin index was found. However, the concentration of total TIMP-2 correlated negatively with the concentration of potassium and positively with the concentration of urea in the blood. It may indicate the potential mechanisms which are involved in the regulation of the production, secretion or degradation of metalloproteinases, and suggest a reason for changes in blood levels of matrix metalloproteinases.

CONCLUSIONS

In conclusion, the results of MMP-2 and TIMP-2 levels determined by ELISA were significantly different in serum, plasma or tissue homogenates, as indicated in literature. It is likely that the type of the ELISA tests used and the laboratory techniques and procedures of collecting and preserving the material have influenced the results. Simultaneously, lack of data regarding the forms of proteins found (free or bound, inactive or active) impedes the comparison of results published by different researchers. The results of our study indicate that determining the level of MMP-2 and TIMP-2 in the sera of patients with colorectal cancer was not clinically useful.

Conflicts of interest

None declared.

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