

Quantitative Estimation of Matrix Metalloproteinases 2 and 7 (MMP-2, MMP-7) and Tissue Inhibitors of Matrix Metalloproteinases 1 and 2 (TIMP-1, TIMP-2) in Colorectal Carcinoma Tissue Samples

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Abstract. *Background:* An essential step in the process of tumor invasion and metastasis involves the degradation of tissue barriers in the extracellular matrix (ECM), particularly in the basal membrane (BM). Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs), in particular MMP-2, MMP-7, TIMP-1 and TIMP-2, play an important role in the process of ECM and BM degradation in connection with tumor invasion. *The aim of our study was to assess the levels of MMP-2, MMP-7, TIMP-1 and TIMP-2 mRNA expression in colorectal carcinoma tissue samples and to correlate them with the stage of the disease. Patients and Methods:* The study included samples of tumor tissue of 38 patients with colorectal carcinoma and samples of tissue of 11 patients with benign disease. The expression levels of mRNA MMP-2, MMP-7, TIMP-1, TIMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as housekeeping gene, were quantified in tissue samples using the method of reverse transcription real-time PCR. *Results:* The levels of mRNA expression of MMP-2, MMP-7 and TIMP-1 were significantly higher in tumor tissue samples than in the control tissue ($p < 0.0005$, $p < 0.0007$ and $p < 0.0004$). In addition the presence of mRNA MMP-2, MMP-7, TIMP-1 and TIMP-2 in tumor tissue samples in these parameters was significantly higher than in the control tissue ($p < 0.003$, $p < 0.0001$, $p < 0.0001$ and $p < 0.05$). *Conclusion:* This pilot study demonstrated that a significant difference in the level and in the presence of mRNA MMP-2, MMP-7 and TIMP-1 expressions between tumor

colorectal and control colorectal tissues might be helpful for the prognosis of colorectal cancer.

Colorectal carcinoma is the second most common cancer in the Czech Republic among both males and females, and is one of the most frequent cancers worldwide. Approximately 98% of all colon carcinomas are adenocarcinomas. All colon carcinomas gradually grow through the intestinal wall into the surrounding structures and infiltrate lymphatic and blood vessels. Degradation of the extracellular matrix (ECM) and infiltration through the basal membrane (BM) are the necessary steps for tumor invasion. Matrix metalloproteinases (MMPs) and inhibitors of matrix metalloproteinases (TIMPs) play an important role in the process of degradation of the ECM and BM in relation to tumor invasiveness (6, 13). Individual MMPs have been identified that show increased expression and matrix degradation in tumors (3, 10).

MMP-2 (type IV collagenase; 92 kDa) degrades molecules of collagen IV, which is the main component of the BM, as well as some other collagens (types V, VII and X), and fibronectin, laminin and elastin (9). The activity of MMPs depends on the balance between the level of active enzyme and tissue inhibitor of matrix proteinases. TIMP-2 binds specifically and noncovalently to the pro-form of MMP-2 (7) and inhibits the activity of the activated enzyme. MMP-7 (matrilysin, pump-1; 28 kDa) cleaves a number of substrates including collagen types IV and X, elastin, fibronectin, gelatin, laminin and proteoglycans, being regulated mainly by TIMP-1 and TIMP-2.

The aim of our study was to assess MMP-2, MMP-7, TIMP-1 and TIMP-2 and the ratio MMP-2/TIMP-2 mRNA expressions in colorectal carcinoma tissue samples with the real-time PCR method, and to correlate the values obtained with the clinical status of the patients.

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Table I. Stages of malignancy of colorectal cancer patients according to UICC (International Union Against Cancer) classification.

Stage	Number of patients
I	2 (5%)
II	14 (37%)
III	11 (29%)
IV	11 (29%)

Patients and Methods

Patients. The study included 38 patients with a primarily detected colorectal tumor (age 33-89 years, mean 70.1) at different stages of malignancy, with distribution according to the UICC (International Union Against Cancer) classification, as shown in Table I, and 11 patients with benign disease of the colon (age 19-70 years, mean 45). Histologically there were acute forms of Crohn's disease and ulcerative colitis.

Tissue samples. The tumor tissues were obtained from 38 patients with colorectal carcinoma (group TUM). From 19 of these patients, normal colon tissue was obtained from areas between the resection line and the tumor itself (group NORM). Tissue samples from patients with benign disease of the colon comprised group BENIGN (n=11). All samples were histologically verified. Tissue samples were taken during surgery and, after surgical resection, these were frozen to -70°C and kept at this temperature until used.

Quantitative estimation of mRNA using RT real-time PCR.

RNA extractions: Total RNA was isolated from 100 mg of tissue using the RNeasy Total Isolation System Promega kit (Promega Corporation, USA). The amount of isolated total RNA was assessed spectrophotometrically and kept at -70°C.

Reverse-transcriptions: Three µg of isolated total RNA were used for reverse transcription (RT), which was performed with Superscript II Reverse Transcriptase (Life Technologies, USA) and oligo d(T)₂₁ as a primer.

Real-time polymerase chain reaction: Primers for MMP-2, MMP-7, TIMP-2 and TIMP-1 were developed in our laboratory (MMP-2: forward primer, 5'-CTTTGCTGGAGACAAATTCTGG-3'; reverse primer, 5'-GTATGGGACATTCCTCTGATCC-3'; reverse primer, 5'-CCAATGAATGAATGAATG GATG-3'. TIMP-2: forward primer, 5'-ATGCACATCACCTCTG TGA-3'; reverse primer, 5'-CTCTGTGACCCAGTCCATCC-3'. TIMP-1: forward primer, 5'-AGACCTACACTGTTGGCTG TGAG-3'; reverse primer, 5'-GACTGGAAGCCCTTTTCAGAG-3');, custom synthesis (GeneriBiotech, Czech Republic). Primers for GAPDH were synthesized as published in the manual of Corbett Research (7) (GAPDH: forward primer, 5'-GAAGATGGTG ATGGGATTC-3'; reverse primer, 5'-GAAGGTGAAGGTCG GAGT-3'). PCR fragments of MMP-2, TIMP-2 and GAPDH were inserted into the pGEM vector (Promega Corporation), cloned and sequenced using the ALFexpress sequencer (Pharmacia Biotech, Sweden). pGEM plasmids with cloned fragments of MMP-2, TIMP-2 and GAPDH were diluted to concentrations of 10³-10⁸ molecule copies per µl and used as standards (Figure 1). One µl of the RT product of each

Table II. The values of MMP-2, TIMP-2, MMP-7 and TIMP-1 mRNA levels of expression in control and colorectal tissue samples (Wilcoxon two-sample test).

mRNA	MMP-2/ GAPDH	TIMP-2/ GAPDH	MMP-7/ GAPDH	TIMP-1/ GAPDH
Group TUM range 25% - 75%	0 - 0.00035	0 - 0.0028	0 - 3.2560	0.00002 - 2.6
Group NORM+ BENIGN range 25% - 75%	0 - 0	0 - 0.0001	0 - 0	0 - 0.6665
Group TUM vs. Group NORM + BENIGN [p-value]	0.0005	0.0549	0.0007	0.0004

sample was used for real-time PCR. We performed real-time PCR using a Rotor-Gene apparatus (Corbett Research, Australia). DNA amplification was monitored with 0.5x Sybr-Green I (Molecular Probes, USA), which intercalates a double-strand DNA molecule. The size of the PCR amplification product for GAPDH was 226 bp, for MMP-2 163 bp, for MMP-7 180 bp, for TIMP-2 177 bp and for TIMP-1 130 bp. The specificity of the reaction was verified by the melting curve and agarose gel electrophoresis (Figures 2, 3). The quantification was performed as absolute (as number of DNA copies per sample). In all samples, GAPDH (housekeeping gene) expression was also assessed. The results are presented as relative values, ratio of the number of copies in the assessed gene (MMP-2, MMP-7, TIMP-2 or TIMP-1) and GAPDH.

Statistical analysis. The statistical analysis was performed by using SAS 6.2 software and the STATISTICA program. In the compared groups the test difference was calculated using the Wilcoxon Two-Sample Test. A p-value less than 0.05 was taken as statistically significant.

Results

The quantitative differences in levels of MMP-2, TIMP-2, MMP-7 and TIMP-1 mRNA expressions (MMP-2/GAPDH ratio) between group TUM, group NORM and group BENIGN were examined. Because it was found that the MMP-2, TIMP-2, MMP-7 and TIMP-1 mRNA levels were not significantly different in normal tissue samples between group NORM and group BENIGN ($p < 0.2231$, $p < 0.4615$, $p < 0.2691$, $p < 0.2932$) these samples were used as a control group. The MMP-2, TIMP-1 and MMP-7 mRNA levels were significantly elevated in tumor tissue samples (group TUM) in comparison to control tissue samples, group NORM + group BENIGN, ($p < 0.0005$, $p < 0.0004$, $p < 0.0007$). However, significant changes in TIMP-2 expression were found ($p < 0.0549$) (Table II).

Table III summarizes the presence of MMP-2, TIMP-2, MMP-7 and TIMP-1 mRNA in tumor tissue (group TUM) and in control colon tissue (group NORM + group BENIGN).

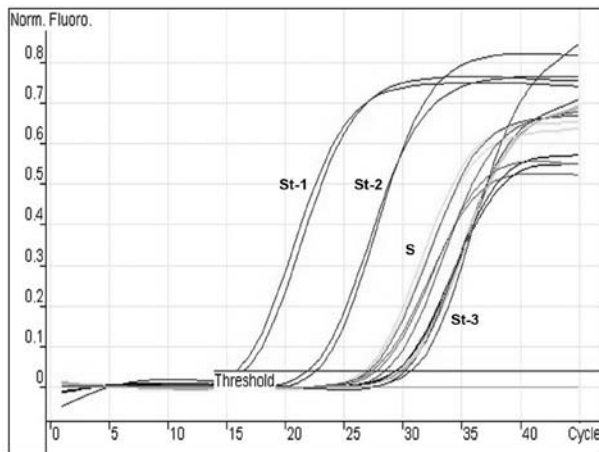


Figure 1. Amplification curves of the TIMP-2 gene in typical cases. St-1=standard 10⁹; St-2=standard 10⁶; St-1=standard 10³; S - samples.

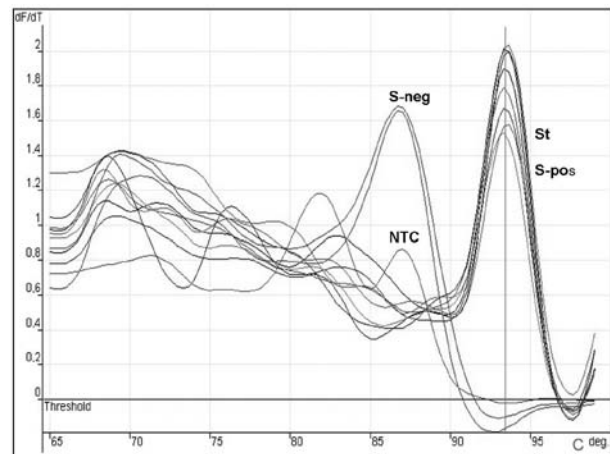


Figure 2. Melting curves of the TIMP-2 gene in typical cases. St=standards; S-pos = TIMP-2 - positive samples; S-neg = TIMP-2 - negative samples; NTC = no template control.

Table III. Relative frequencies of the assessed mRNA MMP-2, TIMP-2, MMP-7 and TIMP-1 parameters in samples of tumor tissue (group TUM) and non-tumor tissue (group NORM + group BENIGN).

Group		detected mRNA	not detected mRNA
	N	MMP-2 N (%)	MMP-2 N (%)
Group NORM + Group BENIGN	30	1 (3%)	29 (97%)
Group TUM	38	16 (42%)	22 (58%)
	N	TIMP-2 N (%)	TIMP-2 N (%)
Group NORM + Group BENIGN	30	8 (27%)	22 (73%)
Group TUM	38	21 (55%)	17 (45%)
	N	MMP-7 N (%)	MMP-7 N (%)
Group NORM + Group BENIGN	30	5 (17%)	25 (83%)
Group TUM	38	25 (66%)	13 (34%)
	N	TIMP-1 N (%)	TIMP-1 N (%)
Group NORM + Group BENIGN	30	14 (47%)	16 (53%)
Group TUM	38	35 (92%)	3 (8%)

MMP-2 mRNA was present in 42% (16 out of 38) of tumor tissue samples and was not detected in 58%. In control tissue, MMP-2 mRNA was only detected in 3% of cases (1 out of 30) (Table III). The presence of MMP-2 mRNA was significantly higher ($p < 0.003$) in tumor tissue than in non-tumor tissue

Table IV. The statistical values of presence of mRNA MMP-2, TIMP-2, MMP-7 and TIMP-1 in tumor colorectal tissue and in control groups (test difference between two proportions).

Test difference between two proportions	p-value			
	MMP-2	TIMP-2	MMP-7	TIMP-1
Group NORM + BENIGN vs. group TUM	$p < 0.003$	$p < 0.05$	$p < 0.0001$	$p < 0.0001$
Group NORM vs. group TUM	$p < 0.001$	$p < 0.04$	$p < 0.0002$	$p < 0.0001$
Group BENIGN vs. group TUM	$p < 0.04$	$p < 0.4$	$p < 0.0276$	$p < 0.0047$

(group NORM + group BENIGN). On comparing the presence of MMP-2 mRNA in the tumor tissue group to the healthy tissue (group NORM), a significant difference ($p < 0.001$) was found. A somewhat smaller, but still significant, difference ($p < 0.04$) was found between the tumor tissue (group TUM) and benign tissue (group BENIGN), as shown in Table IV.

TIMP-2 mRNA was present in 55% (21 out of 38) of tumor tissue samples and was not detected in the remainder. In the control tissue, TIMP-2 mRNA was detected in 27% (8 out of 30) (Table III). On assessing the presence of TIMP-2 mRNA in the tumor colorectal tissue (group TUM) in comparison with the control group (group NORM + group BENIGN), the significance was found to be marginal ($p < 0.05$). Comparison between the presence of TIMP-2 in tumor colorectal tissue (group TUM) and healthy colorectal tissue (group NORM) showed a statistically significant

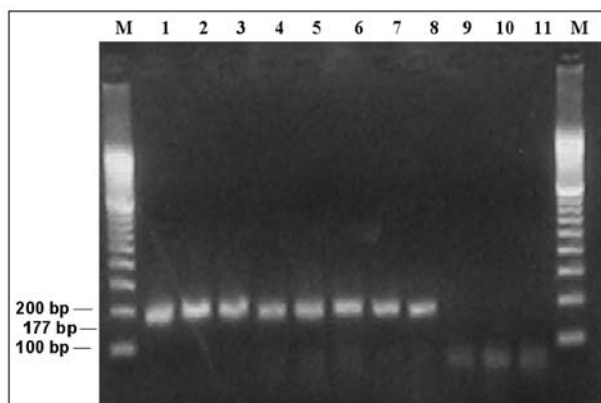


Figure 3. Agarose gel electrophoresis. M=marker; 1-3=standards; 4-8=TIMP-2 - positive samples (177 bp); 9, 10=negative samples; 11=NTC.

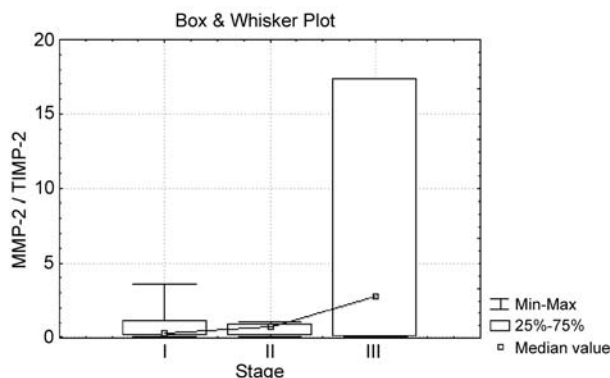


Figure 4. Ratio of MMP-2/TIMP-2 mRNA expressions in tumor colorectal tissues.

difference ($p < 0.04$), but in the tumor tissue (group TUM) and benign tissue (group BENIGN) no significant difference was found ($p < 0.4$) (see Table IV).

MMP-7 mRNA was present in 66% (25 out of 38) of tumor tissue samples and in 17% of the control tissue cases (5 out of 30) (Table III). The presence of MMP-7 mRNA was significantly higher ($p < 0.0001$) in tumor tissue than in non-tumor tissue (group NORM + group BENIGN). On contrasting the presence of MMP-7 in tumor colorectal tissue (group TUM) and healthy colorectal tissue (group NORM), a statistically significant difference ($p < 0.0002$) was recorded and a significant difference was also found for benign tissue (group BENIGN) ($p < 0.0276$) (Table IV).

TIMP-1 mRNA was present in 92% (35 out of 38) of tumor tissue samples and in 47% (14 out of 30) of control tissue samples (Table III). The presence of TIMP-1 mRNA was significantly higher ($p < 0.0001$) in tumor tissue than in non-tumor tissue (group NORM + group BENIGN). Comparison of the presence of MMP-2 mRNA in the tumor tissue group with the healthy tissue (group NORM) gave a significant difference ($p < 0.0001$). On comparison of the presence of TIMP-1 mRNA in the tumor tissue group to the benign tissue (group BENIGN), a significant difference ($p < 0.0047$) was found (Table IV).

The MMP-2/TIMP-2 ratio was correlated with the stage of the tumor disease. As shown in Figure 4, a higher stage of tumor correlated with a higher median value of MMP-2/TIMP-2 ratio (median stage II: 0.29, stage III: 0.69, stage IV: 2.71), but this was not statistically significant ($p < 0.58$).

Discussion

Colorectal carcinoma incidence and mortality in the Czech Republic is one of the highest in the world. One of the reasons is that more than 50% of tumors are diagnosed at an advanced stage of the disease (stage III and IV) because of

late diagnosis (8). This is reflected in our group of patients, where stages III and IV were represented in 60% of cases (23 out of 38 patients).

Assessment of MMP-2, MMP-7, TIMP-2 and TIMP-1 as protein in the serum and in the tissue or as mRNA in the tissue and in the lymph nodes, and their correlation with stage of disease, has been published in numerous studies, but the results are not completely unambiguous (15-20). We used quantitative RT PCR as the most sensitive method for the assessment of specific mRNA for detection of differences in the expression of genes between tumor tissue and control tissue.

In this study, the levels of MMP-2, MMP-7 and TIMP-1 mRNA expressions were significantly higher in tumor tissue than in non-tumor tissue. These results are in agreement with previous reports. Murashige *et al.* and Collins *et al.* reported that MMP-2 mRNA was overexpressed in colorectal cancer tissue and correlated with Dukes' stages (4, 11). Baker *et al.* and Murashige *et al.* reported that the levels of TIMP-1, MMP-2 and MMP-9 mRNA were significantly higher in primary colorectal cancers than in their adjacent normal tissues (1, 11), but Tutton *et al.* found no significant changes in mRNA expression of MMP-2 compared to normal tissue at any stage of colorectal cancer (14).

Whereas we did not register a significant difference in the level of TIMP-2 mRNA expression between the tumor and control tissue, Baker *et al.* observed that TIMP-2 expression was greater in the normal colon (1). We found a significant increase in MMP-7 mRNA in colorectal cancer, similar to Brabletz *et al.* who reported that MMP-7 is overexpressed in 80% of human colorectal cancers (2).

We showed that the presence of MMP-2, TIMP-2, MMP-7 and TIMP-1 mRNA was significantly higher in tumor tissue than in non-tumor tissue. While the mRNA for MMP-2 was detected in the colorectal cancer tissue only, the mRNA for TIMP-2, MMP-7 and TIMP-1 was also detected in non-tumor

tissue. We did not observe a significant difference in the presence of TIMP-2 mRNA between the colorectal cancer tissue and benign tissues ($p < 0.4$).

We proved that a higher stage of tumor approximates to a higher median value of the MMP-2/TIMP-2 ratio (median stage II: 0.29, stage III: 0.69, stage IV: 2.71). Collins *et al* reported that the MMP-2/TIMP-2 ratio was increased in tumor colorectal tissue compared to normal tissue (4). Ornstein and Cohn observed that the TIMP-2/MMP-2 ratio was 2-fold lower and the TIMP-2/MT1-MMP ratio was 1.5-fold lower in tumors compared to normal mucosa. These results suggest that the balance between genes that activate and inhibit MMP-2 is shifted towards activation in colon tumors (12).

The main function of MMPs is the removal of the ECM during tissue resorption, while individual MMPs have different, possibly contradictory, roles in angiogenesis. MMP-2 and MMP-7 have recently been shown to be capable of cleavage of plasminogen to form angiostatin, which specifically inhibits the proliferation of endothelial cells. TIMPs are the major endogenous regulators of MMP activities in the tissue, but TIMPs exhibit an additional biological function. TIMP-1 and TIMP-2 have mitogenic activity on a number of cell types, whereas overexpression of these inhibitors reduces tumor cell growth. These biological activities are independent of MMP-inhibitory activities. This suggests that the relationship between the expression of these genes and clinicopathological variables is complicated and further studies are needed.

The results of this pilot study show that the assessment of MMP-2, TIMP-2, MMP-7 and TIMP-1 mRNA might be helpful for the estimation of prognosis of colorectal cancer. Abnormal expression of these genes may also provide a useful target for new chemoprevention and adjuvant treatment strategies.

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