Verification Of Biological Characteristics Of Resection Lines Of Colorectal Carcinoma By Quantitative Expression Of MRNA CEA And TIMP-1

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Citation

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Abstract

Introduction: Colorectal carcinoma is a lifestyle-related disease with greater incidence in countries with a higher living standard. The standard surgical radical treatment requires removal of a segment of the colon containing the tumour with lymph nodes and vascular blood supply. Resections vary according to the location of cancer. However, according to the generally applied principle the aboral distance of the resection line from the tumour must not be less than 5 cm (or 2 cm in the case of rectal carcinomas) and the oral distance must be at least 10 cm. We opted for verification of biological characteristics of resection lines of colorectal carcinoma by determining mRNA of two different genes - mRNA of the carcinoembryonic antigen (CEA) and the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). Quantitative determination of mRNA of these genes was carried out with the aim to establish the extent to which expression of those genes, whose presence or increased expression is typical for tumour tissue, is present in colorectal carcinoma resection lines.

Methodology: The study includes 45 patients who underwent surgery for colorectal carcinoma. Colorectal carcinoma tissue and a sample of tissue between the resection line and the tumour were obtained from these patients. Total RNA was isolated from these samples, restricting transcription was carried out and the level of mRNA was determined applying the method of real-time PCR, CEA genes, TIMP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as gene housekeeping.

Results: We have identified a statistically significant difference in the levels of mRNA CEA expression between the tumour tissue and the resection lines, regardless of the tumour location ($p < 0.0001$). CEA was detected in 6 samples of the resection lines (6/45). CEA was hardly detected in the resection lines. We have also observed a statistically significant difference in the levels of TIMP-1 expression between the tumour tissue and the resection lines ($p < 0.0436$). However, TIMP-1 was detected in all resection line samples. Assessment of the levels of gene expression according to individual locations did not reveal any statistically significant differences.

Summary: We have nearly not detected the presence of CEA typical for tumour tissue in the resection line tissue. Although mRNA TIMP-1 was present in all resection line tissue samples, its levels throughout the colorectum were significantly lower than the levels in the tumour tissue.

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INTRODUCTION

Colorectal carcinoma is a lifestyle-related disease with greater incidence in countries with a higher living standard. It is the most common cancer of the gastrointestinal tract and

the second most frequent solid tumour in the Czech Republic – after lung carcinoma in men and breast carcinoma in women. The incidence of this disease increases with age and the fact that the Czech Republic is at the top of the worldwide charts for this disease in all age groups is very disturbing. Although colorectal carcinoma is easily accessible for examination, almost one half of patients seek medical advice in advanced stages of the disease with affected lymph nodes and the occurrence of distant metastases.

The standard surgical radical treatment or colorectal carcinoma requires radical removal of a segment of the colon containing the tumour with lymph nodes and vascular blood supply. Resections vary according to the location of cancer. However, according to the generally applied principle the aboral distance of the resection line from the tumour must not be less than 5 cm (or 2 cm in the case of rectal carcinomas) and the oral distance must be at least 10 cm. Vascular blood supply to the resection lines and removal of regional lymph nodes also need to be taken into account when deciding on the extent of a resection ([12]).

We opted for verification of biological characteristics of resection lines of colorectal carcinoma by determining mRNA of two different genes – mRNA of the carcinoembryonic antigen (CEA) and the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). Quantitative determination of mRNA of these genes was carried out with the aim to establish the extent to which expression of those genes, whose presence or increased expression is typical for tumour tissue, is present in colorectal carcinoma resection lines. Cerna et al observed increased expression of mRNA CEA in tumour colorectal tissue compared to the reference tissue sample ([4]). Some authors have previously studied the presence of tumour cells in lymph nodes and tumour tissue according to mRNA CEA ([10]). Furthermore, numerous authors have observed increased expression of TIMP-1 in tumour tissue compared to normal tissue ([1, 16, 24]). TIMP-1 is an inhibitor of matrix metalloproteinase, which contributes to the regulation of activity of certain matrix metalloproteinases. However, its mitogenic activity seems to prevail in tumour tissue ([19]).

PATIENTS AND METHODS

The study includes 45 patients (aged 40-79 years, median age: 63 years, 31 men and 14 women) with primarily detected colorectal carcinoma with histological verification in various stages of malignity and without the presence of metastases. The number of patients diagnosed with rectal carcinoma is 23 and the number of patients diagnosed with colon carcinoma is 22. Colorectal carcinoma tissue samples and aboral resection line tissue samples were obtained from these patients during surgical treatment. The collected samples were frozen at -70°C following the surgery and then processed using the real-time (RT PCR) method. Total RNA was isolated from 100 mg of individual tissue samples using the RNAgent Total Isolation System Promega kit (Promega Corporation, USA). 3 μg of the isolated RNA was used for reverse transcription (RT); oligo dT and the Superscript II reverse transcriptase (Life Technologies, USA) were used as a primer. 1 μl of cDNA obtained in this manner was used to quantify selected mRNA using the real-time PCR method and the Rotor-gene device (Corbett Research, Australia). The method including primer sequence is described in publications ([4, 19]).

Statistical processing was carried out by the so-called sign test, a statistical nonparametric test using paired data; p-values lower than 0.05 were considered statistically significant.

RESULTS

The existence of a statistically significant difference between the resection line and the tumour tissue when determining mRNA for TIMP 1 (p-value 0.0436) for colorectal carcinoma regardless of the location was confirmed (see table 1). However, a statistically significant difference between colorectal carcinoma samples and resection line samples in the case of rectal carcinomas when determining mRNA for TIMP-1 was not confirmed (p- value 0.1338) – see table 2. Similarly, no statistically significant difference between colorectal carcinoma samples and resection line samples in the case of colon carcinomas was observed when determining mRNA for TIMP-1 (p- value 0.2632) – see table 3. This contrast is contributed to the smaller number of samples in individual groups (samples of tumours located in the rectum and in the colon), while both groups combined produce a statistically significant number of samples. Nonetheless, the presence of TIMP-1 in the resection line was confirmed, although at lower levels.

Figure 1

Table 1: Levels of TIMP 1 - tumour tissue / resection line

	Tumour tissue	Resection line
Lower quartile (25%)	44903.00	5846.50
Upper quartile (75%)	265492.00	116870.75
p-value	0.0436	0.0436
Number of patients	45	45

Figure 2

Table 2: Levels of TIMP 1 - tumour tissue / resection line – rectum

	Tumour tissue	Resection line
Lower quartile (25%)	44903.00	1237.00
Upper quartile (75%)	284116.00	182992.00
p-value	0.1338	0.1338
Number of patients	23	23

Figure 3

Table 3: Levels of TIMP 1 - tumour tissue / resection line – colon

	Tumour tissue	Resection line
Lower quartile (25%)	41137.00	9937.00
Upper quartile (75%)	265492.00	112462.00
p-value	0.2632	0.2632
Number of patients	22	22

When determining the CEA, a statistically significant difference between the resection line and the tumour tissue was confirmed regardless of the location (p-value lower than 0.0001), proving the absence of tumour tissue from the resection line- see table 4.. Furthermore, a statistically significant difference between the resection line and the tumour tissue in rectal carcinomas was documented when determining the levels of CEA in tumours and resection lines (p-value 0.0005) – see table 5, and a statistically significant difference between the resection line and the tumour tissue in colon carcinomas was confirmed when determining the levels of CEA in tumours and resection lines (p-value 0.0042) – see table 6, which proves the absence of tumour tissue from the resection line in both groups. The levels recorded in the following tables show that mRNA for CEA is virtually non-existent in resection lines, unlike the levels of mRNA for TIMP-1.

Figure 4

Table 4: Levels of CEA - tumour tissue / resection line

	Tumour tissue	Resection line
Lower quartile (25%)	0	0
Upper quartile (75%)	25823.50	0
p-value	< 0.0001	< 0.0001
Number of patients	45	45

Figure 5

Table 5: Levels of CEA - tumour tissue / resection line - rectum

	Tumour tissue	Resection line
Lower quartile (25%)	0	0
Upper quartile (75%)	28518.50	0
p-value	< 0.0005	< 0.0005
Number of patients	23	23

Figure 6

Table 6: Levels of CEA - tumour tissue / edge of the resected tissue – colon

	Tumour tissue	Resection line
Lower quartile (25%)	0	0
Upper quartile (75%)	25823.50	0
p-value	< 0.0042	< 0.0042
Number of patients	22	22

There is no statistically significant difference between the levels of monitored markers (CEA, TIMP-1) in individual stages of cancer (p- value 0.7778 for CEA and 0.5684 for TIMP-1) – see table 7. Likewise, division of samples according to grading (p-value 0.3720 for TIMP-1 and 0.4608 for CEA), tumour size (T1-T4) (p-value 0.4828 for TIMP-1 and 0.2261 for CEA) or lymph node positivity (N0-N2) (p-value 0.5464 for TIMP-1 and 0.9274 for CEA) does not reveal any statistically significant differences.

Figure 7

Table 7: Levels of markers in individual stages of cancer

	Stage I	Stage II	Stage III	Stage IV	p-value
TIMP-1 Quartile 25%-75%	41137.00 – - 50066.50	45865.50 – - 284116.00	30421.25 – - 225186.00	140140.00 – - 356721.50	0.5684
NormTIMP 1 Quartile 25%-75%	0.7536 – - 3.5753	0.7641 – - 5.7205	0.9052 – - 10.2193	1.8974 – - 8.5134	0.8971
CEA Quartile 25%-75%	0 – 8235.50	0 – 28518.50	0 – 24393.00	7380.00 – - 9055.00	0.7778
Norm. CEA Quartile 25%-75%	0 – 0.0703	0 – 0.2687	0 – 0.4654	0.0297 – - 0.1589	0.7854
Number	10	21	12	2	

DISCUSSION

Carcinoembryonic antigen (CEA) or CACAM 5 (carcinoembryonic antigen related cell adhesion molecule 5) CEA described in 1965 is one of the tumour markers studied over the longest period of time ([7]). It is formed during foetal development and to a limited extent also in normal adult tissue. However, its considerable expression in tumours makes this antigen a very important marker.

CEA is a part of the immunoglobulin supergene family ([22], [23]). It is a glycoprotein with high contents of sugars (approximately 55%). Its molecular weight is 180–200 kDa. Sugar chains are attached to the polypeptide chain, which comprises seven Ig domains attached to the cell surface by a

phosphatidylinositol bond, by an N–acetylglucosamine bond to asparagine. However, the physiological function of CEA has not been explained completely. According to the generally accepted opinion derived from in vitro experiments these molecules are intercellular multifunctional molecules contributing to cell adhesion ([3], [25]). Some studies have shown that the N-domain of these molecules is responsible for the adhesion ([18], [20]).

CEA is present especially in the tissue of colon and rectum carcinomas. Its presence is linked to well-differentiated structures of epithelial cells of these tumours, the antigen being attached to the apical surface of the cells. Other GIT carcinomas producing CEA include carcinomas of the stomach, pancreas, oesophagus and biliary tract. The production of CEA occurs mainly in well or moderately differentiated tumours also in the case of these locations.

Tissue inhibitors of matrix metalloproteinases (TIMPs) are the main endogenous regulators of the activity of matrix metalloproteinases (MMPs) in tissues. MMPs play a role in migration of extracellular matrix cells through remodelling of the extracellular matrix (ECM), and cell proliferation, apoptosis or morphogenesis can occur through changes of the extracellular matrix induced by metalloproteinases. MMPs also play a role in the process of degradation of the ECM and BM in connection with tumour invasiveness ([5], [6], [9], [21]). The regulation of matrix metalloproteinases occurs at various levels – at the level of their transcription, their activation and through specific tissue inhibitors of metalloproteinases (TIMPs) ([2], [9]). Four tissue inhibitors of matrix metalloproteinases - TIMP-1, TIMP-2, TIMP-3 and TIMP-4 have been discovered so far. TIMP-2 forms specifically non-covalent bonds with pro-form MMP-2 and inhibits its enzymatic activity. TIMP-1 regulates the activity of MMP-7. Studies have shown that increased expression of TIMP-1 and TIMP-2 in vivo suppresses metastases , while numerous authors have documented increased expression of TIMP-1 in tumour tissue ([8],[11],[14],[17]).

The findings in this context show that expression of TIMP 1 in colorectal carcinomas is higher in tumours without documented lymphatic metastases than in tumours with metastases in lymph nodes ([15]). However, our study did not confirm this fact. This discrepancy may be due to the smaller number of patients monitored under this study.

Most authors have detected increased expression of mRNA for TIMP-1 in tumour tissue compared to normal tissue, although isolated studies contradict this claim. However, this variance may be due to the character of the so-called normal tissue, i.e. whether the comparison uses tissue from a healthy colon or colon tissue which is free of tumour cells but is obtained for example from a resection line. Our study confirmed the presence of TIMP-1 in the resection line, although the levels were lower than those in tumour tissue. We explain this phenomenon in view of the low recurrence rate in anastomoses carried out in our institute as a protective response of those colon cells that have not been affected by tumour, when the colon as an organ responds to the increased production of matrix metalloproteinases caused by the tumour by increasing the production of TIMP-1 because the level of TIMP-1 is increased not only in all colon cancers but also in inflammatory diseased of the colon ([13]).

The study confirmed that when the surgical principles for colon resection in cases of colorectal carcinoma are observed, the resection line does not show any signs of the presence of tumour cells – mRNA for CEA is not present.

The level of mRNA for TIMP-1 is present in the resection line at lower levels than in tumour tissue and this is due to the role TIMP-1 plays in the colon. Its level increases not only in all colon cancers but also in inflammatory diseased of the colon. The question whether the expression of mRNA for TIMP-1 is also increased outside the resection line will be subject to further research, as will be a potential comparison with samples of colon unaffected by a carcinoma or inflammation. Although the role of TIMP-1 as a prognostic marker remains a subject for further studies, all available studies suggest that the use of TIMP-1 in colorectal carcinoma screening is unsuitable due to the significant non-specificity of this marker.

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