Parameters of Biological Activity in Colorectal Cancer

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Abstract. *Background: The aim of this study was to measure several parameters in patients with early-stage colorectal cancer (CRC) and to evaluate them for their utility in routine clinical practice. Patients and Methods: Pre-operative serum levels of the following parameters were measured in 174 patients with CRC (clinical stage I-III): carcinoembryonic antigen (CEA), carbohydrate antigen CA 19-9, proliferative marker thymidine kinase (TK), tissue polypeptide antigen (TPA), tissue polypeptide-specific antigen (TPS), interleukin-6 (IL-6), interleukin-10 (IL-10), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), vascular endothelial growth factor (VEGF), C-peptide, insulin, adiponectin and leptin. The control group consisted of 50 patients who were undergoing a complete preventive medical examination and in these patients at the time of blood collection there was no evidence of any cancer disease. Results: Significant increase of the following parameters was found in patients with CRC: CEA, CA 19-9, TPA, IL-6, IL-10, TIMP-1, C-peptide, insulin and adiponectin. Only two of these, CA 19-9 and adiponectin, represent highly unfavorable prognostic factors. If elevated, they affect both progression-free interval and overall survival. Conclusion: Based on our results, we can conclude that none of the measured parameters fulfills the criteria for use for screening nor for primary diagnosis of CRC. Some of the parameters are important for prognosis estimate: Elevated CA 19-9 is related to an unfavorable prognosis, in terms of cancer recurrence and mortality rate. Angiogenetic factor VEGF represents a prognostic factor important for OS. CEA represents a parameter which is related to disease progression. Interleukins seem to be prospective complementary tumor markers. Adiponectin may be used for estimation of advanced stage of cancer and for estimate of risk of cancer recurrence.*

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Colorectal cancer (CRC) is considered as the most frequent cancer disease based on prevalence and mortality rates (1, 2). CRC represents 12.1% of all cancer cases in men and 13.7% in woman in the Czech Republic (3).

Most cases of CRC are diagnosed very late in the Czech Republic: 45% of cases in males and 47% in females are diagnosed in clinical stage III or IV. This is a very unsatisfactory situation, as earlier diagnosis allows better therapy outcome. The Czech Republic holds the first position in mortality for CRC worldwide (52.7 deaths per 100,000 inhabitants) (3).

Some experimental studies are involved in understanding the complex characteristics of carcinogenesis and of the metastatic cascade process (4). Detailed description of genes and their protein products (5) that affect the metastatic process are very important, particularly for new drugs and therapy approaches that would result in regulation of different phases of cellular mitosis and growth.

Many new laboratory tests and techniques such as multiplex methods or microarrays for early detection of CRC have been developed recently. Not many of them have been implemented for daily routine clinical practice as yet. The aim of this study was to measure a large spectrum of parameters in patients with non-mestastatic CRC and to evaluate their utility for routine practice, as they may eventually play a regulatory role in cancer development. Most of the parameters currently evaluated in relationship to cancer have already been associated with other clinical conditions (*e.g.* inflammation or autoimmune reactions). Therefore they are generally called parameters of biological activity instead of tumor markers.

Patients and Methods

Cancer patient group. A total of 174 patients with CRC operated on at the Surgical Department of Thomayer's Teaching Hospital in Prague and at the Department of Surgery, Teaching Hospital in Pilsen up to 2006 were included in order to obtain a full 2-year follow-up period. Inclusion criteria included clinical stage I-III (according to the UICC criteria) (6) and R0 resection (*i.e.* no residual tumor). Patients were monitored for the follow-up period at the Department of Oncology and Radiotherapy of Thomayer's Teaching Hospital in Prague and Teaching Hospital in Pilsen. Date of progression was established as the date of first clinical examination where relapse or progression was confirmed (using physical examination, laboratory assessment or imaging techniques). All of the patients were assessed for their current clinical status at the termination of the clinical study. Only 36 patients died during the study and only 24 patients had a progression. This can be explained by the fact that patients with clinical stage IV were excluded. All of the patients completed a 2-year follow-up period, some of them have even completed a 5-year period.

All the patients were assessed for medical history of metabolic diseases and cancer and the presence of different risk factors for CRC. Basic descriptive characteristics of the group of patients are shown in Table I. Serum samples were collected prior to surgery.

Control group. The control group consisted of 50 individuals who were undergoing a complete medical examination at the Department of Preventive Cardiology. At the time of blood collection, there was no evidence of any cancer disease. In order to rule out polymorbidity of these individuals, the mean age of this group is lower than the mean age of pathological group, but the body mass index (BMI) and other metabolic conditions were corresponding. Basic descriptive characteristics of the control group are also shown in Table I.

Laboratory analysis. Blood for the laboratory assessment was collected from the cubital vein in the morning after an 8-hour fasting period. Sera were separated by centrifugation at 1700× *g* 10 min and all specimens were separated into several aliquots, immediately deep frozen and stored at –75˚C until laboratory analysis. All the parameters were measured at the Department of Nuclear Medicine in Pilsen, using commercially available immunoassay kits. Most of the tests were also analyzed using multiplex analysis (xMAP technology using a Luminex 100 instrument; Luminex Corp., USA), therefore a large spectrum of tests were performed on every blood sample. The list of measured parameters is shown in Table II.

Additional routine biochemical laboratory tests were performed in order to rule out the presence of clinical conditions that may affect the serum levels of the measured parameters (*i.e.* serious renal or liver failure).

Statistical analysis. Statistical analysis was performed using Statistical Analysis Software (S.A.S.) release 8.02 and STATISTICA software, release 5.1. Basic descriptive statistic parameters for the total group of patients were estimated: mean, standard deviation, median, minimum and maximum. Wilcoxon two-sample test was used for comparison of distribution of individual parameters. Spearman's correlation coefficient was used for correlation between individual parameters. Sensitivities and specifities in the control group were calculated for optimal reference intervals (cut-off) for individual parameters. Overall survival (OS) and progression-free interval (PFS) were calculated using the Kaplan-Meier method.

Table I. *Basic descriptive statistics of pathological and control groups.*

		CRC N=174	Control N=50
Basic parameters			
Female	(N/%)	111/63.8%	23/46.0%
Male	(N/%)	63/36.2%	27/54.0%
Age (median)	(years)	66.00	50.00
BMI (median)	(kg/m^2)	27.08	28.48
Metabolic status			
Hyperlipoproteinemia	(N/%)	50/28.7%	27/54.0%
Diabetes mellitus	(N/%)	31/17.8%	0/0.0%
Hypertension	(N/%)	85/48.9%	19/38.0%
CRC location			
Colon	(N/%)	81/47.7%	-
Rectum and sigmoid	(N/%)	89/52.4%	-
CRC histology			
Adenocarcinoma	(N/%)	166/96.4%	-
Mucinous	(N/%)	5/2.9%	-

Results

Results of all the measured parameters are shown in Table III for both control and CRC patient groups. Correlations between the individual parameters were calculated for both groups separately. Statistically significant correlations are shown in Table IV. For prognosis estimation, the Cox regression model was used and the results for OS are shown in Table V and the results those for PFS is shown in Table VI.

This study was unique based on the number of patients included in this study in early-stage CRC. Most other published data include a high percentage of patients with metastatic CRC. This study is also unique from the point of the large spectrum of the measured parameters. It represents one of the first projects where most of the parameters were measured using multiplexed technology. Most of the data on multiplexed technology are related to ovarian, breast and prostate cancer (21-23). We found a significant increase of CEA in the CRC group. This parameter can be considered as a prognostic factor for PFS but not for OS. CA 19-9 was significantly elevated in the CRC group and is an unfavorable prognostic factor for both PFS and OS. The elevation of TPA may be related to cell mitosis and cytoskeleton disintegration. Elevated levels above cut-off significantly increase the risk for cancer recurrence. It seems that TPA is more specific for CRC than is TPS. Interleukins IL-6 and IL-10 were both significantly elevated in the group of CRC. This supports the theory of their potential inflammatory role in cancer

Table II. *Parameters used for laboratory analysis*.

Group	Parameter	Manufacturer	Method	Units
Oncofetal marker	CEA	Beckman Coulter	CLIA	ng/ml
Mucinous marker	CA 19-9	Beckman Coulter	CLIA	IU/ml
Cytokeratin marker	TPA	DiaSorin	IRMA	IU/l
	TPS	IDL Sweden	IRMA	IU/l
Adhesion molecules	I-CAM	Linco-Millipore	xMAP	ng/ml
	V-CAM	Linco-Millipore	xMAP	ng/ml
Interleukins	IL 6	Linco-Millipore	xMAP	pg/ml
	IL 10	Linco-Millipore	xMAP	pg/ml
MMP inhibitor	TIMP-1	R&D Systems	EIA	ng/ml
Metalloproteinase	MMP-9	Linco-Millipore	xMAP	ng/ml
Angiogenetic factor	VEGF	Linco-Millipore	xMAP	pg/ml
Metabolic parameters	C-peptide	Linco-Millipore	xMAP	pM
	Insulin	Linco-Millipore	xMAP	pM
	Adiponectin	Linco-Millipore	xMAP	µg/ml
	Leptin	Linco-Millipore	xMAP	pM

CLIA: Chemiluminescence immunoassay; EIA: enzyme immunoassay; IRMA: immunoradiometric assay; xMAP: multiplex assay.

Table III. *Comparison of parameters of biological activity between colorectal cancer patients (N=174) and control group of healthy individuals (N=50). Wilcoxon test*.

Parameter	Units	Control group N=50			Colorectal cancer group N=174			*p*-Value
		Mean	Std. dev.	95th percentile	Mean	Std. dev.	95th percentile	
CEA	ng/ml	1.92	±1.35	4.30	5.98	±12.58	24.10	0.0388
CA 19-9	IU/ml	8.08	±7.72	24.90	25.03	±60.41	96.10	0.0003
TK	IU/l	7.22	±4.05	18.30	6.84	±9.34	22.00	0.0021
TPA	IU/l	24.50	±38.37	57.00	61.87	±68.35	229.00	<0.0001
TPS	IU/l	51.26	±52.05	172.00	82.89	±127.14	248.00	0.0995
I-CAM	ng/ml	126.88	±44.42	228.45	119.58	±49.28	219.18	0.1566
V-CAM	ng/ml	1049.25	±213.43	1463.77	1051.65	±258.56	1504.91	0.9944
IL-6	pg/ml	3.71	±11.69	11.52	18.14	±25.11	74.84	<0.0001
IL-10	pg/ml	1.24	±1.55	3.46	10.27	±21.02	39.21	<0.0001
TIMP-1	ng/ml	145.01	±22.79	188.20	180.16	±51.99	282.00	<0.0001
MMP-9	ng/ml	468.62	±190.12	911.67	529.10	±290.74	1033.32	0.2646
VEGF	pg/ml	134.51	±83.73	324.02	209.43	±217.19	668.61	0.1665
C-Peptide	pM	273.24	±167.87	546.20	590.47	±1029.00	22.74	0.0087
Insulin	pM	73.55	±88.02	294.68	226.07	±625.90	1058.40	0.0138
Adiponectin	µg/ml	12.82	±4.90	25.00	17.61	±6.51	28.87	<0.0001
Leptin	pM	509.90	±357.73	1258.83	896.91	±1104.00	3897.58	0.4727

development. TIMP-1 was significantly elevated in the CRC group. This is related to its biological proliferative and anti-apoptotic activity. C-Peptide and insulin changes are related to insulin resistance and result in cellular proliferation and apoptosis inhibition. Our results from adiponectin related to the fact that only patients with early stage of CRC were included. Serum levels of adiponectin correlated with prognosis (PFS).

We did not find any significant changes of TPS, adhesion molecules, angiogenetic factors or leptin.

We did find significant differences between the control and CRC groups in correlations between the individual parameters: In the CRC group, there was a correlation between CEA and CA 19-9, TK and CA 19-9, TPA, TPS, TIMP-1 and C-peptide, *i.e.* between all the parameters that play an important role in the process of proliferation. No similar correlation was found in the control group. In the control group, correlations, between cytokeratines and adhesion molecules were found. Correlation between metabolic parameters was found in both groups. There was

Table IV. *Statistically significant correlations between individual parameters.*

Correlation	Colorectal cancer		Control group	
	r	*p*-Value	r	*p*-Value
CEA *vs*. CA 19-9	0.33762	0.0002		NS
CEA *vs*. ICAM	0.30048	0.0009		NS
CEA *vs*. C-Peptide		NS	0.40191	0.0042
TK *vs*. CA 19-9	0.33167	0.0032		NS
TK *vs*. TPA	0.62628	0.0003		NS
TK *vs*. TPS	0.47800	0.0001		NS
TK *vs*. TIMP	0.43420	0.0020		NS
TK *vs*. IL 10		NS	0.44551	0.0012
TK *vs*. C-Peptide	0.56949	0.0013		NS
TPA *vs*. TPS	0.41444	0.0002	0.67567	0.0001
TPA *vs*. VCAM		NS	0.51118	0.0001
TPS *vs*. VCAM		NS	0.61434	0.0001
ICAM *vs*. VCAM		NS	0.38724	0.0055
TIMP-1 *vs*. Leptin	0.40264	0.0074		NS
TIMP-1 *vs*. Insulin		NS	0.36812	0.0085
IL6 *vs*. Adiponectin	0.41313	0.0059		NS
IL 10 *vs*. Leptin		NS	0.47938	0.0004
Leptin *vs*. BMI	0.56952	0.0001	0.53677	0.0001
Leptin *vs*. C-Peptide	0.54461	0.0001		NS
Leptin *vs*. Insulin	0.51235	0.0001	0.37016	0.0081
C-Peptide *vs*. Insulin	0.69972	0.0001	0.54326	0.0001
C-Peptide *vs*. BMI	0.26885	0.0011	0.45591	0.0009
Insulin *vs*. BMI	0.27728	0.0007	0.49673	0.0002

Table V. *Cox regression model for overall survival (univariate analysis) arranged according to p-value.*

Parameter (unit)	Parameter estimate	Standard error	Hazard ratio	Chi-square	*p*-Value
N	0.6774	0.2009	1.969	11.3708	0.0007
T	0.6884	0.2416	1.991	8.1216	0.0044
Grading	0.6408	0.2313	1.898	7.6739	0.0056
CA 19-9 (IU/ml)	0.0037	0.0017	1.004	4.5078	0.0337
Age (years)	0.0422	0.0212	1.043	3.9646	0.0465

Table VI. *Cox regression model for progression-free interval (univarate analysis) arranged according to p-value.*

Parameter (unit)	Parameter estimate	Standard error	Hazard ratio	Chi-square	*p*-Value
Age (years)	–0.0681	0.2886	0.934	5.6434	0.0183
CA 19-9 (IU/ml)	0.0046	0.0020	1.005	5.5118	0.0189
CEA (ng/ml)	0.0264	0.0120	1.027	4.8419	0.0278
TPA (IU/ml)	0.0048	0.0022	1.005	4.7413	0.0294
Adiponectin (μg/ml)	–0.3561	0.1679	0.700	4.4973	0.0339
ICAM (ng/ml)	0.0095	0.0045	1.010	4.4430	0.0350

a significant correlation between leptin, C-peptide and insulin in the CRC group. More significant correlations were found between metabolic parameters and BMI in the control group. Based on our results, we can conclude that there are different mechanisms regulating cellular growth between the two groups.

When evaluating the sensitivities and specifities of the individual parameters, we found the best sensitivity to be for IL-10 (higher than 50% at 95% specificity). This could be potentially considered for use as a complementary marker. We found very low sensitivity for CEA and CA 19-9, even at 90% specificity. This fully corresponds to the literature data (19, 20).

Discussion

The article from Hundt *et al*. from 2007 provides a complete review of clinical trials published up to 2007 (7). From this review, it can be seen that there have been many different parameters of biological activity implemented for CRC. Different studies included different sizes of patient groups, different cut-offs of the parameters and different sensitivity and specificity. No official guidelines for the interpretation of such new tests have been issued as yet. Compared to this review article, our clinical study represents one of the largest based on both the size of the patient group and the large spectrum of the parameters. Many discussions have been already published on conventional tumor markers and CRC, therefore we focused on new parameters of the biological activity of tumors.

We found significantly higher serum levels of TIMP-1 in the CRC group. This may be explained by the fact that most of our patients were enrolled in the very early stages of CRC, when TIMP might predominate over the effect of MMP. Data from other clinical studies provide very inconsistent results. Similar results were obtained by Holten-Andersen in 2002 (8). Some authors claim a stimulatory effect of TIMP-1 on cellular growth (9, 10), others describe its inhibitory effects on apoptosis (11, 12).

Metabolic parameters have been studied for many years as risk factors for CRC development (13). The European Prospective Investigation into Cancer and Nutrition (EPIC) has confirmed that insulin resistance (defined by increased C-peptide serum levels) is associated with an increased risk for CRC development (14). Insulin regulates energy metabolism, stimulates cell proliferation and inhibits apoptosis. Elevated circulating serum levels of insulin result in increased bioactivity of insulin-like growth factor-1 (IGF-1) and a decrease in its binding proteins. There have only been few publications about the serum levels of insulin and C-peptide at the time of cancer development. We have found significant differences in the serum levels of insulin and C-peptide between the CRC and control group, but we found no relationship between elevated serum levels and PFS or OS.

Many publications have been dedicated to adiponectin serum level and its relationship to increased risk for CRC. It was proven that the lower the serum level of adiponectin, the higher the risk for CRC development (15). There are only few publications related to the serum level changes in patients with CRC. Our results show that patients with CRC have higher serum levels of adiponectin compared to the healthy control group. This appears to be slightly controversial compared with some literature data. Ferroni *et al*. from 2007 showed that low serum levels are found particularly in advanced CRC and this predicts an increased risk for disease recurrence (16). The median value of our control group corresponds to the median in this publication (12.4 *vs*. 13.1 μg/ml). Our pathological group of patients with CRC included only patients with early stage CRC; therefore, we also found a low recurrence rate in this group. We also found that the higher the serum level of adiponectin, the better the survival and the lower the risk for disease recurrence. Our finding corresponds to those from another study, where the authors found that adiponectin inhibits growth of cancer cells on tissue cultures *via* AMP-activated protein kinase activation (17). Therefore it seems that serum levels of adiponectin are important for perspective estimate and for estimate of disease stage, although such a test has not been performed routinely. Some publications on the dynamic changes of serum levels in the postoperative period have appeared recently. Low serum levels of adiponectin in the postoperative period increase the risk of postoperative inflammatory complications in CRC (18). There was very low incidence rate of inflammatory complications in own study and no serum levels were measured postoperatively.

Table IV shows all statistically significant correlations, where the *p*-value was lower than 0.01. There was no parameter with a correlation coefficient higher than 0.8000. Based on these results, we can conclude that no parameter could be replaced by another, but the existence of significant correlation supports the theory of a regulatory mechanism between the parameters. Correlation between the parameters of biological activity was found only in the CRC group. In particular, correlation between parameters related to the proliferative activity were found (TK *vs*. TPA, TK vs.TPS, TPA *vs*. TPS; see Table IV).

Better correlation between metabolic parameters was found in the healthy control group, but the correlation between leptin and C-peptide or insulin was more significant in the CRC group than in the control group. This is related to the insulin resistance and its potential role in CRC development.

Based on our results, we can conclude that none of the measured parameters fulfills the criteria for use for screening nor for primary diagnosis of CRC. Some of the parameters are important for prognosis estimate: Elevated CA 19-9 is related to an unfavorable prognosis, in terms of cancer recurrence and mortality rate. Angiogenetic factor VEGF represents a prognostic factor important for OS. CEA represents a parameter which is related to disease progression. Interleukins seem to be prospective complementary tumor marker. Adiponectin may be used for estimation of advanced stage of cancer and for estimate of risk of cancer recurrence.

During the past years, many new tests have been developed and introduced for experimental practice for early detection of CRC. But only few have been used for routine clinical practice to date. Knowledge about the biological activity of tumors could potentially help in the whole process from cancer prevention, through primary diagnosis to cancer therapy. It could help to individualize therapy choice at the time of primary diagnosis. This would enable the optimal

health care of the cancer patient. Using the knowledge of the biological activity of tumor in clinical practice should help to restore the natural balance between the stimulatory and inhibitory growth factors.

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