Differential expression of IGF-1 mRNA isoforms in colorectal carcinoma and normal colon tissue

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Abstract. The insulin-like growth factor (*IGF)-1* gene consists of 6 exons resulting in the expression of 6 variant forms of mRNA (IA, IB, IC, IIA, IIB and IIC) due to an alternative splicing. The mechanisms of *IGF-1* gene splicing and the role of local expression manifested by IGF-1 mRNA variants in colorectal carcinoma (CRC) have not been extensively investigated. Therefore, the aim of our study was to analyse the expression of IGF-1 mRNA isoforms [A, B, C, P1 (class I) and P2 (class II)], as well as the protein expression in CRC and control samples isolated from 28 patients. The expression of Ki-67 was also analysed and clinical data were obtained. For this purpose, we used quantitative real-time PCR (qPCR) and immunocytochemistry. The expression of mRNAs coding for all splicing isoforms of *IGF-1* was observed in every tissue sample studied, with a significantly lower expression noted in the CRC as compared to the control samples. The cytoplasmic expression of IGF-1 protein was found in 50% of the CRC and in ~40% of the non-tumor tissues; however, no significant quantitative inter-group differences were observed. The expression of the *IGF-1* gene in the 2 groups of tissues was controlled by the P1 and P2 promoters in a similar manner. No significant differences were detected in the expression of the IGF-1 A and B isoforms; however, their expression was significantly higher compared to that of isoform C. No significant differences were observed between the expression of Ki-67 mRNA in the CRC and control tissue even though the expression of the Ki-67 protein was higher in the CRC compared to the control samples. Ki-67 protein expression was associated with the macroscopic and microscopic aspects of CRC. A significant positive correlation was found between the local production of total mRNA and isoform A and the expression of Ki-67 mRNA, although only in the non-tumor tissues. In CRC samples, the local expression of the total IGF-1 mRNA and all splicing isoforms of IGF-1 mRNA decreased as compared to the normal colon tissues, although however, with conservation of both gene promoter activities and with the continued principal splicing IGF-1 mRNA isoforms.

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Introduction

The role of the insulin-like growth factor (IGF) system in carcinogenesis, including colorectal cancer (CRC) has been extensively described in the literature (1-4). The local overproduction of IGF-1 and IGF-2 has been found in 'endocrine-dependent' cancers (breast and prostate) and other tumors (lung, colon). This suggests that both proteins may play a role in neoplastic growth *in situ* (4-6). Epidemiological data have indicated that high serum concentrations of IGF-1 and low levels of the IFG binding protein 3 (IGF BP-3) are associated with an increased risk of several types of carcinoma (including colon) which are common in economically developed countries (3,4,7,8). Nevertheless, both earlier results (9) and recent prospective studies using meta-analysis have pointed toward a relatively modest positive association of CRC risk with serum IGF-1 concentration (10). Thus, the role of increased serum IGF-1 levels as key regulators of CRC risk seems controversial (2,3,9,10,11). New factors related to IGF-associated metabolic pathways are being investigated in order to determine the risk of CRC development and to prevent the development of colon cancer.

IGF-1 is a peptide growth factor which stimulates postnatal somatic growth and maintains lean tissue mass as an endocrine hormone. It has also been demonstrated to exert autocrine and paracrine effects in several tissues (12,13). In the circulation, over 90% of IGF-1 is bound to IGF BP-3 forming a 150 kDa complex which is not able to cross the vascular endothelium. By contrast, free IGF-1 is able to diffuse into tissues and perform its biological functions (13,14).

The *IGF-1* gene is present in the human genome as a single copy with 6 exons and 5 introns (15,16). The exons of *IGF-1* are alternatively spliced in multiple transcripts encoding specific blood-circulating and tissue-specific isoforms of the IGF-1 peptide (17,18). The alternative splicing regulates the expression of the 2 promoters, P1 or P2, which results in 2 different classes of IGF-1 isoforms, i.e., class I (1) and class II (2), (19,20). Furthermore, alternative splicing gives rise to at least 3 subsets of RNA transcripts at the 3' end of the gene, each encoding 3 distinct carboxy-terminal portions of the unique E-domain extension-peptide (E-peptide) as well as the 3'-untranslated region (3'UTR) (15,21-23). Exon 3 encodes parts of the signal peptide and the mature peptide which is common to all isoforms, while exon 4 encodes the rest of the mature peptide and the proximal part of the E-domain. The expression and composition of nucleotides in exons 5 and 6 determine the formation of isoform variants: A (Ea), B (Eb) and C (Ec) within classes I and II (17) (Fig. 1). The predominant transcript, IGF-1Ea, has exon 4 spliced directly to exon 6. The insertion of exon 5 results in 2 transcript variants in humans: IGF-1Ec (IGF-1b in rodents) [mechano growth factor, (MGF)] and IGF-1Eb (17,18,24). The variant designated as IGF-1Eb has only been observed in humans and it contains mRNA with exon 5 spliced to exon 4 (25). It comprises ~10% of the total *IGF-1* expression (24).

The expression of IGF-1 mRNA isoforms determines various isoforms of the IGF-1 protein (17,18). Peptide Ea which is composed of 35 amino acids is spliced out from the C terminal region of premature IGF-1 (i.e., pre-protein). It may be responsible for both mitogenic (26) and inhibitory effects on cancer cell growth (27). Furthermore, peptide Eb, which is composed of Eb1 and Eb2 peptides may exert mitogenic effects on IGF-1 independently; however, the activity of both peptides differs in various types of tissue (28). Peptide Ec (MGF) was originally identified in the liver, while higher amounts have been found in muscle following training (29,30). The isoform plays a critical role in the development, growth and repair of the skeletal muscle (18,29,30) and it protects cardiomyocytes from oxidative and hypertrophic stress via SirT1 activity (31). The mature molecule of IGF-1 is encoded exclusively by exons 3 and 4. IGF-1 secretion to the vascular bed is represented by the peptide variant of IGF-1Ea (23).

In mammals, the transcription of the *IGF-1* gene is controlled by 2 promoters (P1 and P2) which are located before exons 1 and 2, respectively (16,32). Furthermore, the P1 promoter initiates over 90% of transcripts in mammals (33). It is thought that the P2 promoter encodes the endocrine IGF-1 form which remains under the control of growth hormone (GH) (16). The biochemical mechanisms controlling the usage of *IGF-1* promoters remains unclear.

IGF-1 stimulates cell growth *in vitro* by the activation of proliferation and by the inhibition of apoptosis in cancer cells (2,34-36). The administration of recombinant human IGF-1 (rhIGF-1) has been shown to cause intensive cecal tumor growth, increased tumor mass and a higher rate of hepatic metastases (8). IGF-1 may be responsible for more invasive colon cancer with higher metastatic potential since IGF-1 causes the higher motility and migration of cancer cells and stimulates vascular endothelial growth factor (VEGF) expression (37,38). The complex role of the different IGF-1 isoforms in carcinogenesis has not yet been fully elucidated (39-41).

In the present study, we further investigated the tissue expression of total IGF-1 mRNA, IGF-1 mRNA isoforms (IGF-1A, IGF-1B and IGF-1C) and *IGF-1* gene promoters (P1 and P2). We demonstrate the differences between the CRC samples in comparison to normal colon tissue. The expression of both IGF-1 isoforms and Ki-67 was also compared to clinical data.

Materials and methods

Patients and tissue material. The examined CRC group included 28 patients (22 males, 6 females), 32 to 89 years of age (mean, 65±11 years) from the Department of General, Gastroenterological and Endocrinological Surgery, Poznan University of Medical Sciences in Poznań, who were diagnosed and subjected to surgery between September 2010 and September 2011. A total of 28 paired specimens of colorectal tumor and non-tumor tissues were obtained during surgical treatment. For CRC, colon mucosa and, depending on the depth of tumor invasion, submucosal layers ~15 cm from the tumor site served as the control tissues. No additional tissue was removed from the patients apart from that which is normally removed during surgical procedures. The tumor samples consisted of 1 adenocarcinoma *in situ*, 3 adenocarcinomas with *muscularis propria* invasion (pT2), 23 adenocarcinomas with *muscularis propria* invasion into pericolorectal tissue (pT3), and 1 adenocarcinoma with tumor penetration to the surface of the visceral peritoneum [pT4 in the tumor-node-metastasis (TNM) classification of the International Union Against Cancer]. Two patients had metastases to the liver. The locations of the colorectal tumors were divided into proximal colon (cecum, ascending and transverse colon) and distal colon (descending, sigmoid colon and rectum). Macroscopic types were divided into protruded type (height of tumor ≥3 mm) and flat type (height of tumor, <3 mm). The clinicopathological characteristics of the colorectal tumors are shown in Table I.

The tissue samples were stored in RNA Stabilization Solution (RNAlater®, Applied Biosystems) at -80˚C until use. Additionally, formalin-fixed paraffin-embedded tumor specimens of 28 CRC and fragments of the confirmed control specimens were obtained from patients. Histopathological analysis of the CRC samples confirmed the presence of 27 colonic adenocarcinomas (20 patients with G2, 7 patients with G3 grade), and 1 patient with colonic adenocarcinoma *in situ*. A total of 17 patients were of Dukes' C stage, 8 of Dukes' B stage, and 3 of Dukes' A stage (Table I). Informed consent was obtained from each subject, and the institutional review committee approved this study.

Immunocytochemical analyses. Tissue sections of 5 μm in thickness were deposited onto SuperFrost/Plus microscope slides. In order to qualify the material for the study, routine staining of the sections with H&E was performed. For detection and cellular localization of IGF-1 and Ki-67 antigen, anti-human mouse monoclonal antibodies (MAbs) specific for IGF-1 (R&D Systems; in dilution 1:500) and the human Ki-67 antigen (clone MIB-1) (DAKO, 1:2) were used, as well as the classical ABC (streptavidin-biotin-peroxidase complex), according to the methods described in the study by Hsu *et al* (42) and in our previous study (41). The preparations were

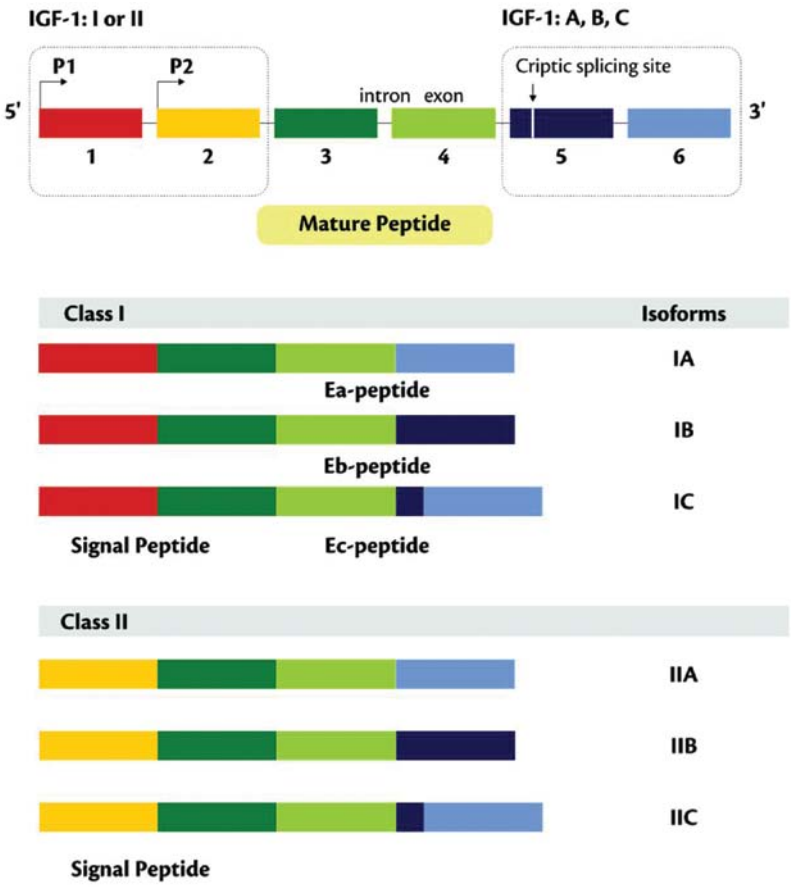

Figure 1. Structure of the human *IGF-1* gene, its mRNA isoforms generated by alternative splicing and encoded precursor peptides. The human *IGF-1* gene is composed of 6 exons. Transcription starts from one of the two promoters (P1 and P2) located in exon 1 and 2, respectively. Exons 1 and 2 are alternatively utilized and comprise class I and II, respectively. Exons 3 and 4 are expressed in all known isoforms. Exon 5 is normally absent (isoform A) but when is included, it forms isoform B or isoform C. mRNAs containing exon 4 spliced directly to exon 6 are designed also as Ea, those containing exon 5 spliced to exon 4 are designed as Eb. The IGF-1Ec splice variant is an exon 4-5-6 variant. All peptide products, derived from pro-IGF-1 are shown (17,18).

counterstained using hematoxylin. Each experiment included internal negative controls in which specific antibodies were substituted by sera of a respective species in 0.05 M Tris-HCl, pH 7.6, supplemented with 0.1% bovine albumin (BSA) and 15 mM sodium azide.

Semi-quantitative evaluation of IGF-1 protein and Ki-67 antigen expression. IGF-1 protein expression was analysed by 2 pathologists employing the semi-quantitative IRS scale, according to the method described by Remmele and Stegner (43), taking into account the number of positive cells (PP) and intensity of the colour reaction (SI). The final score represented a product of scores representing the 2 variables (PPxSI) and ranged from 0 to 12 points. The final value of the PPxSI product ranging between 1 and 2 points characterised a faint, 3-4 a moderate and 6-12 an intense immunocytochemical reaction. In each preparation 10 visual fields of an Olympus BH-2 light microscope were examined, under a x40 objective lens, and the mean scores were calculated.

The expression of Ki-67 proliferation-associated antigen (only clearly labelled cell nuclei were considered), was calculated taking into account the mean proportion of immunopositive cells in 10 light microscope fields. Expression was evaluated using the semi-quantitative scale of Gatter *et al* (44), in which the score of 1 corresponded to up to 10% positive cells; the scores of 2, 3 and 4 corresponded to 11-25, 26-50 and ≥51% positive cells, respectively.

Isolation of total RNA and synthesis of cDNA. The total RNA fraction was prepared using TRI Reagent® Solution (Applied Biosystems) and, it was then purified using the GeneMATRIX Universal RNA Purification kit (EURx). The quantity of total RNA was determined by optical density at 260 nm and its purity was estimated by 260/280 nm absorption ratio, which was consistently >1.8 (NanoDrop® ND-1000; ThermoScientific). RNA integrity was assessed by electrophoresis on a 1% agarose gel with ethidium bromide. All RNA samples were stored in H_2O at -80˚C until use.

Reverse transcription was carried out on 1.0 μg of total RNA following the manufacturer's instructions (First-strand cDNA synthesis kit, Fermentas). Template RNA, random hexamer primers (1 μl) and diethylpyrocarbonate (DEPC)-treated water were mixed together to a total volume of 11 μl and pre-incubated at 65˚C for 5 min in a Biometra thermocycler. The samples were then chilled on ice, centrifuged and moved to fresh PCR tubes in order to prevent permeability of the cup. Subsequently, 5X reaction buffer (4 μl), RiboLock™ RNase inhibitor 20 U/μl (1 μl), 10 mM dNTP mix (2 μl) and M-MuLV reverse transcriptase 20 U/μl (2 μl) were added to the pre-incubated solution, mixed by pipetting and incubated

Table I. Clinicopathological characteristics of the 28 patients with colorectal tumors.

Case	Age	Gender	Location	Macroscopic type	Microscopic type	T	N	M	Grading	Dukes' stage	Astler-Coller stage
1	89	M	P	F	3	3	1	0	3	C	C2
2	78	M	D	P	1	3	2	0	2	C	C2
3	53	M	P	P	1	3	0	0	2	B	B2
4	56	M	D	F	1	3	1	0	2	C	C2
5	75	M	D	P	0	0	0	0		A	A
6	56	F	D	P	1	3	1b	0	2	C	C2
7	68	M	P	P	2	3	2b	0	3	C	C2
8	77	M	P	P	2	3	2b	0	3	C	C2
9	69	M	D	P	2	3	0	0	2	B	B2
10	67	F	D	F	1	3	1	0	2	C	C2
11	51	M	P	P	3	2	1	0	2	C	C1
12	64	F	D	F	1	3	2	0	2	C	C2
13	32	M	P	P	1	3	1	0	2	C	C2
14	64	M	P	F	1	4b	0	0	2	C	C2
15	72	F	P	P	1	3	2	0	2	C	C2
16	58	M	D	F	1	3	1a	0	2	C	C2
17	72	M	D	F	1	2	0	0	2	A	B1
18	63	M	D	P	2	3	2b	0	3	C	C2
19	77	M	P	F	1	3	0	0	3	B	B2
20	55	M	D	F	2	3	1b	1	3	C	C2
21	74	M	P	F	2	3	1a	0	2	C	C2
22	52	M	D	F	1	3	0	0	2	B	B2
23	54	M	D	P	1	3	1a	0	2	C	C2
24	77	M	P	P	1	3	0	0	2	B	B1
25	73	M	D	F	3	2	0	0	2	A	B1
26	63	F	P	P	1	3	0	0	2	B	B2
27	65	F	D	F	2	3	0	1a	3	B	B2
28	75	M	D	P	3	3	0	0	2	B	B2

M, male; F, female. Location: P, proximal; D, distal. Macroscopic type: P, protruded; F, flat. Microscopic type: 0, adenocarcinoma *in situ*; 1, tubulous adenocarcinoma; 2, mucinous/partial mucinous adenocarcinoma; 3, tubulovillous adenocarcinoma.

at 42˚C for 60 min (Biometra thermocycler). Finally, cDNA was stored in H_2O at -20˚C or immediately used for subsequent amplification reactions.

Absolute quantification of mRNA copies

Preparation of standard curves for a copy number determination. cDNA of all IGF-1 isoforms (P1, P2, A, B, C, separately), (Fig. 1) was amplified using standard PCR and specific primers (Table II). PCR products were analysed on a 2% agarose gel in order to confirm their specificity and, then, all PCR products were purified separately using the GeneMATRIX PCR/DNA Clean-up purification kit (EURx). The concentration of each DNA was estimated by measuring the optical density at 260 nm (NanoDrop® ND-1000, ThermoScientific). The weight concentrations were converted to the corresponding DNA copy numbers using the Avogadro constant:

$$\text{DNA (copy)} = \frac{6.02\text{x}10^{23}\ (\text{copies mol}^{-1}) \text{ x DNA amount (g)}}{\text{DNA length (bp) x 660 (gmol}^{-1}\ \text{bp}^{-1})}$$

A 10-fold serial dilution of corresponding DNA and specific primers, ranging from $1\text{x}10^7$ to 10 copies per μl, were used to construct the standard curves. Threshold cycle (C_t) values in each dilution were measured in duplicates and were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated by a linear regression of the plotted points. From the slope of each curve, PCR amplification efficiency (*E*) was calculated according to the following equation: $E = 10^{-1/\text{slope}} - 1$.

Real-time PCR reaction. A total of 1 μl of a given cDNA or DNA was added to the reaction mixture, composed of 12.5 μl 2X Maxima® SYBR-Green/ROX qPCR Master mix (Fermentas), 1 μl specific pair of primer (f.c. 0.3 μM) and 10.5 μl H_2O. Primers for experiments on the expression of vault-related gene and reference genes were taken either from Koczorowska *et al* (40) or designed by ourselves (Table II) and purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

Table II. Primer sequences used in real-time PCR and PCR product sizes.

Target	NCBI accession no.	Product lenghth (bp)	Sequence
Class I (1) IGF-1 P1	NM_001111283	102	5'-CAGCAGTCTTCCAACCCA (F) 5'-CACAGCGCCAGGTAGAAGAGATGC (R)
Class II (2) IGF-1 P2	NM_001111284	101	5'-CACCTACAGTGAAGATGCACACC (F) 5'-CGTCTCCGGTCCAGCCGTGGC (R)
IGF-1 A	NM_001111284	144	5'-TCGTGGATGAGTGCTGCTTCCG (F) 5'-TCAAATGTACTTCCTTCTGGGTCTTG (R)
IGF-1 B	NM_001111285	141	5'-CTCGCTCTGTCCGTGCCC (F) 5'-CCCCTCCTTCTGTTCCCCTCC (R)
IGF-1 C	NM_001111283	126	5'-ACCAACAAGAACACGAAGTC (F) 5'-CATGTCACTCTTCACTCCTC (R)
IGF-1 total	NM_001111283 NM_001111284 NM_001111285	160	5'-CATGTCCTCCTCGCATCTCT (F) 5'-ATACCCTGTGGGCTTGTTGA (R)
Ki-67	NM_001145966	168	5'-CTGCTTGTTTGGAAGGGGTA (F) 5'-AGCCGTACAGGCTCATCAAT (R)
β-ACT	NM_001101	169	5'-TCTGGCACCACACCTTCTAC (F) 5'-GATAGCACAGCCTGGATAGC (R)
B2M	NM_004048	137	5'-CTCACGTCATCCAGCAGAGA (F) 5'-AAGACAAGTCTGAATGCTCCA (R)
GAPDH	NM_002046	199	5'-GAAGGTGAAGGTCGGAGTCA (F) 5'-CTGAGAACGGGAAGCTTGTC (R)
HPRT1	NM_000194	156	5'-CTGAGGATTTGGAAAGGGTG (F) 5'-AATCCAGCAGGTCAGCAAAG (R)
MRPL19	NM_014763	171	5'-ACTTTATAATCCTCGGGTC (F) 5'-ACTTTCAGCTCATTAACAG (R)

The reactions were driven in twin.tec real-time PCR plates with PCR Film (Eppendorf) using Mastercycler ep-realplex^2 (Eppendorf). The PCR program was as follows: i) initial denaturation, 95˚C, 10 min; (ii denaturation, 95°C, 15 sec; iii) annealing 60˚C, 30 sec; and iv) extension 72˚C, 30 sec. The number of cycles was 40-50. Melting curves were made and 2% agarose gel electrophoresis was used to verify the amplification product specificity and size, respectively. All samples were amplified in duplicate or triplicate and in the case when the results varied by >15% the reactions were repeated.

Absolute quantification using real-time PCR. The absolute quantification method was used to quantify mRNA copy numbers of all *IGF-1* mRNA isoforms (P1, P2, A, B, C, separately) (Fig. 1). Absolute quantification determines the exact copy concentration of a target gene by relating the C_t value to a standard curve. Prior to absolute quantification, the C_t values were normalised by comparison to the average of C_t's obtained for 5 housekeeping genes (*β-actin, B2M, GAPDH, HPRT1* and *MRLP19*). Evaluation of alterations in the expression of IGF-1 mRNA isoforms involved a comparison of mRNA copy numbers for those isoforms per μg RNA between tumor and control samples.

Statistical analysis. First, summary statistics were used to summarise the observations (arithmetic mean, standard deviation). Non-parametric statistical analysis of our data was carried out using the Friedman test for multiple dependent samples and the Mann-Whitney test for unpaired samples (tumor and control). Spearman's and Pearson's rank correlation was applied to examine the association between variables. Differences between percentages of IGF-1 mRNA isoforms in the tumor and control samples were assessed by the test for the comparison of fractions. Differences and correlations were assumed to be statistically significant at the level of $p<0.05$. Statistical analysis was performed using the Statistica PL v.10 software.

Results

Immunocytochemical localization of IGF-1 and Ki-67 proteins. Immunocytochemical analyses demonstrated the cellular expression of the IGF-1 protein in 14 out of 28 (50%) samples of CRC. Extensive individual differences were found in the intensity of the reaction, ranging from individual immunopositive cells to an intense, diffuse reaction in the majority of neoplastic cells. In non-tumor samples (control) positive IGF-1-cells were detected in 11 out of 28 (39%) samples. The expression was detected in a cytoplasm of both neoplastic cells manifesting a variable size and shape and normal cells of intestinal crypts and/or connective tissue stroma cells and individual cells of inflammatory infiltrates (Fig. 2A and B).

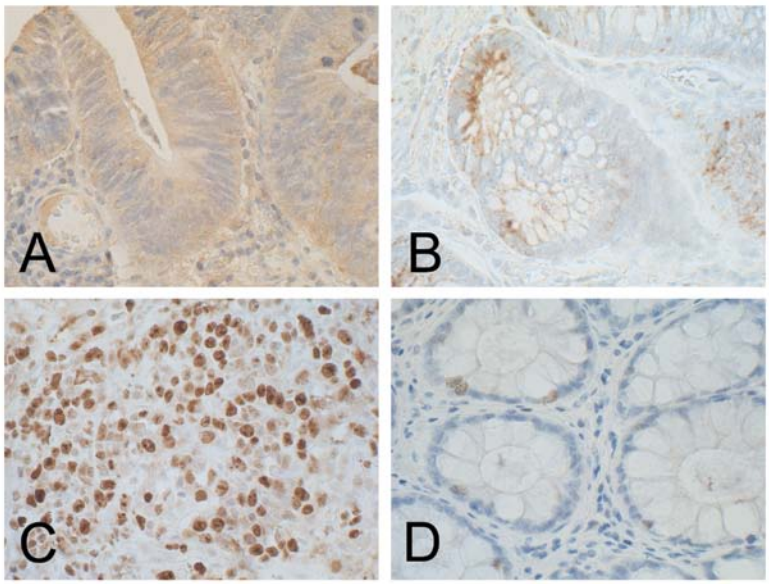

Figure 2. Cytoplasmic expression of IGF-1 protein in (A) a colorectal tissue sample and (B) in a control sample. Ki-67 protein expression in numerous cell nuclei in (C) a CRC sample and (D) in a control colon tissue sample. ABC method. Hematoxylin counterstaining. Objective magnification, x40.

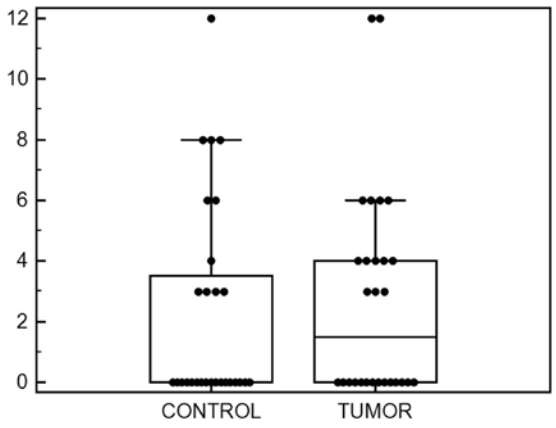

Figure 3. Expression of IGF-1 protein in non-tumor tissue (control), compared to CRC samples. Tissue expression was evaluated in the 12-point IRS scale, as described in Materials and methods (43); p>0.05.

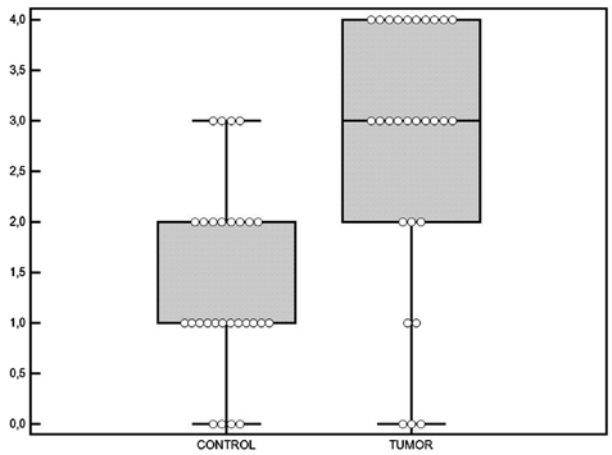

Figure 4. Expression of Ki-67 antigen in the control compared to tumor samples. Tissue expression was evaluated in the 4-point scale as described in Materials and methods (44); p<0.001.

In both groups of examined tissues, the expression of IGF-1 manifested a moderate intensity (with the mean below 3 points in the IRS scale) and no quantitative differences were observed between the control and CRC samples (2.29±3.41 vs. 2.75± 3.48, respectively) (Fig. 3). The nuclear expression of the Ki-67 antigen (Fig. 2C and 2D) was noted in 25 out of 28 (89%) CRC samples and in 24 out of 28 (86%) control tissue samples. A significantly higher expression of Ki-67 was detected in the CRC samples (2.79±1.32) in comparison to the control samples (1.43±0.92) (Fig. 4).

Expression of various IGF-1 mRNA isoforms. All known IGF-1 mRNA isoforms determined by class I (P1), class II (P2), A, B and C transcript expression were identified in the control and cancerous samples using the real-time PCR technique.

Non-tumor samples (control). In the non-tumor samples, statistically insignificant amounts of P1 and P2 mRNA isoforms were found (p>0.05) (Fig. 5). Among the A, B and C isoforms, both A and B (with no significant differences between them) demonstrated a higher expression when compared to isoform C (p<0.01, p<0.05, respectively) (Fig. 5).

CRC samples. Quantitative analysis of the tumor samples demonstrated no differences between the mRNA levels for P1 and P2 isoforms (p>0.05) in the cancer samples. However, when comparing the levels of A, B and C isoforms, isoform A was expressed on the same level as isoform B (p>0.05); however, both isoforms demonstrated a significantly higher expression when compared to isoform C (p<0.001 in both cases) (Fig. 6).

Quantitative analysis of expression of IGF-1 mRNA isoforms in control and CRC samples. The total expression of IGF-1 mRNA class I and class II determined by P1 and P2 promoters respectively, was significantly higher in the control samples when compared to CRC samples (Table III, Fig. 7).

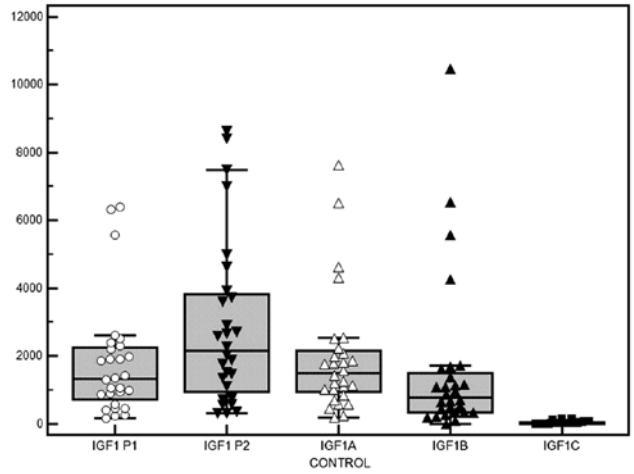

Figure 5. Comparison of quantitative expression involving P1 (class I), P2 (class II), A, B and C *IGF-1* mRNA isoforms within non-tumor tissues (control) (data are expressed as the number of mRNA copies/μg RNA); p>0.05 between P1 and P2; p>0.05 between A and B; p<0.01 between A and C; p<0.05 between B and C.

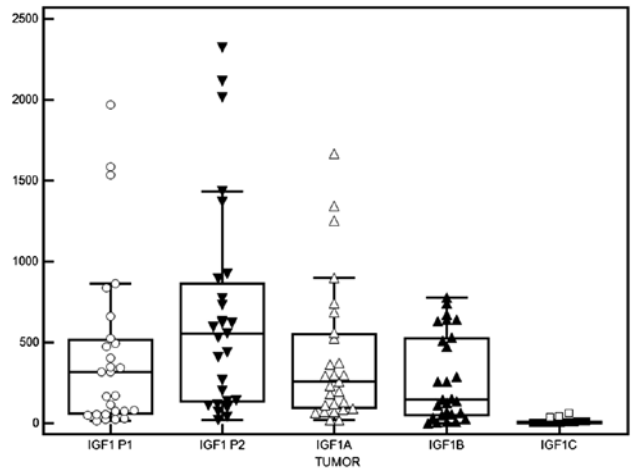

Figure 6. Comparison of quantitative expression involving P1 (class I), P2 (class II), A, B and C *IGF-1* mRNA isoforms within CRC tissues (tumor) (data are expressed as the number of mRNA copies/μg RNA); p>0.05 between P1 and P2; p>0.05 between A and B; p<0.001 between A and C; p<0.001 between B and C.

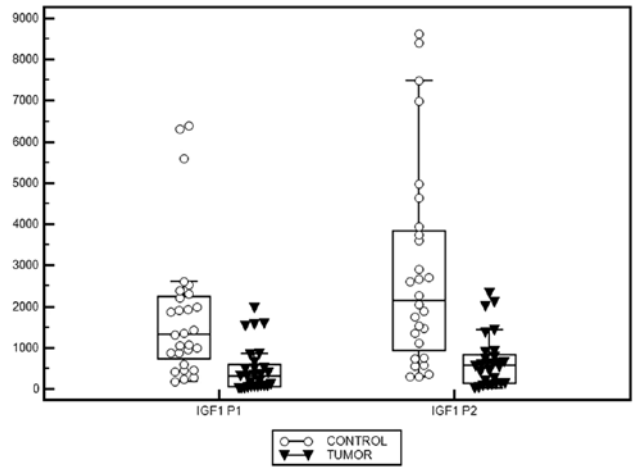

Figure 7. Comparison of quantitative expression involving classes I (P1 promoter) and II (P2 promoter) *IGF-1* mRNA isoforms within control and CRC tissues (tumor) (data are expressed as the number of mRNA copies/μg RNA); p<0.001 between P1 in the control and the tumor tissues; p<0.001 between expression of P2 in control and CRC.

Table III. Quantitative comparison between IGF-1 mRNA isoforms and Ki-67 mRNA (number of copies/μg of RNA) in patients with CRC (tumor) and in non-tumor tissue of the colon (control).

Type of mRNA	Group	Mean ± SD	P-value
IGF-1 P1	Tumor	472±556	<0.001
	Control	1806±1689	
IGF-1 P2	Tumor	673±640	<0.001
	Control	2870±2461	
IGF-1 A	Tumor	1243±4452	<0.001
	Control	1969±1787	
IGF-1 B	Tumor	267±267	<0.001
	Control	1606±2353	
IGF-1 C	Tumor	14±32	<0.001
	Control	40±41	
Ki-67	Tumor	189355±189788	>0.05
	Control	238523±189788	

P-value, level of significance.

Furthermore, all remaining isoforms, i.e. A, B, and C showed a significantly higher expression in the control compared to the CRC samples (Table III). This finding was supported by the expression of total IGF-1 mRNA which was also higher in the non-tumor samples (Fig. 8).

Analysis of *IGF-1* transcription showed no differences in promoter usage between the control and CRC samples. The almost equal contribution of P1 (61% in the control and 59% in the tumor samples) and P2 promoters (39% in the control and 41% in the CRC samples) was arbitrary assigned to 100% IGF-1 expression (Fig. 9).

Isoform A of IGF-1 mRNA was the predominant splicing isoform found in CRC samples (82%). Its expression was significantly higher than that of the isoform obtained for the control samples (55%) (p<0.03). On the other hand, the expression of IGF-1 B isoform was significantly higher (44%) in the control tissue when compared to the CRC samples (17%) (p<0.03). The expression of the IGF-1 C isoform was found at the lowest level representing ~1% participation compared to all isoforms analysed in various types of tissue (Fig. 10).

Expression of Ki-67 mRNA in control and CRC samples. No significant differences were found in the expression of Ki-67 mRNA between the control and CRC samples (Table III). Furthermore, no correlations were found between the mRNA and protein expression of Ki-67 either in the control or cancer

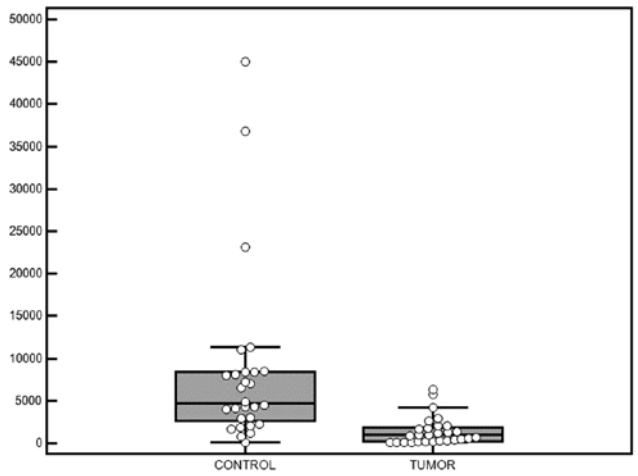

Figure 8. Expression of total IGF-1 mRNA in non-tumor tissues (control), compared to colorectal tumor tissue (data are expressed as the number of mRNA copies/μg RNA); p<0.001.

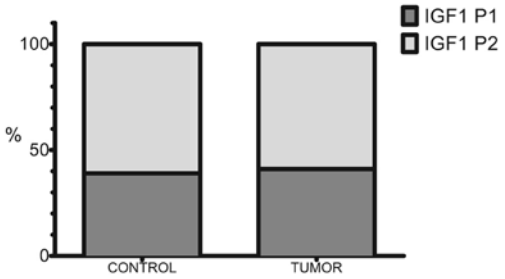

Figure 9. Quantification of P1 and P2 promoter activity in non-tumor samples (control), as compared to that of colorectal cancer samples (tumor). IGF-1 mRNA isoforms A, B and C expression was arbitrarily assigned to 100%.

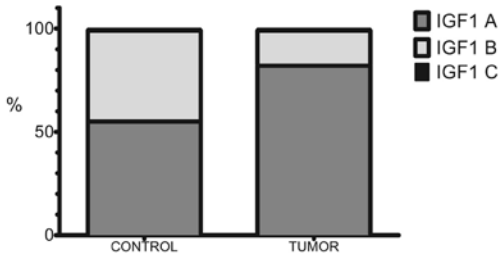

Figure 10. Quantification of IGF-1 mRNA isoform expression (IGF-1 A, IGF-1 B, and IGF-1 C) non-tumor samples, as compared to that of colorectal cancer samples (tumor). IGF-1 mRNA isoforms A, B and C expression was arbitrarily assigned to 100%. The predominant splicing isoform in CRC samples was IGF-1 A (82%). The proportion was significantly higher than proportion of the isoform in control samples (55%) (p<0.03). The expression of IGF-1 B isoform was significantly higher (44%) in control tissue as compared with CRC samples (17%) (p<0.03). The expression of IGF-1 C isoform has amounted to ~1% in both types of tissue examined (p>0.05).

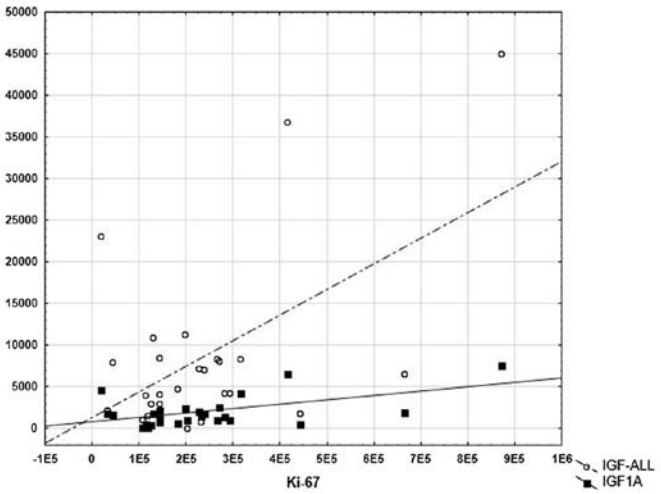

Figure 11. Linear Pearson's correlation between expression of total IGF-1 mRNA and its A isoform on one hand and Ki-67 mRNA on the other in non-tumor samples (r=0.550, r=0.542, respectively, p<0.05). E5, 10^5; E6, 10^6.

samples (r=0.002 for CRC, r=-0.201 for control samples; p>0.05 in both cases) (data not shown).

Correlation between reciprocal expression of different isoforms of IGF-1 mRNA. Highly significant Spearman's correlations were detected between the reciprocal expression of IGF-1 P1 mRNA and P2 mRNA (IGF-1 classes I and II, respectively), between the *IGF-1* P1 and A, B, C isoforms as well as between the *IGF-1* P2 transcript and A, B, C isoforms in the control samples (Table IV). The same significant Spearman's correlations were demonstrated in the cancer samples, except for the correlation between the *IGF-1* P1 promoter mRNA and isoform B mRNA (Table IV).

Correlation between total mRNA and protein expression of IGF-1. No significant correlations were found between immunocytochemically detected IGF-1 protein expression and amounts of total IGF-1 mRNA (r=-0.068 for control samples, r=0.149 for CRC; p>0.05 in both cases) (data not shown).

Correlation between total IGF-1 and Ki-67 mRNA expression. In non-tumor tissues (control) a direct Pearson's correlation was found between the total IGF-1 mRNA and Ki-67 mRNA expression (r=0.550, p<0.05) (Fig. 11). In the case of CRC samples Pearson's correlation between 2 statistical variables proved to be insignificant (r=0.169; p>0.05; data not shown).

Correlation between expression of IGF-1 and Ki-67 proteins. No significant correlations were found between the protein expression of IGF-1 and Ki-67 in any of analysed tissue groups (r=-0.200 for control samples, r=-0.067 for CRC; p>0.05 in both cases) (data not shown).

Correlation between IGF-1 expression (total mRNA, mRNA isoforms and protein) and clinicopathological data. We observed a faint positive correlation (r=0.375, p<0.05) between total *IGF-1* mRNA expression in the control samples and patient age. Apart from this, no significant correlation between the expression of IGF-1 (mRNA and protein) and the gender of the patients was observed in any of the groups (data not shown).

Table IV. Spearman's correlation between reciprocal expression of all examined mRNA isoforms and total IGF-1 mRNA in non-tumor samples (control) and colorectal cancer samples (tumor).

	IGF-1 P1		IGF-1 P2		IGF-1 A		IGF-1 B		IGF-1 C	
Isoform	Control	Tumor	Control	Tumor	Control	Tumor	Control	Tumor	Control	Tumor
IGF-1 P1	-	-	**0.581**	**0.718**	**0.899**	**0.940**	**0.389**	0.316	**0.796**	**0.892**
IGF-1 P2	**0.581**	**0.718**	-	-	**0.788**	**0.766**	**0.914**	**0.811**	**0.770**	**0.749**
IGF-1 A	**0.899**	**0.940**	**0.788**	**0.766**	-	-	**0.624**	**0.394**	**0.851**	**0.901**
IGF-1 B	**0.389**	**0.316**	**0.914**	**0.811**	**0.624**	**0.394**	-	-	**0.696**	**0.433**
IGF-1 C	**0.796**	**0.892**	**0.770**	**0.749**	**0.851**	**0.901**	**0.696**	**0.433**	-	-
IGF-1 total	**0.885**	**0.845**	**0.481**	**0.742**	**0.761**	**0.794**	0.297	**0.518**	**0.714**	**0.829**

Bold numbers denote Spearman's rank correlation coefficients r, at $p \leq 0.05$.

No correlation was observed between GF-1 expression (mRNA and protein) and tumor location, macroscopic and microscopic type of the tumor, grading or TNM classification ($p>0.05$ in all cases, data not shown).

Ki-67 (mRNA and protein) expression and clinicopathological data of CRC patients. A significantly higher expression of Ki-67 protein was found in tubulous adenocarcinoma (3.19±0.91) as compared with mucinous/partial mucinous adenocarcinoma (1.86±1.57) ($p<0.05$). A higher expression of Ki-67 was detected in the protruded macroscopic type of tumor (3.27±0.88) compared to the flat type of the tumor (2.23±1.54) ($p<0.05$). No significant differences were noted in the expression of Ki-67 protein in correlation with tumor location (proximal, distal, $p>0.05$) or grading ($p>0.05$) (data not shown).

No significant differences in Ki-67 mRNA expression were detected in correlation with the macroscopic or microscopic type of carcinoma, tumor localization, grading or TNM classification ($p>0.05$ in all cases) (data not shown).

Discussion

IGF-1 production in the liver demonstrates mainly endocrine activity while IGF-1 synthesis in other tissues acts in a para- and/or autocrine way (12,34). In experimental models of CRC an autocrine release was demonstrated in all IGF system components including IGF-1, suggesting that the IGF signalling pathway plays a role in colorectal carcinogenesis (1,45). The number of IGFs produced reflects the type of epithelial cells and the type of cellular growth (45). Various transcripts and variable IGF-1 precursor peptides act, first of all, as mitogenic factors but they may also inhibit the growth of neoplastic cells (17,18,26,27).

There remains a paucity of data on the local expression of various splicing isoforms of *IGF-1* mRNA in human tumors (39,40) including CRC (41). In this study, the expression of total mRNA and all splicing isoforms of *IGF-1* mRNA was observed in all the examined CRC and control samples. Existing data on the detection of IGF-1 mRNA vary between the absence of the transcript in CRC tissue (46), HT-29 and CaCo-2 cells (47), its detection in a proportion of cases (41-54% in CRC and 60-67% in control samples) (47-49) to its demonstration in all (100%) CRC and normal colon samples (50), which was supported by our results.

Our study demonstrated quantitative differences in the levels of the expression of IGF-1 total mRNA and its specific isoforms in individual CRC patients and control patients. Statistical analysis revealed a significantly lower mean expression of total IGF-1 mRNA and all its isoforms in CRC as compared to the control samples.

Studies on the level of IGF-1 mRNA expression which were obtained using various quantitative molecular techniques in CRC have also reported variable results (46,49-51). The lack of differences in the expression levels of IGF-1 mRNA in 10 colorectal sporadic cancers (Dukes' stage B or C) as well as in the epithelium scraped from normal colon mucosa was revealed by Michell *et al* using northern and western blot analysis (50). However, Tricoli *et al,* using a similar technique (northern blot analysis), reported that ~20% of the primary colon carcinomas showed higher IGF-1 mRNA expression (3- to 5-fold) while normal colonic mucosa demonstrated only background expression of this mRNA (51).

Nosho *et al* applied qPCR as we did, and demonstrated IGF-1 mRNA overexpression in ~54% of 90 human colorectal tumor tissues (49). The transcript was only faintly detected in adjacent non-tumor tissues. The expression of IGF-1 mRNA showed a positive correlation with histopathology but not with age, size, gender, location, or macroscopic type of tumor. In contrast to the patients examined by us, they exclusively analysed an early stage of CRC (adenomas and submucosal pT1 CRC) (49). Moreover, they examined total IGF-1 mRNA expression but not the expression of its isoforms and protein. On the other hand, the expression of IGF-1 mRNA evaluated by Jenkins *et al* was lower in CRC than in control samples (2.6×10^5 vs. 4.7×10^5 copy number/μg total RNA, respectively), which was similar to our results (47).

In interpreting the higher expression of IGF-1 mRNA in control samples, one cannot exclude the theoretical effect of the 'intraluminal' (i.e., dietary) environment, as well as systemic factors on the local production of IGF-1. Thus far, there are no detailed studies available on the regulation of the intestinal IGF system (11).

In our study, the detection of IGF-1 protein using the immunocytochemical approach revealed its almost equal distribution in the CRC and control samples (50% vs. ~40%, respectively) and no quantitative inter-group differences. The mean expression of the IGF-1 protein was low (below 3 points in the IRS scale) in both groups of tissues. Furthermore, the protein expression of IGF-1 did not correlate with total mRNA levels or individual isoforms expression in any of the analysed groups. A previous study using biochemical methods showed that the IGF-1 protein was present in small amounts and was equally distributed in 19 healthy and 10 malignant tissues (46). We were unable to confirm the results of Bustin *et al*, that samples which do not express IGF-1 mRNA have no IGF-1 peptide detectable by immunocytochemistry (48).

The comparative examination of specific IGF-1 mRNA isoforms in CRC samples revealed a novel insight since the local expression of *IGF-1* mRNA isoforms has not been previously examined in this type of tumor. Using identical primers, all known IGF-1 mRNA isoforms were identified in the genital human papillomavirus (HPV)-positive pre-cancerous, cancerous and HPV-negative control epithelial cells of the cervix (40). Studies have demonstrated the upregulation of all isoforms in pre-cancerous lesions when compared to cervical cancer and control tissues. Additionally, the expression of IGF-1 B isoform was higher in cervical cancer than in the control samples (14 and 8%, respectively) (40).

Isoform A of IGF-1 mRNA has prevailed over isoforms B and C (82, 17 and ~1%, respectively) in our study. However, the qPCR technique failed to confirm any significant differences between the expression of isoforms A and B, although both isoforms were significantly more abundant than isoform C of IGF-1 in CRC. Similarly, in the control tissues, even though the expression of isoform B was more pronounced compared to the CRC tissues (44 and 17%, respectively), the accurate quantitative analysis has failed to detect significant differences between the expression of isoforms A and B in the non-tumor tissues. In the CRC and the control samples, isoform C was at its lowest level, which was consistent with the results of Koczorowska *et al* (40). In contrast to our results, the greatest contribution of the P2 promoter in total IGF-1 expression was observed in the cervical cancer samples (31%), but the relative expression levels showed no significant differences in promoter usage between the analysed groups (40). In our CRC samples, the IGF-1 P2 contribution was quantified to 51%, similar to the control tissues (61%). Similar to the results of Koczorowska *et al* (40) no quantitative differences were detected in the mRNA expression manifested by the 2 promoters and, thus, the genomic expression of *IGF-1* seems to remain under the control of P1 and P2 promoters in CRC and in normal tissues of the large intestine. The positive correlation between mRNA IGF-1 P1 expression and A, B (only in control samples) and C and between mRNA P2 and A, B and C indicates that in CRC and non-tumor samples, a parallel expression exists between all the IGF-1 mRNA isoforms.

The results of our study demonstrated no significant differences in the amounts of total IGF-1 mRNA and its isoforms which could be associated with the location of adenocarcinoma, macroscopic or microscopic type of the tumor or the remaining clinical and pathological data. These results are in line with those of a previous study (49). We observed a poor positive correlation between the expression of total IGF-1 mRNA in the control samples and patient age. In other words, the local production of IGF-1 mRNA in the normal colon tissue seems to manifest an increasing tendency with progressing age. Moreover, a positive correlation was documented between the expression of total IGF-1 mRNA and the expression of isoform A and the elevated expression of Ki-67 mRNA in the control tissues. This may point to the role of local IGF-1 production in the stimulation of cell proliferation in the large intestine or to an augmented regenerative potential of normal colon tissues.

The insignificant correlation which we documented between the levels of IGF-1 and Ki-67 transcripts in CRC tissues reflects the analysis of an excessively uniform tissue material (grade 2 in 20 out of 28 patients) and extensive individual differences in the expression of Ki-67 mRNA. We were unable to corroborate the observations of Bustin *et al* concerning the correlation between tissue *IGF-1* mRNA levels and an augmented cell proliferation [examined in the study using the expression of proliferating cell nuclear antigen (PCNA)] in CRC (48).

Even though in our CRC and control tissues, no significant differences were observed between the expression of Ki-67 mRNA in the CRC and control tissue, the tumor samples manifested a significantly higher expression of Ki-67 protein as compared to the control samples. Moreover, a higher expression of the Ki-67 protein was measured in tubulous adenocarcinoma as compared to mucinous/partial mucinous carcinoma and in protruded carcinoma, as compared to the flat macroscopic type of tumor, pointing to a more pronounced cell proliferation in specific microscopic types of CRC. No correlation was observed between Ki-67 and tumor grading and/or CRC location (proximal, distal). No significant correlations were documented between the expression of IGF-1 protein and the expression of Ki-67 antigen in any of the groups examined.

A comparative examination of specific IGF-1 mRNA isoforms (P1, P2, A, B, C) in human CRC and normal colon tissues provided new data. The expression of total IGF-1 mRNA and all isoforms was documented in normal and malignant colorectal tissues. The expression of all transcripts was significantly decreased in CRC, as compared to the control samples. Positive correlations found between: i) mRNA expression of IGF-1 P1 promoter and both A and C isoforms; and ii) P2 and A, B and C isoform expression. This indicates that a parallel expression exists between all *IGF-1* mRNA isoforms in CRC samples. In the control colon tissue material examined, we were able to confirm the existence of a reciprocal correlation between all IGF-1 mRNA variants, as shown in a previous study (41).

Our immunocytochemical analyses confirmed the local cytoplasmic expression of mature IGF-1 protein in only 50% of patients with CRC and in ~40% of normal colon tissues. Statistical analysis failed to corroborate a significant correlation between the local production of total IGF-1 mRNA and its isoforms and the presence of mature IGF-1 protein in the analysed groups of tissues. The increased local production of total IGF-1 mRNA with the progressive age of patients and the positive correlation between the expression of total IGF-1 mRNA and isoform A on one hand and Ki-67 mRNA on the other, may provide evidence for an augmented proliferation and an increased regenerative potential in normal colon tissues.

The results of this study point toward an increased regenerative potential in normal colon tissues which, at least partially, is linked to an elevated expression of total IGF-1 mRNA and its isoform A. In CRC, the local expression of total IGF-1 mRNA and all splicing isoforms of IGF-1 mRNA decreased as compared to normal colon tissues, although however, with conservation of both gene promoter activities and with the continued principal splicing IGF-1 mRNA isoforms.

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