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Immunohistochemical expression and serum level of survivin protein in colorectal cancer patients

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Abstract. Survivin is one of the apoptosis-related inhibitors that is associated with a more aggressive behavior and a poor prognosis in numerous types of malignancies, including colorectal cancer (CRC). The objective of the present study was to perform immunohistochemical tissue analysis of survivin expression and serum analysis of survivin levels in CRC patients. The study group consisted of 55 CRC patients. Survivin expression was assessed by immunohistochemistry in 38 patients using monoclonal antibodies. Color reactions were observed in the nucleus and cytoplasm of the cancer cells. The expression was defined based on the H-score method. The level of survivin was determined using the enzyme-linked immunosorbent assay method. A positive immunoreaction was observed in the tumor tissues of 84.2% (32/38) of patients with CRC, consisting of nuclear (63.2%; 24/38) and cytoplasmic (81.6%; 31/38) expression. The survivin nuclear expression was associated with tumor mass location and the presence of distant metastases (P=0.048 and P=0.026, respectively). Survivin was detected in the sera of 38.2% (21/55) of CRC patients and in 81.8% (18/22) of healthy individuals. Serum protein levels were found to correlate with hematocrit (P=0.035), hemoglobin (P=0.008) and albumin (P=0.045), but not with any of the investigated clinicopathological parameters. The immunohistochemical positive reaction of survivin in the nuclei of cancer cells may condition their proliferative capacity, which is associated with higher risk of developing metastatic foci. Thus, the present study suggests that the expression of survivin may have diagnostic implications in cancer of the colon and thus requires further research. By contrast, the survivin serum level in CRC patients appears to be diagnostically ineffectual for clinical use.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the fourth most common cause of cancer-related mortality worldwide (1). Therefore, scientists have been searching for specific biomarkers that could be applied in the diagnosis and treatment monitoring of CRC patients. However, no such indices have yet been found. Neoplastic biomarkers that may have clinical implications in CRC patients have to go through a number of qualifying stages. At the initial stage, the neoplastic biomarker is usually detected and analyzed scientifically. In order to be clinically valuable, the biomarker has to be assessed in a large retrospective group and then confirmed through prospective randomized research (2). Only when all the aforementioned criteria have been fulfilled can the biomarker be used in clinical practice. The difficulty with obtaining tumor-specific markers is also associated with neoplasia complexity involving proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, angiogenesis, activating invasion and metastases (3). One of the major processes determining cell homeostasis and the development of the immune system is apoptosis, i.e., programmed cell death. In neoplastic diseases, the two signaling pathways are disturbed and the expression of the proteins involved is impaired. Apoptosis is induced via two main routes: The extrinsic or death receptor pathway and the intrinsic B-cell lymphoma 2-dependent pathway (4,5). Thus far, it has been confirmed that cancer cells of the colon show overexpression of FasR and FasL (6,7). Upregulation of FasR expression on cancer cells promotes compatible ligand binding on the surface of T lymphocytes, i.e., cells engaged in the immune response (8). Death domain receptors make the first link of the extrinsic apoptosis signal, activating caspase family proteins and inducing apoptosis. The process of programmed cell death is regulated at several stages by a number of inhibitor of apoptosis proteins (IAPs), of which the best known are livin, X-linked inhibitor of apoptosis (XIAP) and survivin (9).

Survivin is a 16.5-kDa protein consisting of an N-terminal Zn^{2+}-binding baculovirus inhibitor of apoptosis protein repeat domain connected with a 65 Å amphipathic C-terminal α-helix (10). It was initially believed that survivin, together with XIAP, regulated the caspase-dependent apoptotic

pathway, blocked the activity of caspases-3, -7 and -9, and caused their degradation. However, further research has confirmed that survivin does not have a specific motif to bind caspases, and only with the involvement of XIAP may it inhibit caspase-9 (11). Moreover, the protein takes part in the process of mitosis, forming a chromosomal passenger complex with aurora-B kinase, inner centromere protein and Borealin. In contrast to the other IAPs, survivin does not occur in normal differentiated tissue; its presence has been observed in transformed cell types and various types of cancer (12). The function of survivin may vary depending on its location in the cell. The expression of survivin in the nucleus may be responsible for cell proliferation control, while its presence in the cytoplasm regulates cell survival (13). Therefore, the study objective was the immunohistochemical assessment of survivin expression and its serum level in CRC patients.

Materials and methods

Patients. The study group consisted of 55 patients (20 women and 35 men) treated surgically in the Second Department of General and Gastroenterological Surgery in the Medical University of Białystok (Białystok, Poland). The patients ranged in age from 34-86 years (mean, 67.11±1.89 years). The pathological diagnosis confirmed CRC and its stage (tumor-node-metastasis) according to World Health Organization classification (14). Adenocarcinoma was diagnosed in 48 individuals, whereas adenocarcinoma with a mucous component was identified in 7 individuals. The investigated tumors were classified as moderately-differentiated (G2) in 48 patients and poorly-differentiated (G3) in 7 patients. A pT1 tumor was observed in 1 case, a pT2 tumor in 3 patients, a pT3 tumor in 49 patients and a pT4 tumor in 2 patients. At the time of the diagnosis, metastases to the local lymph nodes were observed in 29 out of 55 cases, whereas the presence of metastases to distant organs was noted in 25 out of the 55 studied cases. The control group consisted of 22 healthy volunteers (12 males and 10 females, aged 45-75 years).

Study material consisted of venous blood samples (6 ml) obtained from healthy controls and from the colorectal carcinoma patients prior to the surgery. Blood serum was stored at -80˚C immediately after centrifugation (704 x g) until assays were performed.

The study received the approval of the local Bioethics Committee (Nr. R-I-002-/40/2009). All the participants (study group and controls) signed informed consent forms prior to the examination.

Immunohistochemical assay. The immunohistochemistry (IHC) method was performed in 38 out of the 55 patients with CRC. Formalin-fixed and paraffin-embedded tissue specimens were cut on a microtome into 4-μm sections. The sections were deparaffinized in xylene and hydrated in alcohol (Chempur, Piekary Śląskie, Poland). To visualize the antigens of survivin, the sections were heated in a microwave oven (Laznie WLS084; Adverti, Lodz, Poland) for 20 min in a citrate buffer (pH, 6.0) (Sigma-Aldrich, St. Louis, MO, USA). Next, they were incubated with 3% hydrogen peroxide solution for 5 min in order to block endogenous peroxidase (Chempur). Incubation was then performed with polyclonal rabbit anti-human survivin antibody (cat. no. SAB4501459; dilution 1:200; Sigma-Aldrich) and incubated at room temperature for 60 min. The reaction was performed using the Novocastra Novolink Polymer Detection system (NCL; Novocastra; Leica Biosystems, Milton Keynes, UK). A color reaction for peroxidase was developed with chromogene diaminobenzidine. Positive and negative controls were performed according to the manufacturer's protocols (Novocastra; Leica Biosystems). Counterstaining was performed with hematoxylin.

Immunohistochemical staining was evaluated by two independent pathologists who were blinded to the clinical information. Protein expression was found in the nuclei and the cytoplasm of the tumor cells, and graded separately in an identical manner. Expression was determined using the semi-quantitative method and defined with regard to the intensity of staining (0, absent; 1, weak; 2, moderate; and 3, strong) and the percentage of positive tumor cells. The H-score was derived by summing up the percentages of cell staining at each intensity and then multiplied by the weighted intensity of staining. Score values ranged from 0-300. The study group was divided into negative cases (H-score <150) and positive cases (H-score >150).

Enzyme-linked immunosorbent assay (ELISA). Survivin level was analyzed in 55 patients using the Human Survivin Quantikine ELISA kit (cat no. DSV00; R&D Systems Inc., Minneapolis, MN, USA). Serum samples were prepared according to the manufacturer's protocols. Prior to the assay, the samples were 100-fold diluted with Calibrator Diluents. A monoclonal antibody specific for survivin had been pre-coated onto a microplate and incubated with the serum samples. After the first washing, an enzyme-linked polyclonal antibody specific for survivin was added to the wells. Following the second wash, a substrate solution was added. Next, the color development was stopped. All products were enclosed within ELISA kit. The reaction measurement was based on the intensity of the sample color and automatically measured using an Epoch Microplate Spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA) at an absorbance of 450 nm. The measurement of optical density was converted to survivin concentration (pg/ml) based on a standard curve, which was established using Gen5 Data Secure (BioTek Instruments, Inc.). All the specimens were assayed twice. No statistically significant differences were found between the measurements. The minimum detectable dose (MDD) of survivin ranged from 1.58-9.96 pg/ml. The mean MDD was 4.44 pg/ml.

Statistical analysis. Statistical analysis was conducted based on the STATISTICA 10.0 program (Dell Statistica, Tulsa, OK, USA). In order to compare the two groups, the Mann-Whitney U test was applied. Correlations between the parameters were calculated by the Pearson's correlation coefficient tests. $P<0.05$ was considered to indicate a statistically significant difference. The missing data was removed in pairs. The analysis of the receiver operating characteristic (ROC) curve was performed using MedCalc statistical software (MedCalc Software, Ostend, Belgium).

Results

Immunohistochemical localization of survivin in CRC tissues and its correlation with clinicopathological features.

Table I. Correlations between survivin expression in the tumor tissue and the clinicopathological parameters in patients with colorectal cancer.

	Survivin expression					
	Nuclear			Cytoplasmic		
Parameter	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value
Age, years			0.464			0.657
≤60	6 (15.8)	6 (15.8)		3 (7.9)	9 (23.7)	
>60	18 (47.4)	8 (21.1)		9 (23.7)	17 (44.7)	
Gender			0.587			0.337
Male	10 (26.3)	5 (13.2)		6 (15.8)	9 (23.7)	
Female	14 (36.8)	9 (23.7)		6 (15.8)	17 (44.7)	
Localization			0.048			0.838
Colon	18 (47.4)	6 (15.8)		8 (21.1)	16 (42.1)	
Rectum	6 (15.8)	8 (21.1)		4 (10.5)	10 (26.3)	
Adenocarcinoma type			0.156			0.293
Non-mucinous	10 (26.3)	5 (13.2)		6 (15.8)	9 (23.7)	
Mucinous	14 (36.8)	9 (23.7)		6 (15.8)	17 (44.7)	
Grade of malignancies			0.866			0.959
2	22 (57.9)	11 (28.9)		11 (28.9)	22 (57.9)	
3	3 (7.9)	2 (5.3)		2 (5.3)	3 (7.9)	
Tumor size, cm			0.446			0.958
<5	14 (36.8)	8 (21.0)		6 (15.8)	14 (36.8)	
>5	9 (23.7)	7 (18.4)		5 (13.2)	13 (34.2)	
pT stage			0.289			0.888
1	1 (2.6)	0 (0.0)		0 (0.0)	1 (2.6)	
2	0 (0.0)	4 (10.5)		1 (2.6)	3 (7.9)	
3	22 (57.9)	10 (26.3)		11 (28.9)	21 (55.3)	
4	1 (2.6)	0 (0.0)		0 (0.0)	1 (2.6)	
Lymph node metastasis			0.552			0.168
Absent	8 (21.0)	9 (23.7)		6 (15.8)	10 (26.3)	
Present	16 (42.1)	5 (13.2)		6 (15.8)	16 (42.1)	
Distant metastasis			0.026			0.546
Absent	17 (44.7)	5 (13.2)		9 (23.7)	13 (34.2)	
Present	7 (18.4)	9 (23.7)		3 (7.9)	13 (34.2)	

Data was analyzed by Pearson's correlation coefficient test. Missing data has been removed in pairs.

The expression of survivin was observed in the nuclei and cytoplasm of the tumor cells. A positive immunoreaction in the tumor tissue was observed in 84.2% (32/38) of patients with CRC, including nuclear expression in 63.2% (24/38) and cytoplasmic expression in 81.6% (31/38). Moreover, the detailed immunohistochemical analysis confirmed 23 subjects as Nuc^+/Cyt^+, 8 subjects as Nuc^-/Cyt^+, 6 subjects as Nuc^-/Cyt^- and 1 subject as Nuc^+/Cyt^- (Fig. 1). There was a positive correlation between the nuclear and cytoplasmic expression of survivin in the CRC tissues (P=0.020, R=0.374). The statistical analysis of survivin expression showed that a positive protein reaction in the tumor nuclei was associated with tumor mass localization and the presence of distant metastasis (P=0.048, R=0.323 and P=0.026, R=0.360, respectively) (Table I). The nuclear immunoreactivity of survivin was frequent in tumors located in the rectum and in cases with distant metastasis.

Serum survivin level in CRC and its correlation with clinico-pathological and morphological blood parameters. Survivin antibodies were detected in the sera of 38.2% (21/55) of CRC patients and in 81.8% (18/22) of healthy individuals. The positive cases were defined as having a protein level >0 pg/ml. The mean value of survivin levels in the sera of the patients with CRC was 1.844 ng/ml (range, 0-11.746 ng/ml), which was significantly lower than that in the healthy control group (mean, 4.954; range, 0-23.415 ng/ml) (P=0.016) (Fig. 2). Moreover, positive survivin concentration was observed in the sera

Table II. Correlations between the survivin levels in serum and the clinicopathological parameters in patients with colorectal cancer.

		Survivin (pg/ml)			
Parameter	n	Mean	Range	Coefficient	P-value
Age, years					
≤60	19	1.402	0.000-7.856	0.081	0.412
>60	36	2.116	0.000-23.415		
Gender					
Male	35	1.969	0.000-23.415	0.028	0.304
Female	20	1.723	0.000-10.449		
Localization					
Colon	33	1.616	0.000-11.746	0.040	0.132
Rectum	22	1.946	0.000-23.415		
Adenocarcinoma type					
Non-mucinous	48	1.727	0.000-23.415	0.014	0.981
Mucinous	7	1.896	0.000-7.856		
Grade of malignancies					
2	48	1.759	0.000-23.415	-0.072	0.984
3	7	0.793	0.000-3.967		
Tumor size, cm					
<5	20	2.102	0.000-23.415	-0.134	0.682
>5	18	1.003	0.000-7.856		
pT stage					
1	1	0.000	0.000	0.058	0.661
2	3	0.000	0.000		
3	49	1.817	0.000-23.415		
4	2	0.686	0.000-0.970		
Lymph node metastasis					
Absent	17	1.271	0.000-11.746	0.101	0.897
Present	21	2.085	0.000-23.415		
Distant metastasis					
Absent	30	1.060	0.000-7.856	0.221	0.892
Present	25	2.901	0.000-23.415		
IHC expression of survivin in cancer cells					
Nuc^+/Cyt^+	23	2.010	0.000-0.856	-0.310	0.440
Nuc^+/Cyt^-	1	0.000	0.000		
Nuc^-/Cyt^-	6	0.025	0.000-0.077		
Nuc^-/Cyt^+	8	0.228	0.000-1.373		

Pearson's correlation coefficient test. Missing data has been removed in pairs. Nuc, nuclear expression, Cyt, cytoplasmic expression.

of T3 and T4 CRC patients. The results are shown in Table II. The sensitivity and specificity of the serum survivin level were 57 and 82.6%, at a cutoff value of 13.042 pg/ml. The area under the ROC curve (AUC) for survivin showed that the protein exhibited moderate diagnostic power (AUC, 0.729; P=0.0002).

Survivin level in the sera of the CRC patients was not found to correlate with any clinicopathological parameters (Table II). However, the serum protein level was associated with the morphological blood parameters as follows: Hematocrit (P=0.035, R=0.404), hemoglobin (P=0.008, R=0.487) and albumin (P=0.045, R=-0.477) (Table III).

Discussion

Survivin is overexpressed in malignancies located in organs such as the lungs, liver, ovaries, stomach, breasts and prostate (12). The present study observed the positive expression of survivin in 84.2% of CRC patients. These findings are

Table III. Correlations between the level of survivin protein in the serum and the morphological blood parameters.

	Survivin		
Parameter	n	Coefficient	P-value
Red blood cell count	29	0.262	0.177
White blood cell count	29	-0.199	0.309
Platelets	29	-0.190	0.333
Hematocrit	29	0.404	0.035
Hemoglobin	29	0.487	0.008
Sodium	29	-0.038	<0.01
Potassium	29	0.063	<0.01
Prothrombin time	29	-0.083	0.678
INR	28	0.022	0.916
Total proteins	20	-0.290	0.228
Albumin	18	-0.477	0.045
Aspartate transaminase	22	0.065	0.780
Alanine transaminase	22	0.166	0.471
Glucose	13	-0.316	0.292
Urea	26	-0.159	0.447
Creatinine	23	-0.144	0.523

Blood parameters were not measured in all 55 patients. Pearson's correlation coefficient test. Missing data has been removed in pairs. INR, international normalized ratio.

consistent with the observations reported in the studies by Kalliakmanis *et al* (15), and Choi and Chang (16), which noted the positive expression of survivin in 88.3 and 83.3% of CRC cases, respectively. Hernandez *et al* (17) confirmed the presence of this protein in 93% of patients with colon cancer, while Xi *et al* (18) observed its expression in 60.7% of CRC patients. Survivin has different functions depending on its location. In the nucleus, the protein regulates cell growth, whereas its distribution to the cytoplasm determines the viability of cancer cells (13). In the present study, the positive expression of survivin was more common in the cell cytoplasm (81.5% of cases) compared with the cell nucleus (63.1% of cases) in the CRC patients. Ponnelle *et al* (19) also used IHC and confirmed a higher incidence of positive survivin expression in the cytoplasm (41% of cases) compared with its localization in the nucleus (39%) of colon adenocarcinoma cells. The data are, however, inconsistent with the results published in studies by Shintani *et al* (20) and Qi *et al* (21), which found that positive survivin expression was more frequent in the nucleus than in the cytoplasm of cancer cells in CRC patients.

Survivin as an apoptotic protein may condition tumor aggressiveness and tumor cell invasiveness, including lymph node involvement and distant metastases (22). It has been shown that survivin may determine, via different signaling pathways, the capacity of prostate and breast cancer cells to metastasize (22,23). Single studies have confirmed this potential of survivin in CRC patients. Chu *et al* (24) and Xiaoyuan *et al* (25) observed a positive correlation between survivin overexpression and lymph node metastasis in CRC patients. Furthermore, Shen *et al* (26) found that recombinant adenovirus reduced survivin expression and when linked with fluorouracil, blocked the cancer cell metastasis of CRC. Lymph node involvement is associated with a poor prognosis in CRC patients, since the risk of invasion into distant organs

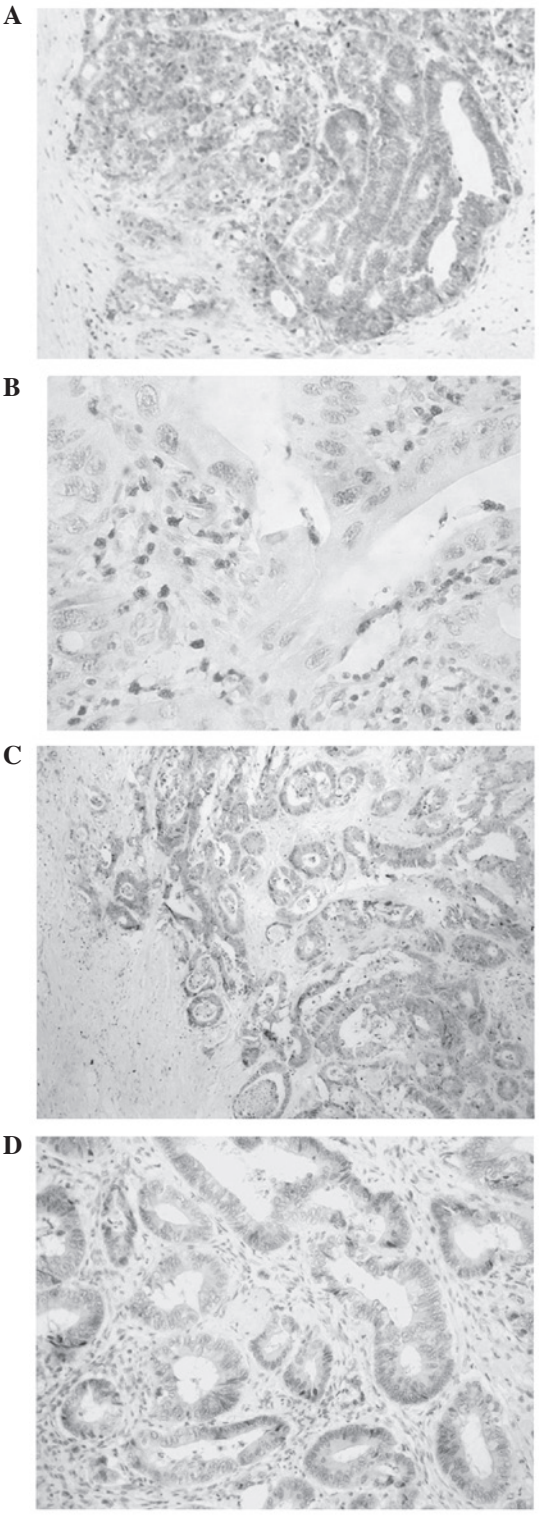

Figure 1. Immunohistochemical expression of survivin in CRC tissues. (A) Positive nuclear and cytoplasmic reactions for survivin were observed in 23 cases (magnification, x20). (B) Positive expression in the tumor nuclei only was found in only 1 case (magnification, x40), (C) whereas a cytoplasmic reaction only was present in 8 cases (magnification, x20). (D) No survivin expression was observed in 6 cases (magnification, x40).

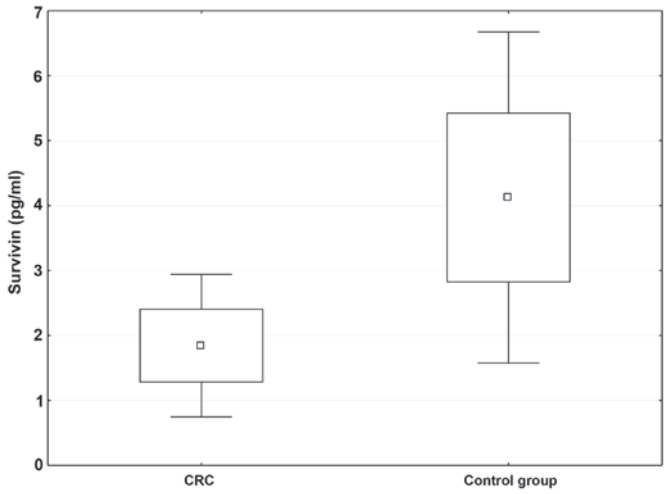

Figure 2. Survivin levels in the sera of colorectal cancer patients and healthy controls. Mann-Whitney U test. Missing data has been removed in pairs.

is increased. Li *et al* (27) noted that the immunohistochemical reaction of survivin was associated with the presence of metastases and disease relapse in CRC patients. Moreover, Lee *et al* (28) showed that the positive expression of survivin was closely associated with primary tumor and distant metastasis categories, and with tumor stage. The present detailed analysis revealed a positive correlation between survivin located in the nucleus of cancer cells in CRC patients and distant metastases and tumor location. The findings contradict those reported in the study by Qi *et al* (21), which noted that nuclear survivin expression was associated with a lower incidence of distant metastasis. Nuclear survivin location is responsible for cell cycle progression. It has been proven that the nuclear overexpression of survivin increases the activity of cell growth and the passage of the cells to the S phase of the cell cycle, and results in a decline in the percentage of the G0/G1 phase cells (29). The positive nuclear survivin expression in CRC cells appears to indicate the growth of tumor mitotic activity and increases the risk of developing metastatic foci, including those to distant organs.

The serum level of survivin was measured in CRC patients using ELISA in the present study. The protein was found only in ~38% of CRC cases and in 81% of healthy subjects. Moreover, the protein level was statistically significantly lower in the CRC patients than in the healthy controls. Yagihashi *et al* (30) assessed serum survivin in various cancers of the digestive system. Survivin antibodies were noted in 39.7% of all cancers studied, including 33% cases of CRC, hepatoma and esophageal cancer, 40% of gastric cancer cases, 42% of pancreatic ductal adenocarcinoma cases and 43% of biliary-tract cancer cases. Similar results were reported in the study by Wang *et al* (31), which confirmed the presence of survivin in the sera of 31.5% of CRC patients. In turn, Rohayen *et al* (32) observed purified recombinant survivin reactions only in 8.2% of CRC patients. The present study failed to confirm any correlations between serum survivin level and the chosen clinicopathological parameters in the CRC patients. However, the serum level of survivin in the CRC patients was found to positively correlate with hematocrit and hemoglobin in the peripheral blood, and negatively correlate with albumin level. The aforementioned observations, together with the present findings, suggest that the serum level of survivin in various digestive system malignancies, particularly CRC, exhibits little diagnostic value and will not have any clinical implication in the future.

In conclusion, the immunohistochemical assessment of survivin in the tissues of CRC patients revealed a major role of protein localization in cancer cells. A positive reaction for survivin in cancer cell nuclei may condition their proliferative potential, which is associated with a higher risk of developing metastatic foci. The assessment of survivin expression requires further detailed studies as it may aid in the diagnosis of CRC in the future. However, in our opinion, the serum level of survivin in CRC patients appears to be diagnostically ineffectual for clinical use.

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