Original paper

Correlation study of GAPDH, Bcl-2, and Bax protein immunoexpression in patients with colorectal adenocarcinoma

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Abstract

Introduction: Colorectal cancer (CRC) is the third and second most commonly diagnosed cancer worldwide in males and females, respectively. Despite prominent progress in diagnosis and treatment, the recurrence rates are still high. A tumour hypoxic environment leads to an increase in glycolytic metabolism. The crucial intermediate component of glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), could play a significant role in cancer progression. An increased level of GAPDH has been described in oncogene-induced transformation and anti-apoptotic function. In other studies, GAPDH has been involved in apoptosis induction.

Aim: We examined colorectal adenocarcinoma samples to assess the immunoexpression of GAPDH protein. We also evaluated the correlation between the expression of GAPDH protein and apoptotic parameters including expression of Bcl2 and Bax.

Material and methods: Paraffin sections were incubated for 60 min with primary antibody against GAPDH, Bcl-2, and Bax.

Results: Results of our study have shown that GAPDH expression in colorectal cancer is upregulated. We revealed significant positive correlation between expression of this protein and grade and size of tumour, and regional lymph node involvement. In the case of apoptosis-associated proteins, e.g. Bcl-2 and Bax, we found negative correlations between expression of these proteins and grade and size of tumour, lymphovascular invasion, and regional lymph node involvement. Finally, we demonstrated that GAPDH up-regulation is connected with down-regulation in Bcl-2 and Bax.

Conclusions: Up-regulation of GAPDH protein and down-regulation of Bcl-2 and Bax may result in increased of cancer.

Introduction

It is generally accepted that colorectal cancer (CRC) is an important global health problem. It is the third and second most commonly diagnosed cancer worldwide in males and females, respectively. Despite the prominent progress in diagnosis and treatment, the recurrence rates in the case of CRC are still high [1]. Therefore, the discovery of molecules significant to cancer development and metastasis as well as new therapeutic strategies seems to be very promising for improving the prognosis and therapy of CRC patients [2].

More than 50 years ago, Warburg hypothesised that cancer growth is facilitated by tumours producing their energy by aerobic glycolysis. Recent studies aimed at evaluating this hypothesis have revealed that cancerous cells have adopted their metabolism to facilitate the uptake and incorporation of nutrients needed for proliferation. Tumour development and progression are indeed associated with elevated glucose uptake and its aberrant metabolism [3, 4]. It should be pointed out that tumour hypoxic environment leads to an increase in glycolytic metabolism. The crucial intermediate component of glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), could play a significant role in cancer development and progression [5]. GAPDH specifically catalyses the simultaneous phosphorylation and oxidation of glyceraldehydes-3-phosphate to 1,3-bi-

phosphoglycerate by the use of NAD‡ as the electron acceptor. GAPDH is composed of a polypeptide chain of 335 amino acids. Structural studies characterised two regions, namely the glyceraldehydes-3-phosphate catalytic site and the NAD‡ binding site, a primary structure described as the Rossmann fold, which is also needed for activation of other dehydrogenases [6, 7].

As revealed by the studies, an increased level of GAPDH has been described in oncogene-induced transformation, angiogenesis, and anti-apoptotic function [8–11]. In other studies, GAPDH has been involved in apoptosis induction. Nevertheless, the reason for this paradox is poorly understood [12]. For example, differential subcellular localisation of GAPDH may be associated with its opposing biological activities in apoptotic and proliferating hepatocytes. The different functions of GAPDH may also be connected with various levels of post-translational modification of this protein [13, 14]. However, to our knowledge, there are no studies analysing the expression of GAPDH with expression of factors connected with apoptosis, especially in the case of cancer development [15].

Apoptosis is of tremendous current interest to clinicians who study and treat cancer. In the case of colorectal cancer, alterations in apoptosis contribute to both the pathogenesis and resistance to chemotherapeutic drugs and radiotherapy, both of which act, at least in part, by killing cancer cells [16]. Previous studies have reported that GAPDH can trigger mitochondrial oxidative stress-mediated cell death controlled by Bcl-2 family proteins [17]. It should be mentioned that there are two classes of Bcl-2 proteins: pro-apoptotic proteins (Bax, Bad, Bid, Bik) and anti-apoptotic proteins (Bcl-2, Bcl-X$_L$, Bcl-W). While anti-apoptotic proteins are involved in apoptosis by delaying the mitochondrial release of cytochrome-c, the proapoptotic proteins activate such releases [18]. Elucidation of the underlying regulatory mechanism seems to be essential not only to disease aetiology but also to treatment. Several types of therapies for cancer, including cytotoxic therapies, depend primarily upon induction of apoptotic cell death. Some authors suggested that abnormal expression of specific apoptosis-related proteins seems to be a major component of chemo-resistance [19, 20]. It may have significance in the context of hypoxic tumour environment and GAPDH expression, which is a marker of glycolysis.

Aim

We examined 60 cases of colorectal adenocarcinoma samples to assess the immunoexpression of GAPDH protein. We also evaluated the correlation between the expression of GAPDH protein and apoptotic parameters including expression of Bcl2 and Bax.

Material and methods

Collection of tumour samples

Sixty formalin-fixed, paraffin-embedded tissue specimens of primary colorectal adenocarcinoma were retrieved from archival material (Chair and Department of Pathomorphology, Medical University of Silesia, Zabrze, Poland). Paraffin-embedded tissue sections taken from postoperative material were diagnosed using standard haematoxylin and eosin staining, and the histological diagnoses were established according to the current standards.

Immunohistochemical staining

Formalin-fixed paraffin-embedded, 3-μm tissue sections were mounted onto SuperFrost slides and deparaffinised in xylene and ethanol of graded concentrations. Microwave antigen retrieval was then performed on the sections, which involved boiling the sections in 0.01 mol/l citrate buffer (pH 6.0) in a domestic microwave oven 10 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in water, and nonspecific staining was blocked by incubation with 5% bovine serum albumin in PBS for one hour at room temperature. Sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated for 60 min with polyclonal rabbit primary antibody against GAPDH (GeneTex; catalogue number GTX100118; dilution 1 : 3000), monoclonal mouse primary antibodies against Bcl-2 (Dako; clone: 124; dilution 1 : 200), and polyclonal rabbit primary antibody against Bax (Dako; code A3533; dilution 1 : 200). The En-Vision method (Dako En-Vision Kit/ Alkaline Phosphatase detection system) was used according to the manufacturer's instructions. The bound primary antibody was detected using the new Fuchsin Substrate System (DAKO A/S). The primary antibody was omitted from negative control slides. To suppress non-specific staining due to endogenous alkaline phosphatase activity, levamisole was used at a final concentration of 0.2 mM. The sections were counterstained with Mayer's haematoxylin.

Evaluation of the IHC variable

Immunohistochemical reactions for GAPDH, Bcl-2, and Bax were classified into four groups according to the intensity of immunohistochemical reaction: 0 – negative; 1 – weak; 2 – moderate; and 3 – strong. Heterogeneity was defined as the proportion of cancer cells showing a positive reaction to the total number of cancer cells and was graded from 0 to 3 by assessment: 0 demonstrated negative staining; 1 represented less than 10%; 2 represented 10–50%; and 3 represented

more than 50% of cancer cells with positive reaction. The results of intensity of staining and heterogeneity were combined and scored as follows: 0 represented negative; 1 and 2 represented low; 3 and 4 represented moderate; and 5 and 6 represented high expression. All sections were independently analysed by two experienced pathologists with no prior knowledge of clinicopathological parameters under a light microscope, and the images were recorded by digital camera.

Statistical analysis

All statistical analyses were done with the use of Statistica 10 software. To assess differences between the groups, the Mann Whitney U test and Kruskal-Wallis test was used. Results were considered statistically significant at $p < 0.05$. The relationships between GAPDH immunoreactivity and the expression of Bcl-2 and Bax were compared using Spearman correlation coefficients.

Results

GAPDH immunoexpression

The results of immunohistochemical analysis of GAPDH protein expression are summarised in Table I and Figure 1 A–C. The positive expression of this protein in colorectal adenocarcinoma samples was observed in 43 (88.4%) patients; 19 (31.66%) patients demonstrated weak staining (1+) with anti-GAPDH antibody whereas 16 (26.67%) patients and 18 (30%) showed moderate (2+) and strong staining (3+), respectively.

Statistical analysis revealed no correlation between GAPDH immunoreactivity in the main mass of tumour and clinicopathological parameters such as age, gender, tumour location, depth of invasion, and lymphovascular invasion (Table II). Interestingly, a statistically significant relationship was found between GAPDH immunoexpression and grade of tumour (Kruskal Wallis, $p < 0.001$). According to the data presented in Table II, higher expression of GAPDH correlated with poorly differentiated adenocarcinoma (Spearman, $p < 0.001$). Moreover, significant correlation was detected between GAPDH immunoreactivity and size of primary tumour (Mann-Whitney U test, $p = 0.011$). Tumours exceeding 10 cm tended to display higher immunoreactivity (Spearman, $p = 0.011$). A significant association was seen between regional lymph node involvement and GAPDH immunoexpression, suggesting higher expression level (56.67%) in "positive patients" (Mann-Whitney U test, $p < 0.001$).

Bcl-2 immunoexpression

The results of immunohistochemical analysis of Bcl-2 protein expression are shown in Table II and Figures 1 D, E). Bcl-2-positive staining was observed in 48 (80%) patients; 16 (26.67%) patients demonstrated weak staining (1+) whereas 13 (21.67%) and 19 (31.66%) patients revealed moderate (2+) and strong (3+) staining, respectively.

The results of our study demonstrated no connection between Bcl-2 immunoreactivity and clinicopathological parameters including age, gender, and tumour location (Table III). Importantly, a statistically significant correlation was revealed between Bcl-2 protein immunoexpression and grade of tumour (Kruskal-Wallis, $p < 0.001$). The higher expression of Bcl-2 was associated with well-differentiated cancer (G1) (Spearman $R = -0.580$; $p < 0.001$). Furthermore, significant correlation was detected between Bcl-2 immunoreactivity and depth of invasion (Kruskal-Wallis, $p < 0.001$; Spearman $R = -0.573$, $p < 0.001$). Interestingly, statistical differences were found between the group with T1 and T3 invasion and between the group with T1 and T4 invasion. In both cases the higher level of Bcl-2 immunoreactivity was demonstrated in patients with T1 invasion. Moreover, differences were also shown between the group with T2 and T3 and between the patients with T2 and T4 invasion. In such cases, the patients with T2 demonstrated higher levels of Bcl-2 protein expression. Correlation was also detected between Bcl-2 immunoreactivity and size of primary tumour (Mann-Whitney U test, $p = 0.011$). Tumours not exceeding 10 cm tended to display higher immunoreactivity (Spearman $R = -0.565$, $p = 0.011$). Statistically significant decrease in Bcl-2 immunoexpression was observed in relation to lymphovascular invasion and regional lymph node involvement. Bcl-2 expression was significantly lower in patients with positive lymph node status in comparison to those without such involvement.

Bax immunoexpression

The results of immunohistochemical analysis of Bax expression are summarised in Table III and Figures 1 F, G. The positive expression of Bax was observed in 48 (80%) patients; 21 patients demonstrated weak staining whereas 12 (21%) and 15 (25%) patients revealed moderate (2+) and strong staining (3+), respectively.

Statistical analysis demonstrated no association between Bax protein immunoreactivity and age, gender, tumour location, and lymphovascular invasion. A significant correlation was found between Bax expression and grade of tumour (Kruskal-Wallis, $p < 0.001$). The higher expression level of Bax protein is characteristic for patients with well-differentiated carcinoma (G1) (Spearman $R = -0.388$, $p = 0.002$). Moreover, significant correlation was detected between Bax immunoreactivity and depth of invasion (Kruskal-Wallis,

Table I. The association of GAPDH protein expression with clinicopathological features in CRC

Parameter		GAPDH expression				Statistical analysis
		Negative	Weak	Moderate	Strong	
Age [years]	≤ 60	3	9	7	8	$Z = -0.070$, $p = 0.944$
		11.11%	33.33%	25.93%	29.63%	$R = -0.0004$, $p = 0.998$
	≥ 61	4	10	9	10	
		12.12%	30.30%	27.27%	30.30%	
Gender	Females	1	9	10	9	$Z = 1.241$, $p = 0.215$
		3.45%	31.03%	34.48%	31.03%	
	Males	6	10	6	9	
		19.35%	32.26%	19.35%	29.03%	
Location of tumour	Proxima colon	4	6	3	7	$H (2.60) = 0.258$,
		20.00%	30.00%	15.00%	35.00%	$p = 0.879$
	Distal colon	2	6	8	5	
		9.52%	28.57%	38.10%	23.81%	
	Rectum	1	7	5	6	
		5.26%	36.84%	26.32%	31.58%	
Grade of tumour	G1	3	12	4	3	$H (2.60) = 13.565$, $p = 0.001$
		13.64%	54.55%	18.18%	13.64%	1–3, 2–3
	G2	4	7	5	7	$R = 0.456$, $p < 0.001$
		17.39%	30.43%	21.74%	30.43%	
	G3	0	0	7	8	
		0.00%	0.00%	46.67%	53.33%	
Depth of invasion	T1	1	4	5	3	$H (3.60) = 7.966$, $p = 0.047$
		7.69%	30.77%	38.46%	23.08%	~2–4
	T2	4	8	4	2	$R = 0.234$, $p = 0.072$
		22.22%	44.44%	22.22%	11.11%	
	T3	1	5	5	7	
		5.56%	27.78%	27.78%	38.89%	
	T4	1	2	2	6	
		9.09%	18.18%	18.18%	54.55%	
Size of primary tumour [cm]	≤ 10	4	17	6	7	$Z = -2.549$, $p = 0.011$
		11.76%	50.00%	17.65%	20.59%	$R = 0.327$, $p = 0.011$
	≥ 11	3	2	10	11	
		11.54%	7.69%	38.46%	42.31%	
Lymphovascular invasion	No	4	10	9	5	$Z = -1.536$, $p = 0.124$
		14.29%	35.71%	32.14%	17.86%	
	Yes	3	9	7	13	
		9.38%	28.13%	21.88%	40.63%	
Regional LN involvement	No	5	17	7	1	$Z = -4.814$, $p < 0.001$
		16.67%	56.67%	23.33%	3.33%	
	Yes	2	2	9	17	
		6.67%	6.67%	30.00%	56.67%	

Z – U Mann-Whitney test, H – Kruskal-Wallis test, R – Spearman rank correlation.
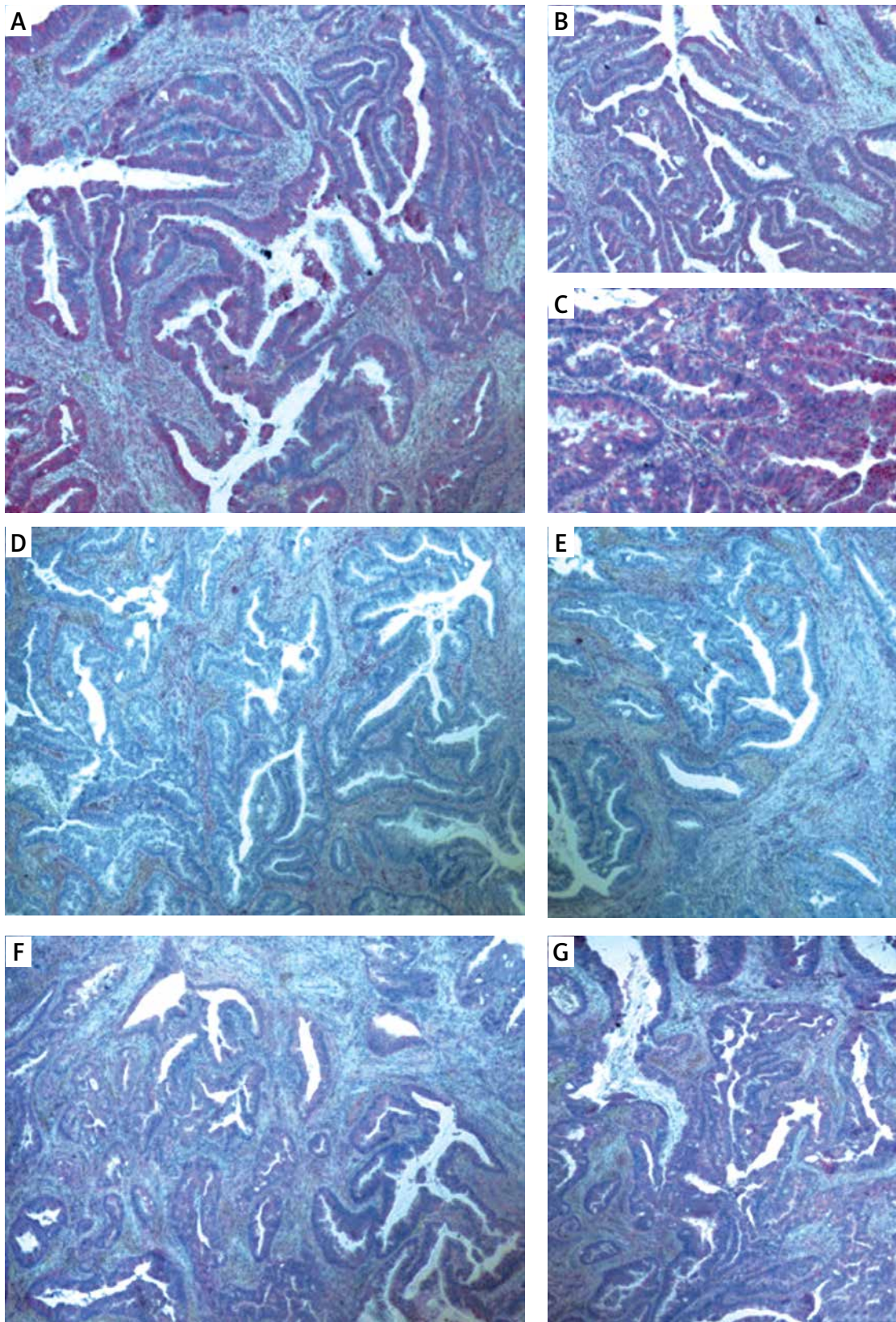
Figure 1. Representative examples of GAPDH (**A**–**C**), Bcl-2 (**D**, **E**) and Bax (**F**, **G**) protein immunoreactivity in G2 adenocarcinoma

Table II. The association of Bcl-2 protein expression with clinicopathological features in CRC

Parameter		Bcl-2 expression				Statistical analysis
		Negative	Weak	Moderate	High	
Age [years]	≤ 60	5	5	6	11	Z = 1.262, p = 0.207
		18.52%	18.52%	22.22%	40.74%	R = −0.111, p = 0.399
	≥ 61	7	11	7	8	
		21.21%	33.33%	21.21%	24.24%	
Gender	Females	4	10	4	11	Z = 0.720, p = 0.471
		13.79%	34.48%	13.79%	37.93%	
	Males	8	6	9	8	
		25.81%	19.35%	29.03%	25.81%	
Location of tumour	Proximal colon	3	5	5	7	H (2.60) = 1.278, p = 0.528
		15.00%	25.00%	25.00%	35.00%	
	Distal colon	5	5	2	9	
		23.81%	23.81%	9.52%	42.86%	
	Rectum	4	6	6	3	
		21.05%	31.58%	31.58%	15.79%	
Grade	G1	2	1	3	16	H (2.60) = 21.241, p < 0.001
		9.09%	4.55%	13.64%	72.73%	1–2, 1–3
	G2	6	6	9	2	R = −0.580, p < 0.001
		26.09%	26.09%	39.13%	8.70%	
	G3	4	9	1	1	
		26.67%	60.00%	6.67%	6.67%	
Depth of invasion	T1	1	1	3	8	H (3.60) = 21.395, p < 0.001
		7.69%	7.69%	23.08%	61.54%	1–3, 1–4, 2–3, 2–4
	T2	2	2	4	10	R = −0.573, p < 0.001
		11.11%	11.11%	22.22%	55.56%	
	T3	6	6	5	1	
		33.33%	33.33%	27.78%	5.56%	
	T4	3	7	1	0	
		27.27%	63.64%	9.09%	0.00%	
Size of primary tumour [cm]	≤ 10	3	4	10	17	Z = 4.343, p < 0.001
		8.82%	11.76%	29.41%	50.00%	R = −0.565, p < 0.001
	≥ 11	9	12	3	2	
		34.62%	46.15%	11.54%	7.69%	
Lymphovascular invasion	No	3	1	9	15	Z = 4.076, p < 0.001
		10.71%	3.57%	32.14%	53.57%	
	Yes	9	15	4	4	
		28.13%	46.88%	12.50%	12.50%	
Regional LN involvement	No	5	0	7	18	Z = 4.289, p < 0.001
		16.67%	0.00%	23.33%	60.00%	
	Yes	7	16	6	1	
		23.33%	53.33%	20.00%	3.33%	

Z – U Mann-Whitney test, H – Kruskal-Wallis test, R – Spearman rank correlation.

Table III. The association of Bax protein expression with clinicopathological features in CRC

Parameter		Bax expression				Statistical analysis
		Negative	Weak	Moderate	Strong	
Age	≤ 60	4	8	7	8	$Z = 1.336$, $p = 0.182$
		14.81%	29.63%	25.93%	29.63%	$R = -0.179$, $p = 0.172$
	≥ 61	8	13	5	7	
		24.24%	39.39%	15.15%	21.21%	
Gender	Females	6	11	6	6	$Z = -0.569$, $p = 0.569$
		20.69%	37.93%	20.69%	20.69%	
	Males	6	10	6	9	
		19.35%	32.26%	19.35%	29.03%	
Location of tumour	Proxima colon	2	6	6	6	$H(2.60) = 2.465$, $p = 0.292$
		10.00%	30.00%	30.00%	30.00%	
	Distal colon	6	7	2	6	
		28.57%	33.33%	9.52%	28.57%	
	Rectum	4	8	4	3	
		21.05%	42.11%	21.05%	15.79%	
Grade	G1	3	4	6	9	$H(2.60) = 9.055$, $p = 0.011$
		13.64%	18.18%	27.27%	40.91%	1–3
	G2	4	9	5	5	$R = -0.388$, $p = 0.002$
		17.39%	39.13%	21.74%	21.74%	
	G3	5	8	1	1	
		33.33%	53.33%	6.67%	6.67%	
Depth of invasion	T1	1	5	3	4	$H(3.60) = 9.529$, $p = 0.023$
		7.69%	38.46%	23.08%	30.77%	~2–3, 2–4
	T2	2	3	6	7	$R = -0.338$, $p = 0.008$
		11.11%	16.67%	33.33%	38.89%	
	T3	5	9	1	3	
		27.78%	50.00%	5.56%	16.67%	
	T4	4	4	2	1	
		36.36%	36.36%	18.18%	9.09%	
Size of primary tumour [cm]	≤ 10	6	8	8	12	$Z = 2.132$, $p = 0.033$
		17.65%	23.53%	23.53%	35.29%	$R = -0.309$, $p = 0.016$
	≥ 11	6	13	4	3	
		23.08%	50.00%	15.38%	11.54%	
Lymphovascular invasion	No	4	8	8	8	$Z = 1.470$, $p = 0.141$
		14.29%	28.57%	28.57%	28.57%	
	Yes	8	13	4	7	
		25.00%	40.63%	12.50%	21.88%	
Regional LN involvement	No	3	4	8	15	$Z = 4.717$, $p < 0.001$
		10.00%	13.33%	26.67%	50.00%	
	Yes	9	17	4	0	
		30.00%	56.67%	13.33%	0.00%	

Z – U Mann-Whitney test, H – Kruskal-Wallis test, R – Spearman rank correlation.

p = 0.023; Spearman R = −0.338; p = 0.008). In this case the higher level of Bax immunoexpression was demonstrated for patients with T2 invasion. Correlation was also detected between Bax immunoreactivity and size of primary tumour (Mann-Whitney U test, p = 0.033). Tumours not exceeding 10 cm tended to display higher immunoreactivity (Spearman R = −0.309, p = 0.016). A statistically significant decrease in Bax immunoexpression was observed in relation to regional lymph node involvement. Bax immunoexpression was significantly higher in patients with negative lymph node (LN) status as compared to those with positive LN status (Mann-Whitney U test, p < 0.001).

Correlations between expression of GAPDH, Bcl-2, and Bax protein

Correlations were tested among GAPDH, Bcl-2, and Bax protein expression. In order to explore this relationship, a significant negative Spearman correlation between GAPDH versus Bcl-2 protein (R = −0.290, p = 0.025) and GAPDH versus Bax (R = −0.450, p < 0.001) was demonstrated.

Discussion

Today it is widely accepted that cancer cells exhibit elevated aerobic glycolysis and rely more on this pathway to produce ATP and metabolic intermediates needed for cell proliferation [17]. GAPDH has been reported to participate in a wide range of cellular processes and may affect the activities of multiple interacting molecules. An increased level of GAPDH mRNA and protein has been detected in pancreatic cancer, lung cancer, and human prostate cancer of late pathological stage, suggesting that enhanced GAPDH expression may show connection with cell proliferation and cancer development [11, 21–25]. Nevertheless, several studies reported that GAPDH has been differentially expressed in renal cancer, breast cancer, prostatic cancer, liver cancer, colorectal cancer, bladder cancer, gastric cancer, melanoma, and glioma [6, 26].

The results of our study demonstrated that in patients with colorectal adenocarcinomas, expression of GAPDH protein was upregulated. We found a significant correlation between the grade of tumour and GAPDH immunoexpression. A significant difference was detected between the patients with G1 and those with G3 tumours. A higher level of GAPDH was observed in G3 patients. A significant correlation was also found between the level of GAPDH immunoexpression and size of primary tumours, and regional lymph node involvement. About 43% of patients with tumour size exceeding 10 cm showed a high level of GAPDH immunoreactivity. Moreover, in the group with positive regional

lymph node involvement about 67% of patients demonstrated high expression of GAPDH protein, whereas in the group with negative status such high a level was described only in 18% of patients. These results may indicate that GAPDH is involved in colorectal cancer progression and metastasis.

Many studies have revealed that cancer is caused by alteration in the regulation and execution of programmed cell death [15]. The high level of GAPDH expression might benefit the relative suppression of apoptosis in cancerous tissues, favouring cancer cell survival and malignancy [6]. Evidence that GAPDH might be involved in apoptosis came from studies on cultured cerebellar neurons, showing that elevated level of GAPDH and subsequent translocation to the nucleus preceded neuronal death stimulated by culture aging or cytosine arabino nucleoside exposure [6]. Therefore, in this work we correlated expression of GAPDH protein with expression of Bcl-2 and Bax at the level of immunohistochemistry.

In this context it should be mentioned that apoptotic signalling pathway is central to conserve a balance between cell death and survival and in keeping genome stability. As a rule, it is thought that the equilibrium between the rates of cell growth and apoptosis sustains intestinal epithelial homeostasis, and this stability is disturbed during cancer expansion [19, 20]. The signalling cascades regulating apoptosis progression have been extensively studied, and both extrinsic and intrinsic pathways have been described for apoptosis activation. The extrinsic pathway is activated by triggering cell death receptors on the cell surface, leading to activation of the apoptotic machinery. Intracellular stimuli including hypoxia and enhanced level of cellular oxidative stress are among the activators of intrinsic mitochondrial pathway [18]. This type of apoptosis is under the control of proteins from the Bcl-2 family. The protein Bcl-2 is a key inhibitor of apoptosis, and its aberrant expression has been demonstrated in a wide range of solid tumours, including colorectal cancer. In non-pathological colon mucosa a high level of Bcl-2 has been found at the level of base cells corresponding to progenitor cells [18]. The high level of Bcl-2 immunoreactivity is also characteristic for adenoma stage, suggesting that abnormal activation of Bcl-2 gene may play a role during early carcinogenesis [27, 28]. Belt *et al.* demonstrated that low level of Bcl-2 expression has been connected with disease reoccurrence, mainly in stage III patients [29]. This finding may indicate that low expression of Bcl-2 is thought to be associated with increased cancer cells death [30]. The results of our study showed a higher expression of Bcl-2 in well-differentiated cancers. In patients with G1 tumours, about

72% demonstrated a high level of expression, whereas in the group with G3, only 7% demonstrated a high level of Bcl2 immunoreactivity. It should be pointed out that there was a negative Spearman correlation between the expression of Bcl-2 protein and grade of tumour, depth of invasion, and size of primary tumours. These results may suggest that Bcl-2 is involved in early stages of colorectal carcinogenesis. Interestingly, in patients without lymphovascular invasion and with negative lymph node status, expression of Bcl-2 was also characterised as high.

Bax protein is a homologue of Bcl-2, which promotes apoptosis. Bax may bind to Bcl-2 forming Bax/Bcl2 heterodimers, or it may bind to itself forming Bax/Bax homodimers. The ratio of Bax to Bcl-2 determines the susceptibility of a cell to apoptosis. Thus, in cells with Bax overexpression, Bax homodimers predominate, and the susceptibility of such cells to apoptotic stimuli is increased [31, 32]. Statistical analysis showed a significant negative correlation between Bax expression and grade of tumour, depth of invasion, size of primary tumours, and regional lymph node involvement. Similar to Sturm *et al.* [33], we also showed that Bax expression in primary tumours decreased from well differentiated (37.52%) to poorly differentiated (20.60%), indicating that Bax expression is involved in tumour differentiation. Pryczynicz *et al.* demonstrated that the frequencies of cases with stronger staining were dramatically decreased in metastases in the regional lymph nodes compared with those of primary tumours [34]. Low expression of Bax has been correlated with greater depth of tumour invasion, lymph vessel invasion, advanced stage, and worse prognosis. Regarding Bax expression in colorectal carcinomas, previous studies have demonstrated that Bax expression is related to marginally better or longer survival [35, 36], whereas in others it has been connected with poorer survival [37]. It is worth noting that Bax promotes apoptosis following genotoxic damage from either chemotherapy or irradiation; therefore, the presence of Bax might be associated with better prognosis [38].

For protein-protein interaction, a statistically significant negative correlation was observed between GAPDH and Bcl-2. Interestingly, in the current study, GAPDH up-regulation is connected with down-regulation of Bcl-2 expression. Similar results have been obtained when GAPDH protein was correlated with Bax. Up-regulation of GAPDH protein and down-regulation of apoptosis-associated proteins may show correlation with increased proliferation and survival of colon cancerous cells, resulting in aggressiveness of cancer. These results reinforce the assumption of similar behaviour of association of Bcl-2 and Bax expression with grade of tumour, depth of invasion, and regional lymph node status. We observed a significant negative correlation between expression of apoptosis-related proteins and such clinicopathological parameters.

Conclusions

Results of our study have shown that that GAPDH, which is the main marker of glycolysis, is upregulated. We revealed significant positive correlation between expression of this protein and grade and size of tumour, as well as regional lymph node involvement. These results may indicate that GAPDH is involved in colorectal cancer progression and metastasis. In the case of apoptosis-associated proteins, e.g. Bcl-2 and Bax, we found negative correlations between expression of these proteins and such clinicopathological parameters as grade and size of tumour, lymphovascular invasion, and regional lymph node involvement. Finally, we demonstrated that GAPDH up-regulation is connected with down-regulation of Bcl-2 and Bax protein expression at the level of immunohistochemical study.

The mechanism of apoptosis in colorectal cancer is a complex process that depends on many factors including hypoxia and oxidative stress. Although a great number of studies have been performed in recent years, the mechanisms implicated in the pathogenesis of colorectal cancer are still not completely known. A better understanding of these issues, especially in the context of altered glucose metabolism and hypoxic tumour environment, could result in more precise assessment of diagnosis and more effective treatment. Studies of this type should be continued, and new insight may in the future result in targeted therapy or possibly prevention.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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