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The prognostic significance of p53, Bax, Bcl-2 and cyclin E protein overexpression in colon cancer – an immunohistochemical study using the tissue microarray technique

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Abstract

In colon cancer, biological markers continue to have a limited prognostic value, the results being controversial. Studies of cell-cycle regulatory proteins and anti-apoptotic proteins aim to identify groups of patients that develop more aggressive tumors and might benefit from an individualized therapy management. The present study evaluates the prognostic role of the p53, Bax, Bcl-2 and cyclin E immunoexpression in colon cancer, using the tissue microarray (TMA) method. Tissue samples were obtained from 31 patients operated for colon cancer, embedded in TMA paraffin blocks and immunohistochemically stained for p53, Bax, Bcl-2 and cyclin E. We evaluated the relationship between the overexpression of these proteins and the clinico-pathological parameters, as well as the effect of these molecular markers on the survival rate. 65.22% of the patients were p53 positive, 39.13% Bcl-2 positive, 78.26% Bax positive and 34.78% cyclin E positive. Bcl-2(+) patients had significantly better differentiated tumors (p=0.043). Significantly poorly differentiated tumors were: Bax(+) patients (p=0.031), Bcl-2(-)/p53(-) patients (p=0.042), Bcl-2(-)/Bax(+) patients (p=0.029), and Bcl-2(-)/p53(-)/Bax(+) patients (p=0.016). The individual expression of the studied proteins did not influence the survival rate. A significantly lower survival rate was found in the following groups of patients: Bcl-2(-)/p53(-) (40% *vs.* 83.3%, p=0.027), p53(-)/Bax(+) (40% *vs.* 83.3%, p=0.027), Bcl-2(-)/p53(-)/Bax(+) (25% *vs.* 84.2%, p=0.003). The current study identified groups of patients with a significantly lower survival rate, which consequently are at an increased risk to develop tumors with a more aggressive biological behavior.

Keywords: colon cancer, prognosis, immunohistochemistry, tissue microarray.

Introduction

Extensively studied over the past years, colorectal cancer remains one of the most frequent cancers in medical practice, being the third most frequent malignant neoplasm in men and the second most frequent in women. In 2008, there were approximately 1.2 million newly diagnosed cases and 608 700 deaths worldwide [1]. Despite the multitude of studies, prognosis in colorectal cancer remains poor and depends on the stage at the time of diagnosis. Biological markers are less useful for diagnosis but they are important in estimating the patient's prognosis or for assessing the risk of recurrence and establishing the therapeutic approach.

In colorectal carcinogenesis, the homeostasis of the intestinal epithelium is disturbed by an imbalance that occurs between the mitosis and apoptosis processes. In the induction of apoptosis, genes encoding cell cycle controlling proteins are involved: p53, Bax, cyclin, etc. Anti-apoptotic proteins (the Bcl-2 protein family) are supposed to induce a less metabolically active state of the cell, thus protecting it from apoptosis.

The normal or wild type of the p53 gene stops the cell cycle in phase G1 to allow for DNA repair. If DNA lesions cannot be repaired, p53 initiates apoptosis, activating the pro-apoptotic Bax gene. If mutations in the cell remain permanent, neoplastic cells accumulate the mutated p53 protein, which can be identified by immunohistochemical techniques. Bcl-2 is a proto-oncogene with anti-apoptotic action encoding proteins that stabilize the mitochondrial membrane function, thus preventing apoptosis [2]. Bcl-2 has mainly been located in the colon epithelial crypts [3, 4], and its expression is reduced with the progression towards the surface intestinal epithelium. The Bax protein is a pro-apoptotic protein belonging to the Bcl family, its activation is induced by p53 and counteracts the anti-apoptotic activity of Bcl-2.

The transition from one cell cycle stage to another is triggered by cyclin-dependent kinases, which become active by coupling with certain proteins specific for each cell cycle stage, termed cyclins. Cyclins D and E are synthesized during the G1 phase of the cell cycle.

This study aimed to analyze the relationship between the expression of certain proteins (p53, Bcl-2, Bax, cyclin E) and clinico-pathological parameters in colon cancer and how the expression of these proteins influences prognosis. The protein expression was evaluated by immunohistochemical methods using the tissue microarray (TMA) technique.

Materials and Methods

This is a retrospective clinico-pathological study performed on the operative specimens of 31 patients who

had undergone surgical resection for colon cancer in the 3rd Surgical Clinic of "Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology in Cluj-Napoca, Romania, between February 2005–May 2006. The patients did not benefit from neoadjuvant chemotherapy. The study was approved by the Ethical Committee of the Regional Institute of Gastroenterology and Hepatology. The diagnosis was confirmed, independently, by two anatomopathologists, and the histological type was established according to the World Health Organization (WHO) classification [5].

Subsequently, the operative specimens of these patients were embedded in TMA paraffin blocks and immunohistochemical determinations were performed in the Department of Pathology, "Prof. Dr. Chiricuţă" Oncological Institute, Cluj-Napoca. At the end of the study (40 months), survival was evaluated using the Computerized Population Register Service.

Preparation of TMA blocks

TMA blocks were created in order to ensure the unitary analysis of the studied specimens, each block containing histological sections from different patients. In the acceptor paraffin block, the neoplastic tissue samples (2 mm in diameter) extracted from each donor block (conventional paraffin block) were introduced. The acceptor paraffin block was introduced into an oven at 37^0C for 30 minutes, to ensure a good adhesion between the neoplastic tissue samples and the acceptor block paraffin. The TMA paraffin block (Figure 1) was sectioned using a Leica microtome, and the sections were Hematoxylin–Eosin (HE) stained or were placed on silanated slides and prepared for immunohistochemical staining (Figures 2 and 3).

Immunohistochemical staining

The silanated slides were incubated in a thermostat at 37^0C for 24 hours, then they were deparaffinized in xylene and rehydrated in alcohol. The antigens were demasked by heating in citric acid solution for 10 minutes. Endogenous peroxidase activity was inhibited by incubation in 0.5–3% hydrogen peroxide (H_2O_2) for 10 minutes. 100 µg diluted primary antibody were deposited on the slides, at 36^0C, for 30 minutes–one hour; after rewashing with tris-buffered saline (TBS) solution, 100 µg biotin-labeled diluted secondary antibody was applied (30 minutes). Then, the Streptavidin–Biotin–Peroxidase complex was applied to convert the colorless chromogen to a colored final product. Finally, the chromogen (DAB – 3,3'-diaminobenzidine) solution was applied in order to visualize the reaction.

The following mouse anti-human monoclonal antibodies were used: p53 (Dako, clone DO-7, 1:250 dilution), Ki-67 (Dako, clone MM1, 1:80 dilution), Bax (Zymed, clone 2D2, 1:200 dilution), Bcl-2 (Dako, clone 124, 1:100 dilution), cyclin E (Novocastra, clone 13A3, 1:40 dilution).

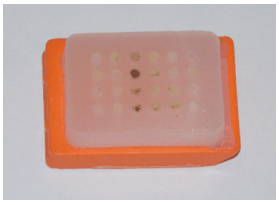

Figure 1 – ***Tissue microarray paraffin block.***

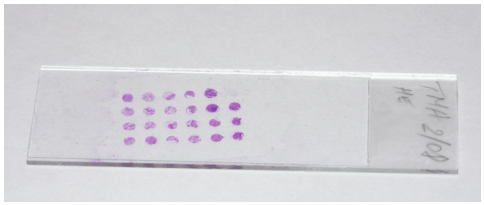

Figure 2 – ***Tissue microarray sections (HE staining).***

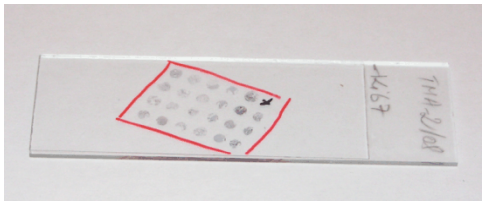

Figure 3 – ***Tissue microarrays containing spots of tissue (immunohistochemical staining).***

Evaluation of the protein expression

The slides were examined with an Olympus CKX41 microscope. First, positive and negative tissue samples were identified and evaluated. P53 and cyclin E stained as a nuclear signal, while Bcl-2 and Bax stained as a cytoplasmic signal. Only the percentage of positive tumor cells (the number of labeled cells out of 100 visible cells in the microscopic field) was assessed: 0–5% negative and >5% positive, without taking into calculation the intensity of immunolabeling, which is in accordance with other published studies [6, 7].

First, the association between the expression of these proteins and certain clinico-pathological characteristics (age, gender, location, histological type, degree of differentiation, etc.) was studied, and subsequently, the effect of these molecular markers on the survival rate.

Statistical analysis

The statistical data processing used both descriptive statistical methods and inferential statistical methods. For describing the characteristics of the group, frequency tables as well as dispersion and centrality indices were used, depending on the data type. Inferential statistics used the *chi*-square or the Fisher test, depending on the case, according to the standard application criteria. Survival data were graphically described using the Kaplan–Meier survival curves, and statistical significance was calculated with the *log*-rank test. Cox regression models were used, with the determination of HR (hazard ratio) and 95% CI (confidence interval). The limit of statistical significance was $p<0.05$. For data processing, the statistical packages SPSS *ver.* 13.0 (Chicago, IL, USA), and MedCalc *ver.* 8.3.1.1 were used.

Results

Characteristics of the patient group

The study group included 31 patients: 15 (48.39%) men and 16 (51.61%) women, with a mean age of 63± 11.71 years. 52% of the tumors (16 patients) were located in the left colon and 48% (15 patients) in the right colon. The tumor size varied between 2.5–8 cm, with a mean value of 4.86±1.428 cm. 51.61% of the tumors were exophytic (16 patients), and the rest of 48.39% (15 patients) were infiltrative and ulcero-infiltrative. Depending on the histological type, 32.2% of the tumors (10 patients) were mucinous colon adenocarcinomas (having more than 25% mucus pools), the rest being considered intestinal adenocarcinomas. According to the *American Joint Committee on Cancer* (AJCC) tumor staging [8], two (4.3%) patients were in Dukes A stage, nine (30.44%)

patients with Dukes B stage, 17 (56.5%) patients with Dukes C stage, and three (8.7%) patients with Dukes D stage. Venous invasion was present in seven (21.74%) patients and perineural invasion in seven (21.74%) patients. Regarding histological grades, the majority of malignant colon tumors (15 – 48.82%) were moderately differentiated, nine (30.44%) tumors were well differentiated, and seven (21.74%) tumors were poorly differentiated. At the time of diagnosis, three (8.70%) patients had distant metastases.

The immunohistochemical expression of p53, Bcl-2, Bax and cyclin E proteins

Among the 31 patients, p53 protein expression (Figure 4) was found in 20 (65.22%) patients, the rest of 11 (34.78%) patients being p53 negative. The immunoexpression of proteins involved in apoptosis was: Bcl-2 positive in 12 (39.13%) patients (Figure 5), and Bax positive in 24 (78.26%) patients (Figure 6). Eleven (34.78%) patients were positive for cyclin E.

The correlations between proteins immunoexpression and clinico-pathological parameters are presented in Table 1.

There were no statistically significant correlations between the individual expression of p53 and cyclin E proteins and any of the studied clinico-pathological parameters (Table 1). A statistically significant difference was noticed between the tumor differentiation degree and the proteins involved in apoptosis: Bcl-2 and Bax. Bcl-2(+) patients (n=12) had significantly better differentiated tumors compared to Bcl-2(-) patients (p=0.043), while Bax(+) tumors (n=24) were significantly more poorly differentiated compared to Bax(-) tumors (p=0.031) (Table 1).

Table 1 – ***Relationship between clinico-pathological parameters and p53, Bcl-2, Bax and cyclin E immunohistochemical expression (univariate analysis)***

	N=31	P53			Bcl-2			Bax			Cyclin E		
		Positive	*Negative*	*P-value*	*Positive*	*Negative*	*P-value*	*Positive*	*Negative*	*P-value*	*Positive*	*Negative*	*P-value*
Gender													
Male	15	11	4	0.469	5	10	0.795	12	3	0.692	4	11	0.469
Female	16	9	7		7	9		12	4		7	9	
Age [years]													
<60	15	9	6	0.879	5	10	0.795	11	4	0.538	7	8	0.304
>60	16	11	5		7	9		13	3		4	12	
Site													
Right colon*	15	9	6	0.879	4	11	0.400	12	3	1.000	7	8	0.304
Left colon**	16	11	5		8	8		12	4		4	12	
Gross appearance													
Exophytic	16	13	3	0.089	6	10	1.000	15	1	0.155	7	9	0.667
Infiltrative / Ulcero-infiltrative	15	7	8		6	9		9	6		4	11	
Size													
<4 cm	13	8	5	0.685	5	8	0.417	12	1	0.339	3	10	0.379
>4 cm	18	12	6		7	11		12	6		8	10	
Histological type													
Intestinal	21	16	5	0.182	9	12	0.657	16	5	1.000	7	14	0.657
Mucinous	10	4	6		3	7		8	2		4	6	
Grade													
G1	9	8	1	0.258	7	2	**0.043**	9	0	**0.031**	3	6	0.909
G2	15	8	6		5	10		8	7		5	10	
G3	7	3	4		0	7		7	0		3	4	
Dukes staging [8]													
A	2	1	1	0.817	2	0	0.260	2	0	0.792	1	1	0.371
B	9	7	2		5	4		7	2		3	6	
C	17	11	6		4	13		13	4		7	10	
D	3	1	2		1	2		2	1		0	3	
Venous invasion													
Present	7	5	2	0.433	4	3	0.280	5	2	0.915	2	5	0.433
Absent	24	15	9		8	16		19	5		9	15	
Perineural invasion													
Present	7	5	2	0.433	4	3	0.280	5	2	0.915	3	4	0.782
Absent	24	15	9		8	16		19	5		8	16	

*Right colon location: cecum and ascending colon; **Left colon location: transverse segment to the sigmoid. *N*: No. of cases; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated. *P*-value <0.05 was considered statistically significant.

Also, the clinico-pathological parameters were correlated with the simultaneous expression of two or more molecular markers. Significant association was noticed between the histological type and certain molecular profiles, such as Bcl-2(-)/cyclin E(+)/Bax(+) patients (n=2) (p=0.005) or the Bcl-2(-)/p53(-)/Bax(+) patients (n=5) when the difference was at the limit of statistical significance (p=0.067) (Table 2).

Table 2 – *Relationship between simultaneous expression of molecular markers and histological type*

Profile	Status	*N*=31	Intestinal type	Mucinous type	*P*-value
Bcl-2 negative p53 negative	Absent profile	24	19	5	0.142
	Profile present	7	3	4	
Cyclin E positive Bax positive	Absent profile	23	18	5	0.318
	Profile present	8	4	4	
p53 negative Bax positive	Absent profile	24	19	5	0.142
	Profile present	7	3	4	
Bcl-2 negative Bax positive	Absent profile	16	13	3	0.193
	Profile present	15	8	7	
Bcl-2 negative p53 negative Bax positive	Absent profile	26	20	6	0.067
	Profile present	5	1	4	
Bcl-2 negative Cyclin E positive p53 negative	Absent profile	29	25	4	**0.005**
	Profile present	2	0	2	

N: No. of cases. *P*-value <0.05 was considered statistically significant.

Analyzing the relationship between the simultaneous expression of the studied proteins and the histological grade, we noticed that the following molecular profiles had significantly more poorly differentiated tumors: Bcl-2(-)/p53(-) (p=0.042), Bcl-2(-)/Bax(+) (p=0.029), Bcl-2(-)/p53(-)/Bax(+) (p=0.016) (Table 3).

Table 3 – *Relationship between simultaneous expression of molecular markers and histological grade*

Profile	Status	*N*=31	G1	G2	G3	*P*-value
Bcl-2 negative p53 negative	Absent profile	24	9	12	3	**0.042**
	Profile present	7	0	3	4	
Bcl-2 negative Bax positive	Absent profile	16	6	10	0	**0.029**
	Profile present	15	3	5	7	
Bcl-2 negative p53 negative Bax positive	Absent profile	26	9	14	3	**0.016**
	Profile present	5	0	1	4	
p53 negative Bax positive	Absent profile	24	8	13	3	0.062
	Profile present	7	1	2	4	
Bcl-2 negative Cyclin E positive Bax positive	Absent profile	27	9	14	4	0.111
	Profile present	4	0	1	3	
Bcl-2 negative Cyclin E positive p53 negative	Absent profile	29	9	14	6	0.152
	Profile present	2	0	0	2	

N: No. of cases; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated. *P*-value <0.05 was considered statistically significant.

Survival analyses

Overall survival (OS) rate was 63.9% at 40 months and mortality rate was 36.1% (Figure 7). The median survival time for the studied group was 32.26±8.735 months.

No significant correlation was found between the individual expressions of the studied markers: p53, Bcl-2, Bax, cyclin E and overall survival (Table 4; Figure 8, A–D).

Patients with the Bcl-2(-)/p53(-) profile had a significant lower survival rate compared to the rest of patients, 40% *vs.* 83.3%, with a median survival time of 26.200±4.151 months *vs.* 37.000±1.959 months (p=0.027) (Figure 9). Also, survival rate was significantly lower for p53(-)/Bax(+) patients (40% *vs.* 83.3%, p=0.027), but especially for Bcl-2(-)/p53(-)/Bax(+) patients (25% *vs.* 84.2%, with a mean number of survival months of 23±3.824 *vs.* 37.158 ±1.867, p=0.003) (Figure 10; Table 4).

Table 4 – *Relationship between OS and p53, Bcl-2, Bax and cyclin E immunohistochemical expression (univariate analysis)*

Profile	Status	*N*=31	OS (40 months)	Age [years] (mean ± SD)	*P*-value
p53	Negative	11	62.5%	30.000 ±3.186	0.326
	Positive	20	80%	36.400 ±2.321	
Bcl-2	Negative	19	64.3%	33.143 ±2.232	0.242
	Positive	12	88.9%	34.333 ±3.457	
Bax	Negative	7	80%	35.800 ±1.073	0.653
	Positive	24	72.2%	34.000 ±2.510	
Cyclin E	Negative	20	80%	34.133 ±2.668	0.466
	Positive	11	62.5%	34.875 ±2.666	
Bcl-2 negative p53 negative	Absent profile	24	83.3%	37.000 ±1.959	**0.027**
	Profile present	7	40%	26.200 ±4.151	
Bcl-2 negative Cyclin E positive	Absent profile	24	83.3%	34.944 ±2.264	0.081
	Profile present	7	40%	31.800 ±3.627	
p53 negative Cyclin E positive	Absent profile	29	76.2%	35.476 ±2.052	0.367
	Profile present	2	50%	25.500 ±5.303	
Cyclin E positive Bax positive	Absent profile	23	76.5%	34.235 ±2.379	0.678
	Profile present	8	66.7%	34.667 ±3.388	
p53 negative Bax positive	Absent profile	24	83.3%	37.000 ±1.959	**0.027**
	Profile present	7	40%	25.400 ±3.737	
Bcl-2 negative Bax positive	Absent profile	16	83.3%	34.667 ±2.641	0.290
	Profile present	15	63.6%	33.363 ±2.847	
Bcl-2 negative p53 negative Bax positive	Absent profile	26	84.2%	37.158 ±1.863	**0.003**
	Profile present	5	25%	23.000 ±3.824	
Bcl-2 negative Cyclin E positive Bax positive	Absent profile	27	80%	35.750 ±2.136	0.084
	Profile present	4	33.3%	29.333 ±5.193	

N: No. of patients; OS: Overall survival rate. *P*-value <0.05 was considered statistically significant.

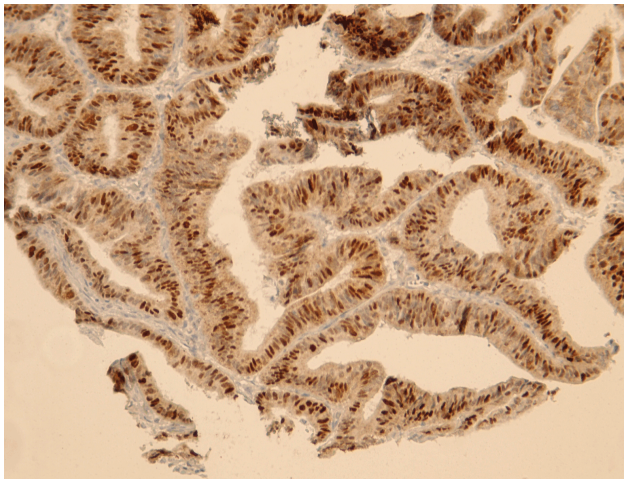

Figure 4 – *Tissue microarray section of colon adenocarcinoma with positive nuclear immunostaining for p53 (×200).*

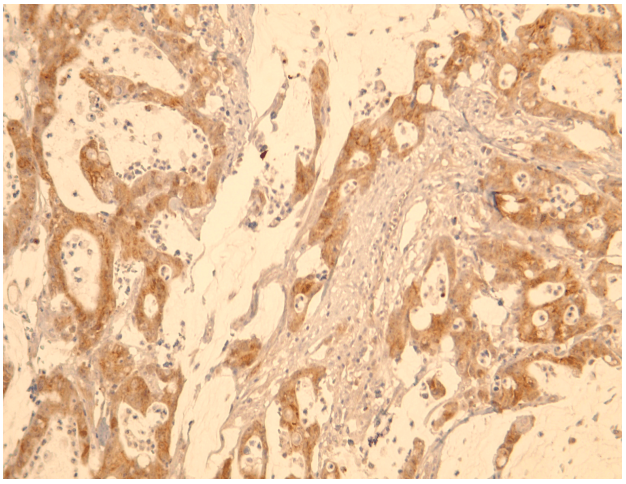

Figure 5 – *Tissue microarray section of colon adenocarcinoma with positive cytoplasmatic immunostaining for Bcl-2 (×200).*

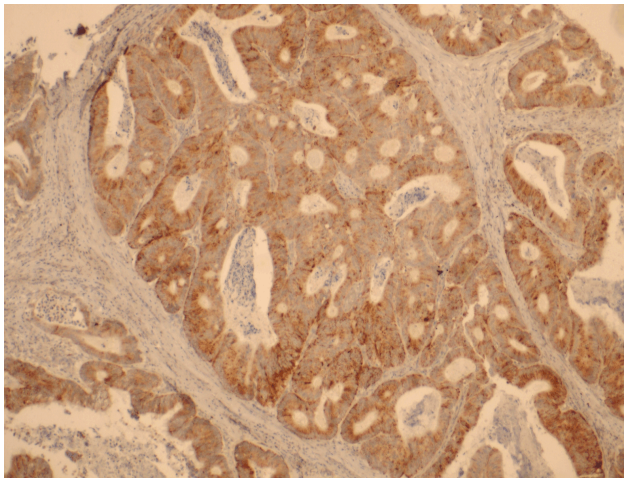

Figure 6 – *Tissue microarray section of colon adenocarcinoma with positive cytoplasmatic immunostaining for Bax (×200).*

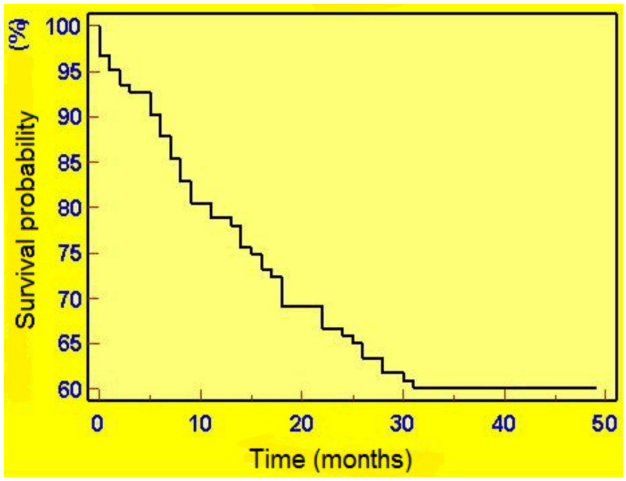

Figure 7 – *Kaplan–Meier overall survival curve.*

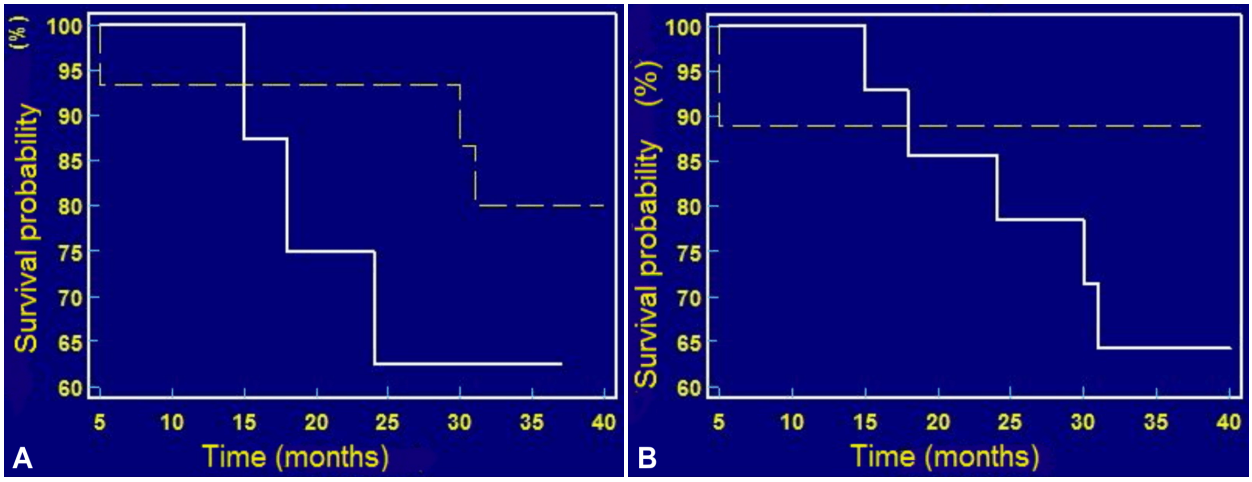

Figure 8 – *Overall survival curve for individual protein expression: (A) p53 overexpression: ── p53 negative; – – – p53 positive; (B) Bcl-2 overexpression: ── Bcl-2 negative; – – – Bcl-2 positive.*

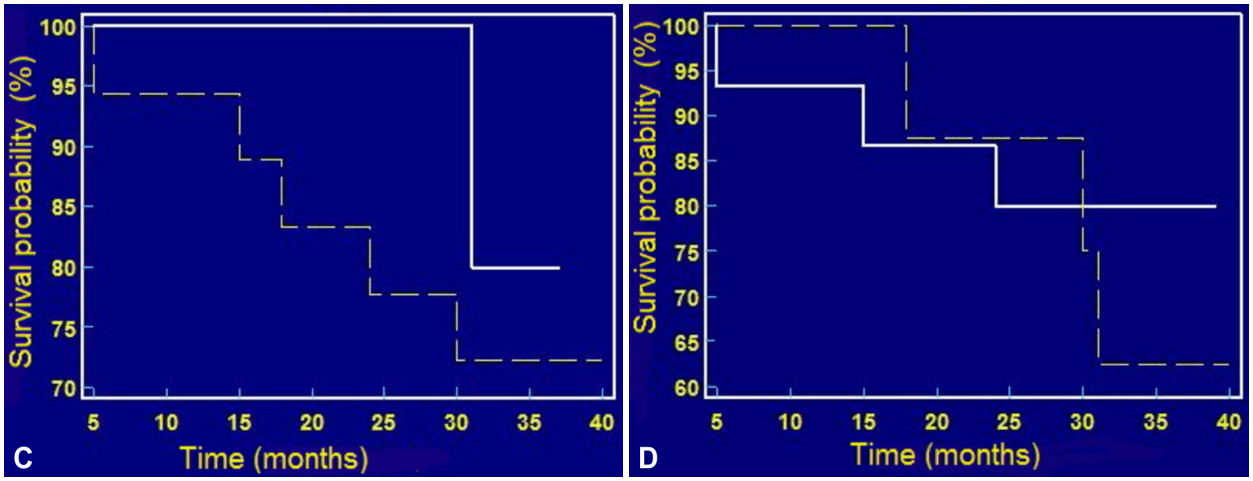

Figure 8 (continued) – ***Overall survival curve for individual protein expression: (C) Bax overexpression: ── BAX negative; – – – BAX positive; (D) Cyclin E overexpression: ── cyclin E negative; – – – cyclin E positive.***

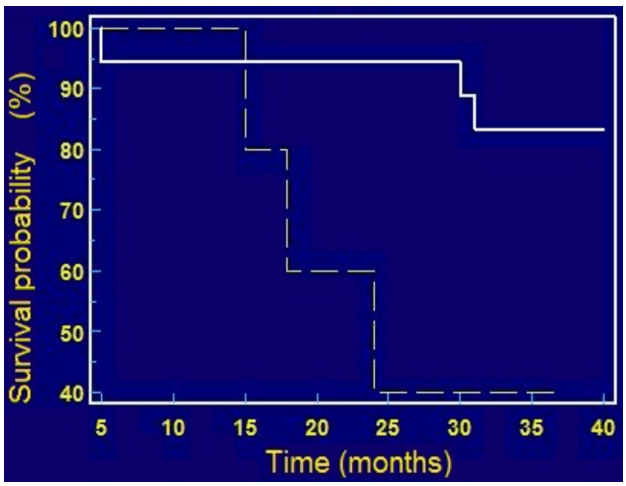

Figure 9 – ***Overall survival curve for Bcl-2(-)/p53(-) profile: – – – Bcl-2(-)/p53(-) molecular profile present; ── Bcl-2(-)/p53(-) molecular profile absent.***

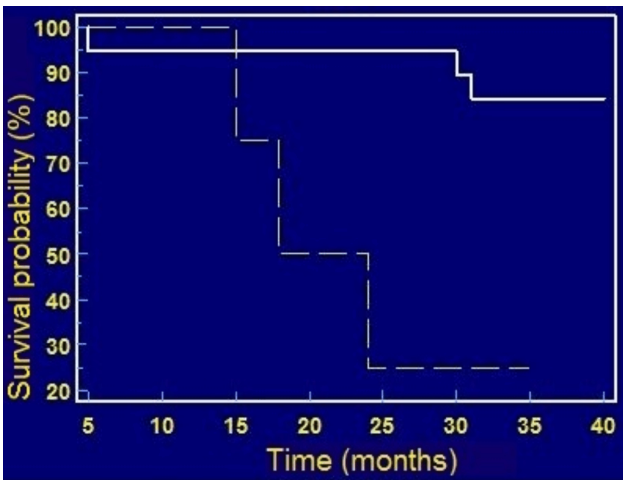

Figure 10 – ***Overall survival curve for Bcl-2(-)/p53(-)/Bax(+) profile: – – – Bcl-2(-)/p53(-)/Bax(+) molecular profile present; ── Bcl-2(-)/p53(-)/Bax(+) molecular profile absent.***

Discussion

Although many studies have been reported regarding tumor molecular markers, these markers continue to be an issue of high current interest and, in colon cancer, they may be useful in estimating prognosis and establishment the therapeutic approach.

Our study aims to evaluate the prognostic role of certain molecular markers (p53, Bcl-2, Bax, cyclin E) in colon tumors, as well as the relationship between these proteins expression and clinico-pathological parameters.

The immunohistochemical analysis of the samples was performed on TMA blocks. The TMA method allows for the uniform and rapid analysis, on the same slide, of tumor tissue samples from different patients, all spots being stained under the same conditions, with identical antibody concentrations [9, 10].

An important characteristic of neoplastic processes is uncontrolled cell proliferation. P53 and Bax proteins are pro-apoptotic, cyclin E controls the cell cycle, and the Bcl-2 protein family is supposed to induce a less metabolically active state in the cell, protecting it from apoptosis.

The mutated p53 protein can be detected immunohistochemically; it accumulates in the tumor cell nucleus. In sporadic colorectal cancer, the frequency of p53 mutations varies between 30–70% [7, 11–16]. In the studied group, the mutated p53 protein occurred with a 65.22% frequency.

In the present study, there were no significant correlations between p53 expression and the studied clinico-pathological parameters. Some authors reported similar results [17–21], and other have shown significant correlations with lymphatic and vascular invasion [22].

Regarding the relationship between the p53 protein expression and OS, p53 had an independent unfavorable prognostic role or is involved in appearance of tumor recurrence in some studies [22, 23]. In contrast, other authors [2, 24–28] did not identify in p53 an independent prognostic factor for colon cancer. In a review of 25 studies analyzed by Macdonald *et al.* on colon tumors [29], 14 studies revealed a correlation between the expression of the mutated p53 gene and a lower survival, eight studies established no correlation between p53 expression and the survival rate, and three studies identified p53 expression as a positive prognostic factor. All these results prove once again that p53 overexpression has a controversial prognostic role.

Our study did not identify a correlation between p53 expression and the OS rate; it was even found that p53(+) patients had a better survival compared to p53(-) patients (80% *vs.* 62.5%), without a statistically significant difference. There are other authors who reported similar results, *i.e.*, an association between p53 expression and better survival [11, 15, 30–33]. This could be explained by the fact that in tumor processes, the mutated p53 protein loses its normal function, reducing apoptosis, which leads to uncontrolled cell proliferation.

The differences between the frequency of p53 immunopositivity in different studies might be due to the different techniques used: fixation methods, antibodies used, antigen extraction methods, threshold values and scores used for interpretation [34], differences between the groups of patients included in the study (regarding location, the histological type, tumor stage, microsatellite instability), as well as to a different biological behavior of tumors [35].

In conclusion, despite the many studies performed, it cannot be stated with certainty that the expression of the mutated p53 protein in the neoplastic cells has an impact on the evolution and survival of patients with colorectal cancer, so it is not recommended to perform this determination as routine in order to detect patients who should receive adjuvant chemotherapy.

The overexpression of the Bcl-2 protein in tumor cells involves a mutation that diminishes or suppresses its mitochondrial membrane-stabilizing role, thus favoring apoptosis [2, 31].

In our study, Bcl-2 protein expression was found in 39.13% of patients, the rest of 60.8% being negative. These data are in accordance with the results published by other authors who found an absence of Bcl-2 protein expression in more than half of the patients with colorectal cancer, the positive reaction being seen in small groups of patients [36, 37].

The analysis of the relationship between Bcl-2 expression and different clinico-pathological parameters in our group of patients showed a statistically significant correlation between the loss of Bcl-2 expression, Bcl-2(-) patients, and low degree of tumor differentiation (p=0.043), result which has also been reported by other authors [38]. Other clinico-pathological parameters did not correlate with Bcl-2 expression, our results being similar to some other published studies [17, 36, 39].

Regarding the prognostic role of Bcl-2 expression, some authors [4, 10, 17, 31, 40] reported an association between Bcl-2 overexpression and good survival, while others [36, 41] showed that the lack of Bcl-2 expression indicated an unfavorable outcome. However, studies performed in larger groups of patients could not identify Bcl-2 as an independent prognostic factor for colorectal cancer [10, 42, 43].

Our data did not show any significant effect of Bcl-2 protein on the survival rate, although Bcl-2(+) patients had a better survival rate compared to Bcl-2(-) patients (88.9% *vs.* 64%). The same findings were presented by other authors [4, 10, 31, 38, 40, 44], who suggested that tumors expressing the Bcl-2 protein have a less aggressive biological behavior.

However, the question arises: Why is the absence of Bcl-2 correlated with a higher tumor aggressiveness and unfavorable prognosis, considering that its role is to inhibit apoptosis? The role of this protein might be more complex than known so far, including an anti-proliferative role [4, 45], which is confirmed by some studies performed on breast adenocarcinoma, where Bcl-2 overexpression was associated with a reduced cell proliferation [17, 46].

Under these circumstances, the simultaneous expression of the mutated Bcl-2 and p53 proteins was studied. Some authors suggest that p53(+)/Bcl-2(-) patients had an unfavorable outcome [4, 47]. In the present study, the Bcl-2(-)/p53(-) profile was statistically significantly correlated with a lower survival rate (40% *vs.* 83.3%, p=0.027) and, also, with a low tumor differentiation degree (p=0.042). 78.36% of the patients had Bax protein overexpression, similarly to other reported results [42, 48–50]. Our study also demonstrated that Bax overexpression was correlated with the histological grade (p=0.031), and, in Dukes D stage, a very low expression of this protein was found. However, Bax expression could not be correlated with other clinico-biological parameters. Other authors reported that Bax expression is correlated with the tumor differentiation degree and the histological type [48].

The significance of the Bax protein as an independent prognostic factor in colorectal cancer is controversial. In our group of patients, there was no statistically significant relationship between the individual Bax protein overexpression and the survival rate. In this aspect, these results agreed with other reports [48, 51, 52], while Giatromanolaki *et al.* [37] reported that Bax(+) patients have a significantly lower survival and a higher recurrence risk.

Some authors reported an unfavorable outcome in p53(+)/Bax(+) patients [4, 42, 47]. In the current study, the p53(-)/Bax(+) profile was significantly correlated with a lower survival rate (40% *vs.* 83.3%, p=0.027), so the activation of the Bax gene is probably not initiated only by the p53 gene, but also by other mechanisms.

In the present study, the concomitant expression of molecular markers revealed that the Bcl-2(-)/p53(-)/Bax(+) molecular profile has been statistically correlated with a lower survival rate (OS 25% *vs.* 84.2%, p=0.003) and with a lower tumor differentiation degree (p=0.016). Tsamandas *et al.* [44] found that Bcl-2(-)/p53(+)/Bax(+) patients had a significant worse prognosis.

Cyclin E is a protein synthesized during the G1 phase of the cell cycle, required for the transition between G1 to S phase. In certain types of cancer (breast, gastric, pulmonary, renal cancer), the authors noticed that overexpression of cyclin E is correlated with poor survival [53]. In colorectal cancer, data regarding cyclin E overexpression are controversial and varies from 9.4% to 92% [53, 54]. The results of the present study revealed cyclin E overexpression in 34.78% cases and no correlation between protein immunoexpression and any of the patients or tumor characteristics. Similar results were reported by other authors [53, 55]. In contrast, Zhou *et al.* correlated cyclin E overexpression with venous invasion [56].

In the current study, there was no significant correlation between cyclin E overexpression and the survival rate. However, some molecular profiles show a trend toward a poor prognosis, the results being at the limit of statistical significance: Bcl-2(-)/cyclin E(+) patients (OS 40% *vs.* 83.3%, p=0.081) and Bcl-2(-)/cyclin E(+)/Bax(+) patients (OS 33% *vs.* 77.3%, p=0.084).

Conclusions

The individual expression of biological markers has a limited prognostic value, which is why their simultaneous expression is the focus of current studies, with the possibility of detecting patients at high risk for developing tumors with a more aggressive biological behavior, who might benefit from an individualized therapy management. In the present study, we identified three groups of patients that were statistically significantly correlated with a lower survival rate: Bcl-2(-)/p53(-)/Bax(+) patients, p53(-)/ Bax(+) patients, and Bcl-2(-)/p53(-) patients. For the immunohistochemical analysis of biological markers, the TMA method was used, allowing the rapid, uniform and cost effective analysis of many tumor tissue sections situated on the same slide. In the future, it is estimated that, in addition to the study of clinico-pathological parameters, the analysis of the molecular or genetic profile of colorectal tumors will dictate the therapeutic decisions.

Conflict of interests

The authors declare that they have no conflict of interests.

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