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The prognostic value of the immunohistochemical aspects of tumor suppressor genes p53, bcl-2, PTEN and nuclear proliferative antigen Ki-67 in resected colorectal carcinoma

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Abstract

This study aim was to identify the relations between tumor suppressor genes (p53, bcl-2, PTEN), nuclear proliferative antigen Ki-67 and epidemiologic, morphologic and histologic patient related factors, in colorectal cancer. *Materials and Methods*: Twenty-two prospectively collected colorectal cancer resection specimens were histologically prepared, using standard paraffin-embedded and Hematoxylin–Eosin staining method; for immunohistochemical study, the Streptavidin–Biotin (sABC)/Horseradish Peroxidase (HRP) method was used. *Results*: P53 was positive in 86.36% of cases, more intense (>50%) in rectal cancer and in women 59.16±9.49-year-old; the G1/2 adenocarcinoma was dominant. Bcl-2 was positive in 18.18% of the cases, in distal colorectal cancer, only in men, 63.5±13.2-year-old. PTEN was positive in 95.45% of the cases; intense positivity was recorded in 12 men and six women, 61.47±11.67-year-old, in rectal topography. Ki-67 was positive in 86.36% of the cases, more intense in four men and four women, 63.45±12.22-year-old, in proximal and advanced colorectal cancers (pT3N1/2 – 62.5%). *Conclusions*: Tumor suppressor genes mutations are often present in colorectal cancer; the intensity of the expression of these mutations varies, which could explain the different prognosis for these patients.

***Keywords*:** colorectal cancer, tumor suppressor genes p53, bcl-2, PTEN, proliferative nuclear antigen Ki-67.

Introduction

Colorectal cancer represents the most frequent site of the digestive cancers, and represents the second leading cause of death of cancer worldwide; an increase in the colorectal cancer incidence have been reported all over the world, especially in men in some cancer registries [1, 2].

In the etiopathogenesis of the colorectal adenocarcinoma are implied environmental factors and a series of genetic alterations of oncogenes with role in tumor cell growth, apoptosis and cell metastasis.

The p53 and bcl-2 tumor suppressor genes play an important role in colorectal cancer development, not only in the adenoma-carcinoma sequence, but also in tumor progression; the patient prognosis seems to be affected by the association between these oncogenes, the best prognosis being recorded for the bcl-2 positive/p53 negative cases [3, 4].

On the other hand, the PTEN tumor suppressor gene is much rare involved in colorectal carcinogenesis (especially in sporadic cancers), but when it is involved seems to affect the response to Cetuximab, an important anti-angiogenetic drug [5, 6].

In the literature the results concerning the prognostic implications in colorectal carcinoma of the described markers are contradictory and also, there are few data how the concomitant expression of bcl-2, p53, PTEN and Ki-67 affects survival in patients with colorectal cancer.

The aim of this study was to analyze concomitant immunohistochemical expression of these markers, and to establish a relation between some clinical related factors (patient's age and gender, tumor location and size), morphological grade, pathologic stage of the tumors (lymph node metastasis, angiolymphatic invasion) and clinical outcome of the patients.

Materials and Methods

The study was performed on 22 colorectal cancer resection specimens, operated in the Surgical Department of "Dr. Alexandru Gafencu" Emergency Military Hospital of Constanţa, between 2005 and 2009. The specimens were harvested intra-operatively, after an initial diagnosis of the disease extension (local extension, regional or distant metastasis). In order to have a quality resection specimen, there were excluded inoperable cases due to local extensive invasion or distant metastasis. Afterwards the resected specimens were fixed in 10% formalin solution, paraffin embedded, sectioned at 4–5 µm, and histologically examined using the standard Hematoxylin–Eosin staining method.

The immunohistochemical reactions were performed by the Streptavidin–Biotin (sABC)/Horseradish Peroxidase (HRP) method (StreptABC, Dako, Denmark). The paraffin-embedded blocks were sectioned at 5-µm on electrostatic charged slides and then deparaffinized. To achieve antigen unmasking epitope the slides were boiled in citrate buffer antigen retrieval to a micro-oven 20 minutes at 350 W. The peroxidase activity was blocked with 3% hydrogen peroxide and then the sections were incubated with primary antibodies overnight: p53 antibody, DO7 clone (Dako), 1:50; bcl-2 antibody, clone 124 (Dako), 1:50; PTEN antibody, clone 28H6 (Novocastra), 1:100; Ki-67 antibody, clone MIB-1 (Dako), 1:50. Subsequently, they were incubated in secondary biotinylated antibody from LSAB+ peroxidase kit (Dako, K0690, Denmark), followed by incubation with Streptavidin HRP (Dako, Denmark), and finally a two minutes incubation with DAB chromogen solution and counterstained with Hematoxylin.

The method of quantifications of Ki-67, p53 and PTEN (which are nuclear immunohistochemical markers) was a semiquantitative one, and the positivity index was determined as the number of positive cells out of 100 countered tumoral cells on microscopic fields investigated with ×40 magnification. According to the intensity of the expression, cases were classified as having a weak (poor) expression (below 50% positivity in the tumoral cells), and intense (strong) expression (positivity above 50% in the tumor).

The pattern of reaction for bcl-2 was cytoplasmic in the tumoral cells, the reaction being considered positive, when we had positive tumoral cells over 5%, and respectively negative, for a percentage of positive tumoral cells <5%. The positive tissue witness for Ki-67 and PTEN was lymphoid associated tumoral tissue, for bcl-2 was also germinal centers of lymphoid follicles, and for p53 the crypt of normal glands of the colonic mucosa.

The immunohistochemical study was performed in the Pathology Department of "Dr. Carol Davila" Central University Emergency Military Hospital of Bucharest. The slides were examined with Leica DMRBE microscope.

All the included patients have given their consent for surgery and subsequent pathologic and immunohistochemical analysis.

All the data were prospectively recorded in a 2003 Excel worksheet, and statistically analyzed using SPSS, ver. 11 for Windows software. In order to establish correlations or compare considered variables *chi*-square test was used; as statistical significance were considered values of $p<0.05$ (95% confidence interval).

The main hypothesis was that patient' related factors, and also morphologic and histologic factors, have an influence on the presence and intensity of the protein markers expression, and subsequently on the stage at the moment of the diagnosis.

Results

This study was performed on 22 human colorectal cancer resection specimens (four proximal colon cancers, eight distal colon cancers and 10 rectal cancer specimens); there were 13 men and nine women, aged between 38 and 79-year-old (62.45±11.31-year-old).

Histologic structure was adenocarcinoma in 17 cases, neuroendocrine carcinoma in two cases, mixed neuroendocrine carcinoma and adenocarcinoma in two cases, and epidermoid carcinoma (one case); mucinous structure was diagnosed in four cases (18.19%). Sixteen cases (72.72%) were G1, G2 or G1+G2, while G3 tumors were encountered in six cases (27.27%). The vascular and neural tumoral invasions were considered very important prognostic parameters. In this study, the vascular invasion was present in 12 cases (six men and six women, 63.67±9.5-year-old), in proximal colon cancer (two cases), distal colon cancer (five cases), and five rectal cancers. Perineural invasion was diagnosed in 10 cases (four men and six women, 63±10.36-year-old), in proximal colon cancer (one case), distal colon cancer (five cases), and four rectal cancers. Both, perineural and vascular invasion was diagnosed in 10 cases (all cases with perineural invasion presented, also, vascular invasion).

P53 immunohistochemical study

In the studied cases, p53 immunohistochemical expression was positive in the tumoral cells (Figure 1) in 19 cases (86.36%) and negative in three cases (13.64%); the intensity of the p53 expression varied between 15% and 80% in the tumoral cells.

The main epidemiologic and morphologic aspects are presented in Tables 1–3.

Table 1 – ***The main results and relations between tumoral markers and patient's age and gender***

	n	%	<60 years	61–70 years	>70 years	Average (± std. dev.)	*p**	Women	Men	*p*
P53++	6	27.27	5	1	0	59.16±9.49		4	2	
P53-/+	13	59.09	4	2	7	65.15±11.23	*0.026*	4	9	*0.57*
P53-	3	13.64	2	1	0	57.33±13.69		1	2	
Bcl-2+	4	18.18	0	3	1	63.5±13.2		0	4	
Bcl-2-	18	81.81	9	3	6	62.22±11.63	*0.24*	9	9	*0.06*
PTEN++	18	81.82	8	5	5	61.47±11.67		7	11	
PTEN-/+ or -	4	18.18	1	1	2	68.66±8.73	*0.375*	2	2	*0.68*
Ki-67++	8	36.36	3	1	4	63.45±12.22		4	4	
Ki-67-/+	11	50	6	2	3	61.45±10.42	*0.341*	3	8	*0.51*
Ki-67-	3	13.64	2	1	0	58±17.77		2	1	

++ – Strong expression; -/+ – Weak expression; - – Negative. *The comparison was done between the younger age group (<60-year-old) and the older age group (>70-year-old).

Table 2 – ***The relations between tumoral markers and tumor's topography and stage***

	Proximal colon	Distal colon	Rectum	*p*	Stage I	Stage II	Stage III	*p*
P53++	0	2	4		1	2	3	
P53-/+	3	6	4		1	4	8	
P53-	1	0	2	*0.09*	2	0	1	*0.19*
Bcl-2+	0	3	1		2	1	1	
Bcl-2-	4	5	9	*0.15*	2	5	11	*0.17*
PTEN++	3	6	9		4	6	8	
PTEN-/+ or -	1	2	1	*0.44*	0	0	4	*0.13*
Ki-67++	1	3	4		1	3	4	
Ki-67-/+	2	5	4		1	3	7	
Ki-67-	1	0	2	*0.65*	2	0	1	*0.19*

Table 3 – ***The relation between tumoral markers and perineural invasion (NI), vascular invasion (VI) and both (perineural and vascular invasion – V&NI)***

	NI+	NI-	*p*	VI+	VI-	*p*	V&NI+	V&NI-	*p*
P53++	3	3		3	3		3	3	
P53-/+ or -	7	9	*0.79*	9	7	*0.79*	7	7	*0.9*
Bcl-2+	1	3		1	3		1	3	
Bcl-2-	9	9	*0.35*	11	7	*0.18*	9	7	*0.26*
PTEN++	6	12		8	10		6	10	
PTEN-/+ or -	4	0	*0.02*	4	0	*0.04*	4	0	*0.03*
Ki-67++	4	5		4	4		4	5	
Ki-67-/+ or -	6	7	*0.93*	8	6	*0.74*	6	5	*0.65*

There were five cases of adenocarcinoma with intense p53 positivity and one case of neuroendocrine carcinoma (five G1 and G2 and one G3 classification), while for negative p53 cases there were two adenocarcinomas and one epidermoid cancer, G2 in all cases.

Intense p53 expression was present in four pT3 and two pT2 tumors; all negative p53 tumors were staged as pT2. Lymph node invasion was present in three pN1 cases with intense p53 positivity, while in p53 negative tumors there was only one case with lymph node involvement.

Bcl-2 immunohistochemical study

Bcl-2 immunohistochemical expression was present (Figure 2) in four colorectal carcinomas, while in eight cases bcl-2 immunohistochemical expression was negative in tumoral cells but positive in peritumoral inflammatory or lymphoid tissue. The main epidemiologic, morphologic and histologic characteristics for bcl-2 positive and bcl-2 negative patients are presented in Tables 1–3.

All bcl-2 positive cases were histologically diagnosed as adenocarcinoma, poor differentiated in one case and well differentiated in other cases; no mucinous structure was encountered in any of these cases.

Two of the bcl-2 positive cases were pT2 staged, while the other two cases were pT3 staged; lymph node invasion was present in only one case, staged pN2.

PTEN immunohistochemical study

PTEN expression was positive (Figure 3) in 21 colorectal carcinoma cases, and negative in one case (4.54%); a poor PTEN expression was encountered in three cases (13.63%). The relations between PTEN characteristics and patients' age and gender, and tumors' topography and stage are presented in Tables 1–3.

All cases with negative or poor PTEN expression were adenocarcinoma, moderate (three cases) or poor differentiated (one case), with over 50% mucinous component in two cases; cases with intense PTEN positivity were well or moderate differentiated in 15 cases, and poor differentiated in three cases (p=0.221).

All cases PTEN negative or with poor PTEN expression were classified as pT3, compared with six pT2, 11 pT3 and one pT4 tumors with intense positive expression. Also, all negative or weak PTEN expression cases presented lymph node involvement, compared with eight cases with lymph node involvement for intense PTEN expression (p=0.077).

All cases with PTEN negativity or with poor PTEN expression presented nervous and lymphovascular invasion; in all cases lack the expression of p53 and bcl-2.

Ki-67 immunohistochemical study results

The immunohistochemical expression of Ki-67 was positive (Figure 4) in 19 cases (86.36%) and negative in three cases; in eight cases, the immunohistochemical expression was intense positive (over 50%).

Tables 1–3 present the results for immunohistochemical expression of Ki-67.

Women with positive Ki-67 immunohistochemical expression (seven cases) had a mean proliferative index of 45±6.45%, while Ki-67 positive men (12 cases) 47±16.03% (p=0.668); also, in Ki-67 positive young patients the mean proliferative index was 44.16±9.17%, while in elderly was 52±16.02% (p=0.363). The mean Ki-67 proliferative index in proximal colon cancer was 43.33±9.46%, in distal colon cancers 45±14.67%, and in rectal cancer 48±14.12% (p=0.688).

Ki-67 negative cases were adenocarcinoma (one case), neuroendocrine carcinoma (one case) and epidermoid carcinoma (one case); all the other cases were Ki-67 positive. The histologic grading was G1 and

G2 in five cases with intense Ki-67 expression (27.77%) and G3 in three cases (50% out of poor differentiated tumors have had intense positive Ki-67 expression); all cases Ki-67 negative were moderate differentiated. Also, well differentiated tumors have had a mean proliferative index of 35±7.07%, moderate differentiated tumors 44±12.51, while poor differentiated tumors have had a mean proliferative index of 50±12.24% (p=0.307).

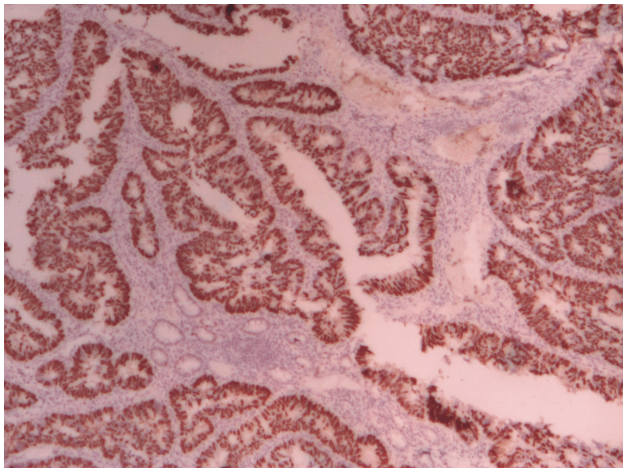

Figure 1 – ***P53 intense positive (90%) in the colonic adenocarcinoma tumoral cells (IHC, ×40).***

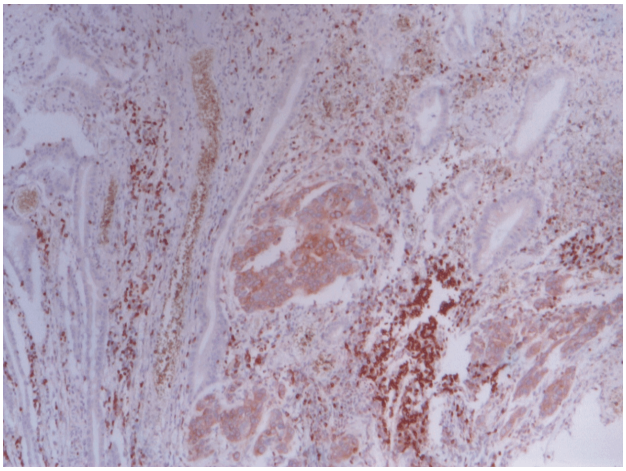

Figure 2 – ***Bcl-2 poor positivity in the tumoral cells and lymphoid infiltrate in a colon adenocarcinoma (IHC, ×200).***

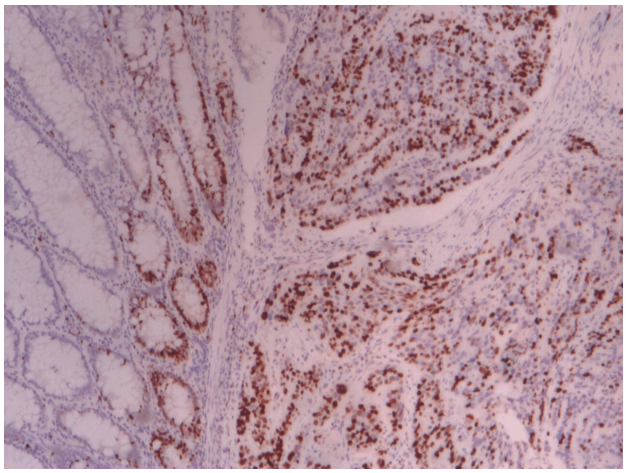

Figure 3 – ***80% positivity of PTEN in the tumoral cell, in a colon adenocarcinoma (IHC, ×200).***

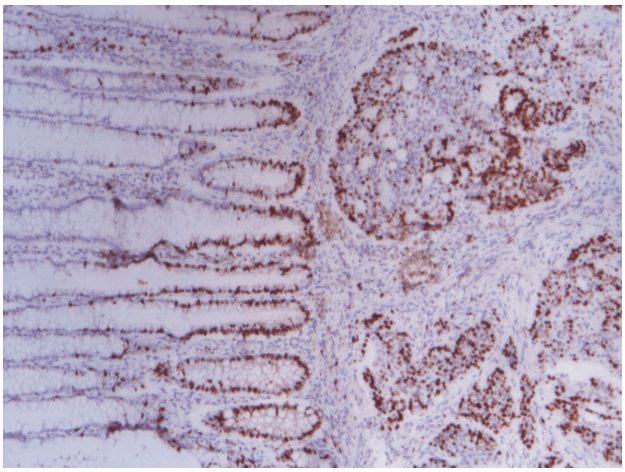

Figure 4 – ***Ki-67 positive (over 50% of the tumoral cells) in a colon adenocarcinoma (IHC, ×40).***

In intense Ki-67 positive cases mucinous structure was present in three cases; also, mucinous tumors had a mean proliferative index of 48.33±2.88%, while non-mucinous tumors 46±13.49% (p=0.363). Cases with perineural invasion had an average proliferative index of 47±12.61%, while in cases with no perineural invasion the mean proliferative activity was 45.55 ±15.09% (p=0.934); also, in cases with vascular tumoral invasion the mean proliferative index was 42±12.7%, while the other cases had a mean proliferative index of 48±14.14 (p=0.208). In the colorectal cancer cases with concomitant vascular and perineural tumoral invasion, the mean proliferative index was 46.87±12.61%, while cases with no vascular and no perineural invasion had a mean proliferative index of 49±13.21% (p=0.432).

Considering the tumoral stage, the Ki-67 negative tumors were classified as pT2 (two cases – 33.33%) and pT3 (one case – 6.67%), while intense positive Ki-67 tumors were staged as pT2 (one case – 16.67%) and pT3 (eight cases – 53.33%). In cases with tumoral extension limited to the colorectal wall (pT1 and pT2) the mean proliferative index was 37±7.5%, significantly lower than in cases with complete bowel wall invasion (pT3 and pT4 – 48±12.78%) (p=0.042).

Intense Ki-67 positive tumors were pN+ in seven cases (out of eight cases), weak Ki-67 positive tumors were pN+ in four cases (out of 11 cases), and Ki-67 negative tumors were pN+ in only one case (out of three cases) (p=0.24). The average proliferative index for pN1 colorectal tumors was 43±7.98%, for pN2 colorectal cancers 45±22.91%, and for pN0 47±14.86% (p=0.706).

P53/bcl-2 correlations

Due to the opposite prognostic significance of the p53 positivity (worse prognosis) and bcl-2 positivity (apparently favorable effect), we have compared bcl-2 positive/p53 low positivity cases with bcl-2 negative/ p53 intense positive cases. There were no bcl-2 positive/ p53 negative cases (possibly the most favorable association) in the studied group.

Out of seven cases with intense p53 positivity, there were two cases associating bcl-2 positivity; the association seems to have a good prognosis, since it was present both in stage I cases (T2N0M0), in two

men with left colon cauliflower adenocarcinoma, with moderate differentiation (G2). Also, in both cases was associated low Ki-67 positivity (40%), and also PTEN positivity.

The two cases with bcl-2 positivity/p53 low expression were diagnosed in advanced stages, with lymph node involvement (N2 in one case), with tumor invading the entire bowel wall, and also with nervous and lymphovascular invasion.

Bcl-2 negative/p53 intense positive cases (five cases) were diagnosed in stage III in 60% of the cases, in 80% of the cases the tumors were beyond the entire bowel wall; also, in 60% of these cases the nervous and lymphovascular invasion were present.

P53/Ki-67 correlations

Intense p53 positivity and intense Ki-67 positivity was present in three cases, two women and a man, with rectal cancers; in two cases the stage was T2N0, respectively T3N1 in one case. All cases associated intense PTEN positivity, and bcl-2 negativity.

Discussion

The tumor suppressor genes play an important role in colorectal carcinogenesis, acting mostly by inhibiting the apoptosis, thus favoring the tumoral cells replication; also, tumor suppressor genes seem to be involved directly in not only the patient's carcinogenesis and prognosis but also influencing the response to the cytotoxic drugs. Thus, knowing that effect, in some patients negatively influenced drugs may be avoided and in such case, other, more effective drugs can be used, leading to a more selective approach over the oncologic treatment.

On the other hand, Ki-67 proliferative antigen represents an important marker of tumoral cell proliferation, a higher index of Ki-67 seeming to correlate with tumoral aggressiveness.

Still, in the modern literature persists some debates, and even some contradictory results were published over the prognostic role of the p53, bcl-2, PTEN and Ki-67; the results of our study also contradict some of the published articles regarding the prognostic role of these genes.

P53 immunohistochemical analysis significance

The p53 tumor suppressor gene mutation determines an abnormal P53 protein, easily detectable immunohistochemical; this abnormal protein is involved in adenoma-carcinoma sequence but also in cancer dissemination and prognosis [7]. In the Zhao's study, the survival of the patients with p53 mutations was significantly lower than p53 negative cases (5 years survival rate of 32.5 *vs.* 71.7%) [3]; these results were confirmed by Watson NF *et al.*, which have demonstrated a 64 month average survival rate in the p53 positive cases compared with 76 month for the p53 negative cases (p=0.024) [4].

The p53 mutation was detected in 19 cases (86.36%), with an intensity of the expression varying between 15% and 80%; this is according to other studies, showing an over 50% colorectal cancer cases with immunohistochemically detected p53 [3, 4, 7]. In 68.42% of the cases, the intensity of the p53 mutation was below 50%. The immunohistochemical expression of p53 was higher in women (49%) than in men (43%), different from Rambau's study [7].

A higher p53 expression was detected in young patients (mean age 59.16±9.49 years), corresponding to other studies [7]; if we take into consideration the worse prognosis of the patients presenting p53 mutations, this could be a factor explaining the worse prognosis of colorectal cancer in young patients.

The p53 positivity was more intense in distal colon and rectal cancer, similar to the results of Rambau PF *et al.*, factor that could explain the different prognosis, but also pathogenic differences, between different locations of colorectal cancer [7].

In contradiction with the previously discussed results are the results of the histologic study, that showed an increased intensity of the p53 expression in well and moderately differentiated cancers (46.92% average intensity for G1 and G2 compared with 40% for G3 cases); also, in no intense positive cases the mucinous structure was present. This could represent a tendency for the p53 mutation to be present in differentiated colorectal cancers. These results contradict somehow the pejorative prognostic significance of p53 mutation, suggesting the implication of many other factors in the prognosis and pathogenesis of colorectal cancer [3, 7].

The intense p53 positivity was associated with an advanced stage now of diagnosis (50% of intense p53 positive cases were diagnosed in stage III) (p=0.19).

Bcl-2 immunohistochemical analysis significance

Bcl-2 is a gene with role in cell proliferation regulation; bcl-2 super expression can inhibit the apoptosis and induce tumorigenesis, and also it seems capable of inhibiting the therapeutic (chemo- or radiotherapeutic) apoptosis, being responsible for some failures of these treatments [8, 9].

The prevalence of the bcl-2 in tumoral cells was of 18.18% of the studied cases, smaller than in other studies [3, 4, 8]; this difference may be explained by the small number of cases in our statistics, but some differences in the immunohistochemical technique and interpretation of data may be also responsible.

The bcl-2 positivity was present only in men, different from other studies that have demonstrated a higher incidence of bcl-2 in women, not always significant. There was no statistical significance regarding age group distribution, nor in our study, or other studies (mean age for positive cases 63.5±13.2 years, compared with 62.22±11.63 years in bcl-2 negative cases) [8, 9]. The small number of cases bcl-2 positive in our study may also be responsible for these slightly differences.

Bcl-2 positivity was encountered in distal colon cancer (37.5% of distal colon cancers) and rectal cancer (10% of rectal cancers); no proximal colon cancers had bcl-2 positivity.

75% of bcl-2 positive tumors were well or moderate differentiated, only one case being poor differentiated, which could explain a better prognosis for bcl-2 positive

cancers [8, 9]. No bcl-2 positive tumors presented mucinous structure, different from Contu's study, which have demonstrated 38.1% of the bcl-2 positive cases with a mucinous structure [9].

75% of the bcl-2 positive cases were diagnosed in stage I and II, with only one case in stage III, similar with the study of Contu PC *et al.*, which found that below one third of the cases with lymph node involvement presented bcl-2 positivity; also, it appears that the incidence of bcl-2 positivity decrease with the transmural tumoral penetration increasing: 50% of the tumors limited to the colorectal wall have had bcl-2 positivity, compared with only 16.67% bcl-2 positivity for cases with complete or beyond colorectal wall invasion. These results could suggest a better prognosis for bcl-2 positive colorectal cancers, sustained by other studies [3, 9]. On the other hand, the study of Zhao DP *et al.*, have failed to identify a better 5 years survival rate for bcl-2 positive tumors compared with bcl-2 negative cancers, if the analysis is performed including all cancer stages [3]. An important conclusion of the Zhao's study is that Dukes B patients with bcl-2 negative tumors could benefit from adjuvant chemotherapy [3].

P53/bcl-2 immunohistochemical analysis significance

Many studies have been made in order to demonstrate a prognostic relation between p53 and bcl-2: it seems that the best prognosis in colorectal cancer is for bcl-2 positive/p53 negative patients, associated with a diagnosis in early stages, and a rare lymph node involvement at the time of the diagnosis; this association appear to be present in 25.9% of colorectal cancers, most of these patients having an average survival period longer than other associations [3, 4].

In our study, due to the small number of cases this association could not be analyzed. On the other hand analyzing bcl-2 positive/p53 positive but with a weak immunohistochemical expression and bcl-2 negative/ p53 intense positive cases the results were inconclusive, the number of cases in advanced stages of the disease being very high (50% *vs.* 60%), similar to the Zhao DP *et al.* study [3].

Regarding the same relation between p53 and bcl-2 in colorectal cancer, Jiang M and Milner J have demonstrated an inhibition for apoptotic activity of the p53 in the cancerous cells induced by bcl-2; suppressing bcl-2 activity resulted in a significant increase of p53 apoptotic activity, still dependent of Bax and caspase 2, as apoptotic essential mediators. This conclusion can lead to a new possible targeted therapy, anti-bcl-2 [10].

PTEN immunohistochemical analysis significance

PTEN (phosphatase and tensin homologue) is a tumor suppressor gene; mutational inactivation of PTEN leads to carcinogenesis and tumor growth stimulation, but also is associated with a poor response to Cetuximab. In colorectal cancer, PTEN mutation is very rarely encountered, in only about 2.7% of the cases [5, 6, 11].

In this study the incidence of PTEN negativity was 4.54% (only one case), and in two cases (9.09%) PTEN expression was weak (below 50%).

The lack of the immunohistochemical detection of PTEN in colorectal cancer is associated with an advanced stage of the disease, with nodal involvement, and also with a significantly lower 5 years survival rate, as demonstrated by Sawai H *et al.* study [12]. In our study, the same tendency to be diagnosed in advanced stages was evident for PTEN with negative or weak expression, even though the statistical significance was not reached: all cases presented the invasion of all the bowel layers, and lymph node invasion, even N2 in one case. The lack of the statistical significance is most probable due to the small number of cases.

Colorectal cancers with negative or poor PTEN expression have had a preferable topography for distal location; even though the statistical significance was reached (p=0.04), this could be determined only by the selection criteria.

There was no relation between the loss of the PTEN expression and age, gender and tumoral degree of differentiation; still, all cases with poor or negative PTEN expression presented lymphovascular and perineural invasion.

Ki-67 immunohistochemical analysis significance

Ki-67 is a nuclear protein that is present in all proliferative cells; there are important proliferative variations in colorectal cancer, as published in many studies [13, 14].

In our study, Ki-67 expression was present in 19 cases (86.36%) with a variation from 30% to 70% (average 46.17±12.43%). There were no significant differences in Ki-67 proliferative activity related to gender in colorectal cancer (p=0.668), and also there are no differences related to age (p=0.36) [13, 14].

Related to tumoral topography, there was a tendency for distal cancers to have a higher proliferative index, as well for poorly differentiated and mucinous tumors; still, none of these cases reached statistical significance. Thus, our results, even contradictorily to those of Nabi U *et al.*, are similar to those obtained by Jansson A *et al.* [13, 14].

There was no statistical significant difference considering the proliferative index Ki-67 and perineural and/or lymphovascular invasion (p=0.93, respectively p=0.208), similar to the study of Jansson A *et al.* [14].

The only histologic parameter that correlates with a higher proliferative index was bowel wall penetration, with 48±12.78% proliferative index for cases with transmural invasion, compared with 37±7.5% for cases with tumors limited to the bowel wall (p=0.042). Lymph node involvement did not correlate with Ki-67 proliferative index (p=0.706).

These results have confirmed the results of Jansson's study [14] and demonstrated that Ki-67 proliferative index do not correlates with clinicopathologic parameters, excepting the transmural tumoral penetration degree. Thus, although Ki-67 was considered as an important predictive parameter in colorectal cancer, these studies, and also Debucquoy A *et al.* study, have

failed to demonstrate the prognostic significance of Ki-67; still, Ki-67 could be used as an estimation parameter to the radiotherapy response in rectal cancer [15, 16].

The predictive value of Ki-67 in neoadjuvant chemo-/radiotherapy settings was confirmed, also, by the study of Kikuchi M *et al.*, which proposed a prediction score, based on Ki-67, Bax and thymidylate synthase expression diminishing [17].

P53/Ki-67 immunohistochemical analysis significance

There is also a presumable favorable prognostic significance in case of negative or weak expression of p53 and weak Ki-67 positivity (seven cases with presumed favorable prognosis) and intense positive p53 and intense Ki-67 expression (presumed poor prognosis). The favorable association was encountered in six men and one woman, 64.5±12.5-year-old, in two proximal colic cancers, three distal colic cancers and two cases of rectal cancer. There was five adenocarcinoma and two neuroendocrine carcinomas, G1/G2 in five cases and G3 in two cases, with vascular tumoral invasion in five cases and perineural invasion in three cases. Four cases presented lymph node invasion, and pT stage was pT2 in two cases, pT3 in four cases, respectively pT4 in one case.

The presumed prognostic negative association was present in two cases of rectal cancer, 58-year-old. The histologic structure was G2 adenocarcinoma, in one case, and neuroendocrine carcinoma in one case. One case presented vascular and perineural invasion. Both tumors were staged pT3, the neuroendocrine carcinoma presenting also lymph node invasion (classified as N1).

Regarding the p53/Ki-67 association, in our study it was not possible to establish a prognostic correlation, because the known invasiveness markers (lymphovascular and perineural involvement, poor differentiation degree, the entire bowel wall involvement and lymph nodes tumoral invasion) were relatively similar, regardless of the presence or negativity of these prognostic markers.

In conclusion, the association had no value, the invasiveness being relatively similar.

Correlation between prognostic markers, age, and gender in colorectal cancer patients

The only prognostic marker associated with a statistically significant difference between age group distribution was p53, with a significant tendency for intense positivity in younger patients (p=0.006): in older patients p53 mutations were absent or weak, while younger patients presented intense positivity for p53 in 55.55% of the cases. Consequently, this could be an explanation for the worse prognosis in young colorectal patients or, at least, it could explain the better prognosis for older patients due to the lack of mutational activity of p53. No other tumoral marker presented statistical significance regarding age distribution, nor patient's gender.

Correlations between tumoral markers, morphologic, and histologic tumoral parameters

There was no prognostic association between the presences of the immunohistochemical detected tumoral markers and tumor's topography; in other words, the different prognosis between different locations of colorectal cancers is not dictated by the presence and importance of the studied markers, but most probably due to other factors.

The presence/absence of the vascular tumoral invasion correlated with tumoral markers expression only for PTEN: all cases without vascular tumoral invasion had intense PTEN expression (p=0.03). Therefore, the intense PTEN expression (presumed favorable prognosis) correlates with other favorable prognostic marker: the absence of the vascular invasion (p=0.04). This results were similar in case of perineural invasion (versus absence of the perineural invasion), and also in the case of a concomitant perineural and vascular invasion, compared with the absence of the perineural and vascular invasion (p=0.02).

No other correlation between vascular and or neural tumoral invasion and the other tumoral markers could be established in our study.

There were also no significant correlations between pTNM stage and immunohistochemical expression of the studied tumoral markers: regardless of the stage, the tumoral marker's expression was relatively similar, with no significant differences.

Conclusions

P53 gene mutations have a higher incidence in colorectal cancer; an intense expression of p53 could be an explanation for the worse prognosis in some cases (young patients, distal colonic cancer), most of these cases being diagnosed in advanced stages of the disease.

Bcl-2 overexpression was rarely present in colorectal cancer; most of the bcl-2 positive cases were diagnosed in early stages, which could represent an explanation for the better prognosis of these cases.

The incidence of the mutational inactivation of PTEN in colorectal cancer was very low; negative or weakly expressed PTEN tumors had a tendency to be diagnosed in advanced stages, and also a tendency to be located on the distal colon.

Ki-67 proliferative index did not correlate with the clinicopathologic prognostic factors, excepting for the tumors with transmural colorectal invasion with a significantly higher proliferative index.

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