ORIGINAL

# Correlation between immunoexpression of BCL-2 protein family with apoptosis index, cellular proliferation and survival in colorectal carcinomas

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## Abstract

**Objectives:** To evaluate the Bcl-2, Bax, Bad and Bak immunoexpression in tumor and nontumorous tissue of 130 patients with colorectal carcinoma submitted to surgery at São Paulo Hospital, EPM/UNIFESP, from 2002 to 2005, and to correlate the immunoexpression data with the apoptotic index (AI, obtained by anti-cleaved caspase 3 and M30 labeling), cell proliferation score (CPS, obtained by Ki-67), immunoexpression of p53 and patient's clinical prognosis. **Results:** Positive correlation was verified between Bcl-2 protein family in tumor and nontumor tissue. Only Bcl-2 protein correlated with IA and CPS in the tumor. Positive correlation was observed between pro-apoptotic proteins and Bcl-2 protein. In the adjacent mucosa, Bcl-2 correlated with Ki-67 and p53, but not with IA. Carcinomas exhibited higher immunoexpression of CPS and IA markers. No correlation occurred between immunoexpression data and patient survival. **Conclusion:** Positive correlation was observed between the pro-apoptotic proteins of the Bcl-2 family and the anti-apoptotic protein Bcl-2. In the adjacent nontumor mucosa, Bcl-2 correlated with Ki-67 and p53, but not with AI. Carcinomas presented greater immunoexpression for CPS and AI markers; however immunoexpression of these markers was not correlated with patient survival.

**Keywords:** apoptosis, c bcl 2 proteins, cell proliferation, colorectal neoplasms, microarray analysis.

## INTRODUCTION

Colorectal carcinoma is an important cause of mortality and morbidity in Western populations[1]. In Brazil, colorectal carcinoma shows high frequency, especially in the Southern and Southeastern states (Ministry of Health, *Ministério da Saúde*, http://www.datasus.gov.br). For 2012, an incidence of 12.05 to 26.35 per 100,000 inhabitants is estimated in the male population and 13.42 to 28.22 cases per 100,000 inhabitants for women (National Cancer Institute, *Instituto Nacional do Câncer*, http://www. inca.gov.br/).

Colorectal carcinogenesis is a multiple and cumulative steps process, with mutational activation of oncogenes associated with inactivation of tumor suppressor genes[2,3] and the participation of several metabolic pathways of cell proliferation, DNA repair and apoptosis[4-7].

Studies involving tumor kinetics suggest that expansion of neoplastic colonic epithelial cells *in vivo* results not only from an increase in the cell proliferation index, but also from a decrease in the apoptosis index[8]. In addition, a lower proportion of apoptotic cells have been reported during the progression from benign tubular adenomas to invasive malignant tumors[9].

The Bcl-2 protein family regulates the distal steps in a highly conserved pathway during the evolution of programmed cell death or apoptosis[10,11]. Some members of this family are cell death inhibitors, like Bcl-2, while others are apoptosis promoters, such as Bax, Bak and Bad. Molecular interactions between pro-apoptotic and anti-apoptotic members, inducing homo- and heterodimers, are crucial events in the control of apoptosis[12,13]. These proteins contain up to four conserved extensions of amino acids homologous domains known as Bcl-2 (Bcl-2 homology (BH) domains)[12,13]. Anti-apoptotic proteins like Bcl-2 and Bcl-Xl show homology to BH domain four (BH1 to BH4). The pro-apoptotic proteins can be grouped into subfamilies 'multi-domain' and 'BH3only'. Multi-domain pro-apoptotic proteins such as Bax and Bak, show homology in the BH1 to BH3 domains, while the BH3-only proteins such as Bid, Bim, Bad, PUMA and Noxa, are structurally similar to the multi-domain family

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members, but the similarity is limited only to the BH3 domain. They act as pro-death flags and subsequently activate pro-apoptotic members BAX and BAK[14,15]. Once activated, BAX and BAK cause mitochondrial dysfunction and lead to the release of pro-apoptotic molecules such as cytochromec[16,17].

Many forms of apoptosis require direct activation of BAX and BAK at the mitochondria by a member of the BID, BIM, or PUMA family of proteins[18]. The relative proportions of these members determine the final sensitivity of cells to death stimulus. The Bcl-2 protein family, mainly Bax and BclX, also play a role in the maintenance of the integrity and fragmentation of mitochondria[19]. Ren et al.[18] showed that the BH3-only proteins BH3-interacting domain death agonist (BID), BCL-2-interacting mediator of cell death (BIM, also known as BCL2L11) and PUMA are essential for homo-oligomerization of BAX and BAK and for the initiation of apoptosis in response to several stimuli.

The intrinsic apoptosis pathway (mitochondrial mechanism), controlled by the Bcl-2 family, promotes activation of a proteolytic enzymes cascade, the caspases family members[20]. The mitochondrial pathway acts through mitochondrial membrane permeabilization, with the release of cytochrome c (electron carrier molecule in oxidative phosphorylation) together with the release of apoptosis induction factor (AIF) and endonuclease G[21,22].

Molecules of cytochrome c activate the caspase--dependent pathway in the cytoplasm involving the association of cytochrome c with pro-caspase 9 and Apaf-1, in strict association with cytokeratin 18[23]. The caspase--cleaved fragment of cytokeratin 18 (M30) has been used as a biomarker of apoptosis[24]. In the presence of ATP, this protein complex cleaves pro-caspase 9, leading to caspase 9 activation, which cleaves and activates pro-caspase 3 and pro-caspase 7, producing active caspase 3 and active caspase 7, respectively[21]. Following this activation, pro--caspase 6 is cleaved by active caspase 3 and additionally by pro-caspase 9[21,25]. Activated caspases 3, 6 and 7 cleave cytokeratin 18, forming the cleaved cytokeratin 18[26]. Activated caspases 3 and 7 cleave the poly- (ADP-ribose) polymerase (PARP), forming the cleaved PARP[27]. Activated caspase 6, subsequently to its cleavage by activated caspase 3[21], cleaves the nuclear lamin A, leading to cleaved lamin A[25] and finally to apoptosis.

Programmed cell death has emerged as a potential target for cancer treatment in diverse stages of tumoral progression. Chemoprevention, immunoregulation and metastasis are prospective targets where apoptosis mechanisms might be used in tumorigenesis prevention and management[28].

In order to understand the complex relationships between these multiple immunomarkers, the immunoexpression of Bcl-2 family proteins (Bcl-2, Bax, Bad and Bak), p53, apoptotic and cellular proliferation index were evaluated in colorectal cancer tissue of patients not previously submitted to radio- or chemotherapy and correlated with patient prognosis.

## MATERIAL AND METHODS

Colorectal cancer tissues of 130 patients seen at São Paulo Hospital, Paulista Medical School, São Paulo Federal University (EPM/UNIFESP), submitted to surgery between 2002 and 2005, and not submitted to radio- or chemotherapy, were included in the study. The patients were operated in temporal sequence, with no genetic or familiar background, and were genetically heterogeneous.

The samples, obtained from surgical specimens, were fixed in 10% formalin and routinely embedded in paraffin for histological analysis. Histological sections of 4 µm thickness were cut from each block and stained by Hematoxylin-Eosin. The microslides were evaluated for diagnostic confirmation and reevaluation of the histopathological findings, including the selection of sites for the removal of cylindrical cores used in Tissue Microarray (TMA) block construction.

TMA blocks were constructed using Beecher™ equipment (Beecher Instruments, Silver Spring, MD, USA) according to the manufacturer's instructions, in the following steps: 1) the selected area in the respective paraffin block was marked; 2) a cylindrical core was created in the receptor block using the apparatus; 3) a 1 mm cylinder of tissue was extracted from the area of interest; 4) the cylindrical tissues obtained from the donating block was transferred to the core in the receptor block; 5), new core positions were created in the receptor block, separated by fractions of 1 mm, such that a collection of tissue samples was created following the matrix arrangement; 6) the quality of the block was assessed before storing. An adhesive tape system (Instrumedics Inc, Hackensak, NJ, USA) was used to guarantee adhesion of the TMA block slices on the slides.

The streptavidin-biotin method was used in the immunohistochemical reaction. The primary antibodies used were monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:1000, monoclonal anti-Bad (H-168) (Santa Cruz Biotechnology) diluted 1:1000, monoclonal anti-Bak, (H-211) (Santa Cruz Biotechnology) diluted 1:2000, monoclonal anti-Bax, (N-20) (Santa Cruz Biotechnology) diluted 1:1500, monoclonal p53 (Bp53-12, sc-263) antibody (Santa Cruz) diluted 1:1500, monoclonal anti-Ki-67 (clone MIB-1, M7240) antibody (DakoCytomation, CA, USA) diluted 1:100, polyclonal anti-cleaved-caspase-3 (AP1027) antibody (Calbiochem) diluted 1:1000, and anti-cleaved cytokeratin 18 (M30 CytoDEATH) antibody (Roche, NJ, USA) diluted 1:500. The amplification system was the LSAB+ System HRP kit (Dako, CA, USA). The color was developed with 3.3`Diaminobenzidine (DAB) (Sigma Chemical Co., MO, USA), which was counterstained with Harris hematoxylin.

Positivity for Bcl-2 protein family occurs in the cytoplasm (Figure 1A-D), for Ki-67 and 53 in the nucleus, and for caspase-3 (Figure 2A) and M30 (Figure 2B) in the nucleus or cytoplasm. Histological sections previously proven as positive for these markers were used as positive controls. Slides omitting the primary antibody were used as negative controls.

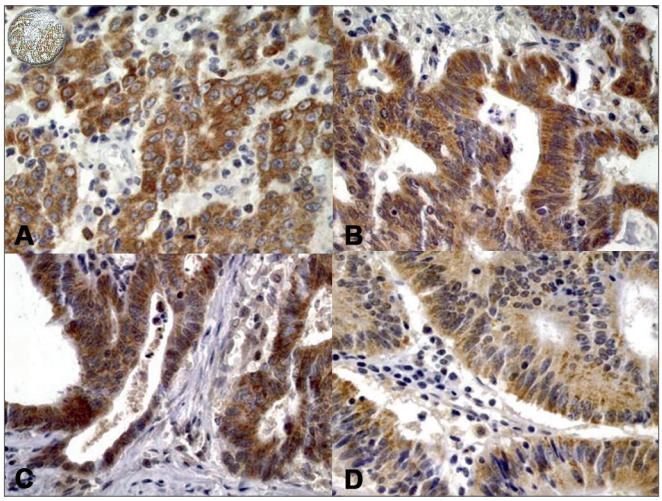


**Figure 1.** (A) Positivity pattern for Bcl-2; (B) Bad; (C) Bak; and (D) Bax. At bottom: lower view of Bcl-2.

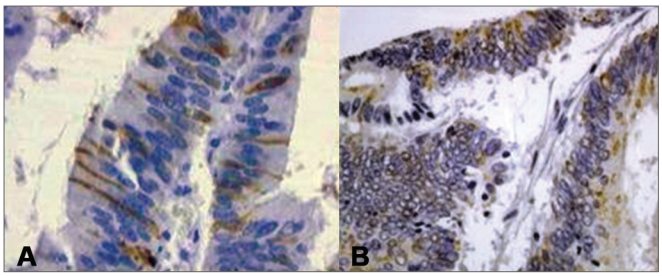


**Figure 2.** (A) Positivity pattern for cleaved caspase-3 and (B) cleaved cytokeratin 18 (M30).

The immunoexpression of Bcl-2, Bax, Bad, Bak, p53 and Ki-67 was evaluated based on stain intensity, scored from 0 to 3, with 0 considered negative, 1 as weak, 2 as intermediate and 3 as strong[29]. The number of positive cells was evaluated on a scale of 0 to 3, where 0 corresponded to 0 to 10% of cells, 1 to 11 to 25%, 2 to 26 to 50% and 3 to more than 51% of cells. The slides were evaluated independently by three experienced pathologists, with no interobserver variability and no information regarding patient's conditions. A score was obtained for each reaction by multiplying the intensity of the reaction by the percentage of positive cells. The immunoexpression of the markers was evaluated, both in tumor tissue and in the adjacent mucosa[30]. The immunoexpression of markers presenting different histological types, such as tubular, mucinous and medullar, were evaluated separately. Evaluation of the apoptotic index (AI) was based on the immunoexpression of cleaved caspase-3 and M30 (cleaved cytokeritin-18). ImageLab™ software and image capture system and an Olympus™ trinocular microscope, model BX-40 were used for evaluation. Two hundred positive cells were counted in "hot-spot" areas[31].

The patients were grouped in low/negative expression and intermediate/strong expression of the markers. The cutoffs for these groups were obtained by plotting the data in histograms and represented the end of the first peak. Patient survival between the two groups was studied by the Kaplan-Meier method and compared by the log-rank test. Time of surveillance was counted from the day of diagnosis to the day this work was finished.

The Spearman Rank-Difference Coefficient of Correlation was used to evaluate the immunoexpression of each marker in the tumor and in the nontumorous mucosa.

To verify the correlation between immunoexpression of the tumor tissue and the nontumorous mucosa, the *t* test for continuous variables was used (cleaved caspase-3, M30 and Ki-67). The Mann-Whitney test was used for the remaining markers. All tests were two-tailed.

Expression of the proteins in the distinct histological types was compared using nonparametric analysis of variance (Kruskal-Wallis test). When this test demonstrated $p < 0.05$, Dunn's post-test for multiple comparisons was used to specify the differences.

The immunoexpression of the different markers between tumors of low/intermediate versus high histological grades was analyzed by the Mann-Whitney test.

The software used for the analyses was Prism 4.0 (Graph Pad Software Inc., San Diego, EUA, 2003) and BioEstat 4.0 (Belém, Brazil, 2005).

## RESULTS

### Bcl-2 protein family in the tumor

The majority of the tumor samples (72%) did not show Bcl-2 immunoexpression. In contrast, Bax exhibited a strong immunoexpression in 81% of cases, with weak or negative staining in 10% and 9%, respectively. Bad and Bak exhibited strong immunoreaction in 49% and 50% of the cases, respectively, and weak or negative staining in the other half.

### Cleaved caspase-3, M30, Ki-67 and p53 immunoexpression in the tumor

Most tumors presented high AI evaluated by cleaved caspase-3 and M30 with percentage of positive cases of 62% and 42%, respectively. Little more than half of all tumors showed low immunoexpression of Ki-67 and p53.

### Correlation of the markers between tumor and adjacent mucosa

Table 1 shows the correlation between Bcl-2, AI, Ki-67 and p53 in tumor tissue. Table 2 shows the same correlation scores in the adjacent mucosa. Markers with positive correlations in their immunoexpression are in bold letters. A direct correlation was observed between Bad, Bax, Bak and anti-apoptotic Bcl-2 in the tumors. Bad and Bcl-2 correlated with AI; therefore, positive correlation was observed between Bad and p53 and Bad and Ki-67. In addition, Bcl-2 also correlated with Ki-67.

**Table 1.** Statistical correlation between Bcl-2 protein family, AI, Ki-67 and p53 in tumoral tissue (Spearmann non-parametric test).

| Tumor | | | | |
|---|---|---|---|---|
| | | *p* | r | IC 95% |
| BAD | p53 | $p < 0.0001$ | 0.3230 | 0.1546 to 0.4731 |
| BAD | Caspase-3 | $p < 0.0001$ | 0.3240 | 0.1558 to 0.4740 |
| Bcl-2 | Caspase-3 | $p < 0.0001$ | 0.3483 | 0.1824 to 0.4950 |
| BAD | Bcl-2 | $p < 0.0001$ | 0.3856 | 0.2236 to 0.5268 |
| BAD | BAK | $p < 0.0001$ | 0.5031 | 0.3578 to 0.6247 |
| CASPASE-3 | M30 | $p < 0.0001$ | 0.5164 | 0.3728 to 0.6359 |
| BAD | Ki-67 | 0.0004 | 0.2926 | 0.1217 to 0.4467 |
| M30 | KI-67 | 0.0007 | 0.2790 | 0.1064 to 0.4353 |
| Bcl-2 | Ki-67 | 0.0009 | 0.2706 | 0.09810 to 0.4273 |
| BAD | BAX | 0.0009 | 0.2778 | 0.1015 to 0.4372 |

**Table 2.** Statistical correlation between Bcl-2 protein family, AI, Ki-67 and p53 in adjacent mucosa (Spearmann non-parametric test).

| Adjacent mucosa | | | | |
|---|---|---|---|---|
| | | *p* | r | IC 95% |
| BAD | Bcl-2 | $p < 0.0001$ | 0.4721 | 0.3179 to 0.6020 |
| BAD | BAK | $p < 0.0001$ | 0.5790 | 0.4448 to 0.6877 |
| CASPASE-3 | M30 | $p < 0.0001$ | 0.3459 | 0.1769 to 0.4950 |
| Bcl-2 | Ki-67 | $p < 0.0001$ | 0.3402 | 0.1706 to 0.4902 |
| BAD | BAX | $p < 0.0001$ | 0.4626 | 0.3040 to 0.5962 |
| BAK | Bcl-2 | $p < 0.0001$ | 0.4693 | 0.3153 to 0.5992 |
| BAK | BAX | $p < 0.0001$ | 0.4368 | 0.2751 to 0.5744 |
| Ki-67 | P53 | $p < 0.0001$ | 0.3365 | 0.1680 to 0.4859 |
| Bcl-2 | p53 | $p < 0.0001$ | 0.3544 | 0.1863 to 0.5024 |
| BAX | Bcl-2 | 0.0001 | 0.3290 | 0.1554 to 0.4829 |

In the adjacent mucosa, correlation was verified between Bad and Bax and Bak, while Bcl-2 correlated with Bax, Bad and Bak. Furthermore, positive correlation was verified between Bak and Bax. No correlation occurred between Bcl-2 protein family members and AI markers in the adjacent mucosa. In contrast, Bcl-2 correlated with both Ki-67 and p53 in the adjacent tissue.

### Immunoexpression of the markers in the tumor and the adjacent mucosa tissue

A significant difference was observed between Bad and Bax immunoexpression in the tumor and adjacent mucosa (Figure 3A-B), with strong Bad immunoexpression in the adjacent mucosa. Significant scores for Bax were verified in the tumor. Similar results were obtained for AI with cleaved caspase 3 and M30 (Figure 4A-B) and for Ki-67 and p53 (Figure 5A-B). No differences were observed in Bcl-2 and Bak reactivity between tumor and adjacent mucosa tissue.

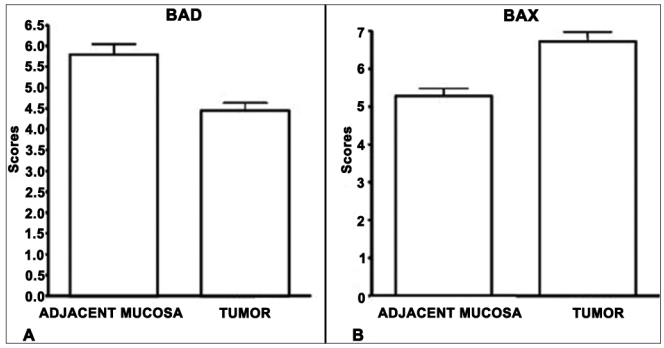


**Figure 3.** Difference between Bad and Bax immunoexpression in (A) tumor and (B) nontumor adjacent mucosa.

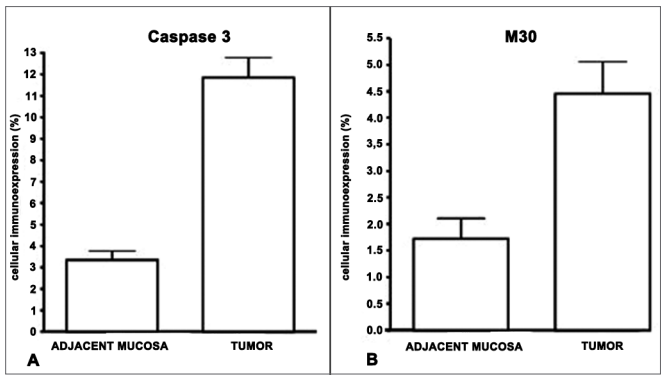


**Figure 4.** Difference between Caspase 3 and M30 immunoexpression in (A) tumor and (B) nontumor adjacent mucosa.

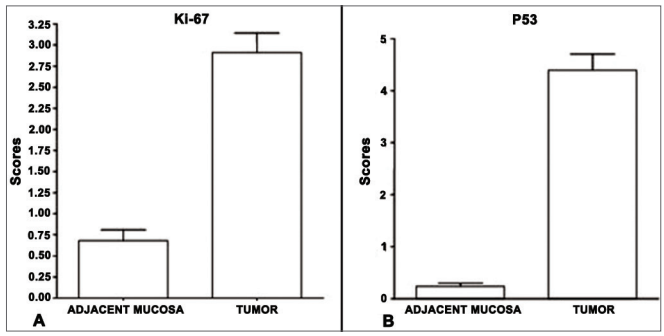


**Figure 5.** Difference between Ki-67 and p53 immunoexpression in (A) tumor and (B) nontumor adjacent mucosa.

**Immunoexpression of markers and tumor grade and histological types**

No difference between the immunoexpression of the markers was observed between low grade and high grade tumors. Regarding the immunoexpression in different histological types, only Bax showed a statistical difference, with strong labeling in tubular adenocarcinomas compared to mucinous adenocarcinomas.

**Correlation between markers and patient survival**

Survival data were possible to be obtained from 107 of the 130 patients. Table 3 presents the cutoff obtained for markers in the histograms. Kaplan-Meier curves showed no differences for patient survival between intermediate/high and low/negative immunoexpression groups.

**Table 3.** Cut-offs and *p*-value from Log-rank test of survival curves constructed by Kaplan-Meier test.

| Markers | Cut-off | *p* | n |
|---|---|---|---|
| Ki-67 | < 4 | 0.6082 | 111 |
| p53 | < 4 | 0.6114 | 111 |
| Bcl-2 | < 2 | 0.6698 | 111 |
| M30 | < 3 | 0.7012 | 110 |
| BAX | < 6 | 0.7904 | 106 |
| BAK | < 6 | 0.9235 | 111 |
| Caspase-3 | ≤ 6 | 0.9333 | 111 |
| BAD | < 6 | 0.9543 | 111 |

## DISCUSSION

Colon epithelium is a unique model of continuous tissue renovation, containing cells in different stages of proliferation and differentiation[32]. It has been proposed that the transformation of normal colorectal epithelium into carcinoma involves the progressive inhibition of apoptosis [32-36]. Apoptotic cells are generally more numerous in malignant tumors than in normal adult tissue[37]. High grade adenomas and colon carcinomas are reported to present greater cellular proliferation and less apoptosis than their respective precursor lesions[33,38-41]. Clinically aggressive tumors show increase in both the mitotic and apoptotic indices[42-44].

The role of proteins involved in apoptosis and the cell cycle has previously been studied in the adenoma--cancer sequence of colorectal tumorigenesis[3,29,45-47]. These studies demonstrated that p53 and Ki67 immunoexpression are greater in tumor samples than in normal mucosa[48]. In this study, greater expression of p53, Ki67 and apoptotic index were observed when comparing tumor and adjacent mucosa samples, demonstrating that cellular proliferation and apoptosis were increased in the tumors. Despite the apparent paradox, the rate of apoptosis and cellular proliferation in tumors regulates the velocity of tumor growth and its induction is related to the therapeutic response[49].

Similar to the present study, low Bcl-2 immunoexpression was reported by Zlobec et al.[50] while studying a series of 87 rectal adenocarcinomas. They observed that 76% of the tumors presented complete absence of Bcl-2 expression and that approximately 72% of these tumors were p53 positive. Krajewaska et al.[29] were the first to evaluate immunoexpression of Bcl-2, Bax and Bak. In 30 colorectal adenocarcinomas and 24 adenomas, Bcl-2 immunoexpression was significantly lower in tumors than in normal mucosa and in adenomas and lower in less-differentiated than in well-differentiated tumors. The results suggested that diminished Bcl-2 expression was a later event associated with the progression of colorectal carcinogenesis. The same was observed for Bak expression. Krajewaska et al.[29] observed no difference in the immunoexpression of this marker compared to normal mucosa or among the different histological tumor grades. In this study, no difference in Bak immunoexpression was verified in relation to histological grade; however, greater immunoexpression was detected in the tumor than in the nontumor mucosa. This finding suggests an ineffective attempt to block tumor progression. Loss of BCL2L10 protein expression shown by immunohistochemistry associated with real-time polymerase chain reaction (PCR) and immunoblotting predicts poor clinical outcome in gastric carcinoma[51]. Data obtained through *in vitro* research suggest that the regulation of endoplasmic reticulum (ER) membrane permeability by Bcl-2 proteins could be an important molecular mechanism of ER stress--induced apoptosis[52]. Wang at al. reported that in a process dependent on the proapoptotic Bcl-2 members, Bax and Bak, exogenously expressed fluorescent protein localized to the ER lumen, is released into the cytosol in cells undergoing ER stress[52]. Upon ER stress induction, endogenous ER luminal proteins are also released into the cytosol in a similar manner accompanied by translocation and anchorage of Bax to the ER membrane. In addition, Bax and truncated-Bid (tBid) mediate a global increase in ER membrane permeability to ER luminal proteins *in vitro*.

No difference between the immunoexpression of Bcl-2 and Bak was observed in nontumoral mucosa and in tumor samples, perhaps due to the fact that the nontumoral mucosa adjacent to the carcinoma already presented certain molecular alterations. Moreover, no differences were observed in the immunoexpression of these markers in the different grades of tumor differentiation, possibly a reflection of the uniformity of the series, composed mainly (more than 96%) of moderate and well-differentiated tumors. Moderately differentiated tumors were grouped with the well-differentiated tumors. Krajewaska et al.[29] classified tumors into well-differentiated and less-differentiated tumors, the latter including moderately differentiated tumors.

Among research groups, wide variation can be observed in the criteria used to classify carcinomas into high and low marker immunoexpression. Our group opted for

the construction of histograms with the values obtained for each marker to define the threshold between high and low immunoexpression. Separation of these groups based on histograms made the threshold definition more natural and representative.

Chen et al.[53] observed increased Bcl-2 expression in the tumor samples compared to the nontumoral mucosa. The authors suggest that Bcl-2 overexpression might be related to high levels of cyclooxygenase-2 (Cox-2) in colorectal cancer, since prostaglandin E2, a metabolite of Cox-2, induces the expression of Bcl-2 and inhibits apoptosis[54]. Considering Bax immunoexpression, the same authors showed increased immunoexpression of this marker in tumors compared to the nontumoral mucosa, similar to the present study. These findings appear paradoxical, since Bax, a pro-apoptotic molecule, neutralizes the function of Bcl-2 by homo or heterodimerization. Indeed, it is known that Bcl-2 overexpression retards or inhibits the conformational alteration of Bax required to induce apoptosis[55]. In contrast to Bcl-2, increased Bax immunoexpression sensitizes the tumor cells to apoptosis induced by several agents[56]. Thus, the equilibrium between Bcl-2 and Bax appears to determine whether or not the cell will be susceptible to apoptosis. In this study, no correlation between immunoexpression of the markers and patient survival was observed. The survival and mortality data evaluated for a period of 37 to 60 months showed that the majority of patients were alive and free of disease. A longer follow-up is required to confirm the present data.

While studying colorectal carcinoma using TMA, El Hameed[57] showed no correlation between the clinical--pathological data and p53 immunoexpression. However, survivin expression was greater in Bcl-2 positive tumors and survival among survivin-positive patients was significantly greater.

Rodel et al.[58] studied survivin, p53, Bcl-2 and AI in colorectal adenocarcinomas of patients submitted to preoperative radio and chemotherapy. When comparing the five-year survival rates, distal metastasis risk, recurrence risk and AI, the authors observed an inverse correlation for survivin in relation to AI and five-year survival. Moreover, a direct correlation between metastasis risk and local recurrence was detected. No correlation was observed between Bcl-2 and survivin or patient survival.

Finally, the positive correlation detected between the immunoexpression of pro-apoptotic proteins Bax, Bad and Bak with the anti-apoptotic protein Bcl-2 in colorectal carcinomas suggests a loss of equilibrium of normal homeostasis. In the nontumor adjacent mucosa, Bcl-2 correlated with Ki-67 and p53, but not with AI, suggesting an immunophenotype with a tendency toward uncontrolled cellular proliferation preceding the apoptosis disturbance.

In conclusion, the carcinomas presented greater immunoexpression for cell proliferation index and apoptotic index markers, findings that could be related to the growth velocity of each particular tumor and appear to be correlated with better long-term prognosis in this group of patients. However, based on the relatively short follow-up in this study, immunoexpression of the markers did not directly correlate with patient survival.

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