ARQGA/1478

ANALYSIS OF THE IMMUNOHISTOCHEMICAL EXPRESSIONS OF p53, bcl-2 AND Ki-67 IN COLORECTAL ADENOCARCINOMA AND THEIR CORRELATIONS WITH THE PROGNOSTIC FACTORS

Hunaldo Lima de **MENEZES**[1], Mário Jorge **JUCÁ**[2], Edmundo Guilherme de A. **GOMES**[3], Benício L. Bulhões B. P. **NUNES**[1], Henrique Oliveira **COSTA**[4] and Delcio **MATOS**[5]

ABSTRACT – ***Context*** - Search of tumors markers that allow treatment with higher survival rates, and indicate the response to treatment and recurrence of cancer – ***Objective*** - To analyze the immunoexpression of the proteins p53, bcl-2 and Ki-67 in colorectal adenocarcinoma and correlate them with the clinical-pathological prognostic factors. ***Method*** - Tissue microarray paraffin blocks were made from colorectal adenocarcinoma tissue resected from 82 patients who had undergone surgery but not chemotherapy or radiotherapy, at "Hospital São Paulo", São Paulo, SP, Brazil, between 2002 and 2005. Thin sections (4 μm) were subjected to immunohistochemical reactions, and immunoexpression staining scores were obtained. The scores were correlated with the degree of cell differentiation, staging, disease-free interval, recurrence, survival and specific mortality. The study variables were analyzed using the chi-square and Kaplan-Meier tests to investigate associations with the markers. The significance of the differences between the curves of the disease-free interval and survival was analyzed using the Logrank and Wilcoxon tests. ***Results*** - The immunohistochemical expression of p53 was positive in 70 tumors (85.4%) and negative in 12 (14.6%). The expression of bcl-2 was positive in 26 (31.7%) and negative in 56 (68.3%). The expression of Ki-67 was positive in 62 (75.6%) and negative in 20 (24.4%). There was no statistically significant correlation between the expressions of these markers separately or in conjunction, in relation to the degree of cell differentiation, staging, disease-free interval, survival and specific mortality. In relation to recurrence, there was a statistically significant correlation with positive expression of Ki-67 ($P = 0.035$). ***Conclusion*** - The immunohistochemical expression of Ki-67 in colorectal cancer is associated with recurrence of this disease.

HEADINGS - Colorectal neoplasms. Adenocarcinoma. Immunohistochemistry. Tumor suppressor protein p53. Proto-oncogene proteins c-bcl-2. Bcl-2 homologous antagonist-killer protein.

INTRODUCTION

There are 943,000 new cases of colorectal cancer per year. It is the second most prevalent malignant neoplasm in the world (after breast cancer), and an estimated 2.4 million people have been living with this diagnosis over the last 5 years. In Brazil, in 2008, it was estimated that 27,000 new cases of colorectal cancer appeared, and it was the fifth most frequent malignant tumor[3, 13].

Since early diagnosis is directly related to treatments with greater survival rates, many tumor markers have been studied because they indicate the presence, extent and response to treatment of this neoplasm, along with predicting recurrences. Carcinoembryonic antigen (CEA) is one of the most extensively studied tumor markers. Despite its low sensitivity for early-stage colorectal cancer (between 20% and 40%), CEA is still considered to be a standard serum tumor marker for following up colorectal cancer patients, with sensitivity greater than 90% for detecting recurrences[6].

An ordered sequence of non-random events leads to the development of colorectal cancer, in which the epithelium goes through an invasive transformation. The progression from intestinal epithelium to the development of invasive carcinoma is estimated to take between 7 and 12 years[15]. This cascade of mutations is correlated with progression of the neoplasm. The

Work performed within the Postgraduate Program on Surgical Gastroenterology, Federal University of São Paulo (UNIFESP) – Escola Paulista de Medicina.
No conflicts of interest to declare. Work performed with own resources.
[1] Postgraduate Program on Surgical Gastroenterology, Federal University of São Paulo, "Escola Paulista de Medicina" (UNIFESP-EPM); [2] Discipline of Coloproctology, School of Medicine, Federal University of Alagoas (FM-UFAL); [3] Department of Surgery FM-UFAL Maceió, AL, Brazil; [4] Department of Pathology, State University of Health Sciences of Alagoas, Maceió, AL, Brazil; [5] Discipline of Surgical Gastroenterology, UNIFESP-EPM São Paulo, SP. Brazil.
Correspondence: Dr. Hunaldo Lima de Menezes - Condomínio Aldebaran Ômega, H-8 – Tabuleiro - 57080-900 – Maceió, AL, Brazil. E-mail: hunaldomenezes@arthurramos.com.br

p53 gene is one of the most prevalent markers in neoplasia, with abnormal presence in 60% to 80% of colorectal tumors. The p53 locus is at 17p13 and it encodes a 53-kDa protein and 393 amino acids that characteristically are expressed when DNA undergoes some type of damage. In such events, the p53 protein binds to the site of the damaged DNA and halts the cell cycle at the G1 phase, thereby activating repair mechanisms for DNA or even apoptosis. Increased quantities of this cell protein are associated with interruption of the cell cycle and programmed cell death (apoptosis). When mutated, p53 transforms cell division into an uncontrolled process and induces the formation of tumors[18]. Loss of the p53 gene is crucial for the transformation of colorectal adenoma into carcinoma. The immunohistochemical expression of p53 may have prognostic value among patients with colorectal cancer, because greater protein detection has been shown in tumors with greater lymph nodal involvement. Moreover, 5-year survival is lower in cases with tumors that are positive for p53, compared with negative cases. Because of this function of detecting DNA abnormalities and consequent cell correction or death, the p53 protein is considered to be a genome guardian and is an important element in preventing tumor development. Its encoding gene is classified as a tumor suppressor gene[26, 29].

While p53 has been implicated in controlling the cell cycle and DNA synthesis and repair, as well as programming cell death, expression of the bcl-2 gene promotes cell survival because it opposes the stimulation of apoptosis. The bcl-2 protein is known for postponing programmed cell death, by inhibiting apoptosis, propagating cell division and potentially contributing towards tumor growth. Many studies have shown that the bcl-2 protein prolongs the survival of a variety of cells, thus blocking apoptosis. Consequently, in normal human tissues, bcl-2 appears only in the tissues in which apoptosis has a role in the development of complex structures or in which it promotes cell renovation[23]. Among the proteins of this family that have been studied most are Bax (pro-apoptotic) and bcl-2 (anti-apoptotic). The latter is super-expressed in colorectal adenomas and carcinomas. Studies have shown the following data regarding bcl-2 expression and apoptosis in colorectal tumorigenesis: in 17 out of 24 colon adenomas (71%) and in 14 out of 21 adenocarcinomas (67%), immunoreactivity of bcl-2 could be detected. In the majority of clinical trials, the expression of cytoplasmic bcl-2 has been associated with better prognosis. However, other studies have shown an association between bcl-2 and low survival[11].

Classically, neoplasia is consequential to cell proliferation disorders. Quantification of cell proliferation activity in neoplasms has been the target of many investigations. Since the development of the Ki-67 monoclonal antibody by Gerdes in 1984, its use has become increasingly popular as a method for measuring cell proliferation. The antigen defined by the Ki-67 monoclonal antibody is a human nuclear protein found in all active parts of the cell cycle, and it is widely used as a proliferation marker. The immunoexpression of Ki-67 has been related to tumor growth in various types of malignant neoplasia. However, its correlation with clinical-pathological parameters has been inconsistent[7].

In this research, the objective was to analyze the immunoexpression of the proteins p53, bcl-2 and Ki-67 in colorectal adenocarcinoma and correlate them with the clinical-pathological prognostic factors.

METHODS

Eighty-two colorectal cancer patients who underwent operations at "Hospital São Paulo", within the Discipline of Surgical Gastroenterology of the Federal University of São Paulo, "Escola Paulista de Medicina" (UNIFESP-EPM), São Paulo, SP, Brazil, between May 2001 and March 2005, were analyzed in a retrospective longitudinal clinical study. Patients were included in the study not only when their operations were elective (with clinical staging established preoperatively) but also when they underwent emergency operations. The other inclusion criteria used were that there should be no incidence of chronic degenerative diseases and that the clinical follow-up should be at least 6 months, so as to not compromise survival. The exclusion criteria were: other concomitant malignant pathological conditions, metachronic colorectal cancer, non-resected tumors, previous use of antineoplastic therapy and lack of postoperative clinical follow-up. The research project was analyzed and approved by the Research Ethics Committee of UNIFESP-EPM, registered under the number CEP 1431/05.

Out of the 82 patients, 44 were women (53.7%) and 38 were men (46.3%). Their ages ranged from 29 to 89 years, with a mean of 59 years. The length of follow-up ranged from 6 to 64 months, with a mean of 35 months. Regarding the location, 27 cases (32.92%) presented a tumor in the right colon, 26 (31.71%) in the rectum, 19 (23.17%) in the left colon and 8 (9.76%) in the transverse colon. There were also 2 cases (2.44%) of synchronous tumors. Sixty-seven elective operations (81.7%) and 15 emergency operations (18.3%) were carried out.

Resection in which no residual disease could be observed macroscopically, and in which the anatomopathological examination of the resected material did not show infiltration of the safety margins, were considered curative. Following these criteria, 69 patients (84.1%) were deemed to have undergone curative surgery and 13 (15.9%), palliative surgery. The staging of the tumors was carried out by means of the international classification for malignant tumors (TNM) of the International Union Against Cancer. This distribution is presented in Table 1.

TABLE 1. Staging of the colorectal tumors according to the TNM classification

Stage	Frequency	%
I	10	12.2
II	32	39.0
III	26	31.7
IV	14	17.1
Total	82	100.0

The disease-free interval was taken to be the time, starting from the curative surgery, during which there was no local or distant tumor recurrence. The disease-free interval ranged from 6 to 64 months.

Recurrence was defined as the return of the neoplastic disease after a disease-free interval, and could occur locally or at a distance. Metachronic tumors were those found in other segments of the colon that were diagnosed during the follow-up and not considered to be metastases of the primary tumor. Recurrence was observed in 18 patients (21.95%) during the follow-up.

Survival was taken to be the time elapsed between the curative surgery and the date of the last consultation, the last telephone contact, or even the patient's death. The minimum survival period used by this study was 6 months, and survival ranged from 6 to 64 months.

According to the recommendations of the American College of Pathology[4], well and moderately differentiated tumors were grouped together and called a low degree of malignancy, while the poorly differentiated and undifferentiated were called high grade of malignancy.

Specific mortality was defined as the percentage of deaths that occurred as a result of the colorectal cancer. In this sample, there were 16 deaths up to September 2007, thus representing a mortality rate of 19.5%.

Anatomopathological reports were surveyed within the Discipline of Pathological Anatomy of UNIFESP-EPM. The colorectal carcinoma tissue and adjacent non-tumoral mucosal tissue from the surgical specimens were fixed in 10% formalin and routinely processed by means of the method of paraffin embedding for histological analysis. Histological sections of 3 μm in thickness from each block, stained using hematoxylin-eosin, were reviewed by three pathologists to confirm the diagnosis, reevaluate the histopathological findings and select the sites for extracting cylinders of tissue for building up a tissue microarray sample.

For the immunohistochemical reaction, the streptavidin-biotin method was used. This is based on the capability of streptavidin to bind to biotin. The primary antibodies used in the study were: anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology) diluted at 1:1000; anti-p53 monoclonal antibody (Bp53-12, sc-263) (Santa Cruz) diluted at 1:1500 and anti-Ki-67 monoclonal antibody (MIB-1 clone, M7240) (DakoCytomation) diluted at 1:100. The amplification system that was used in the reaction was the LSAB+System kit, HRP (Dako, CA, USA). To develop the reaction, the substrate used was hydrogen peroxide (H_2O_2), plus the chromogenic agent 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co., USA), with final counterstaining using Harris hematoxylin.

The positivity pattern for anti-protein primary antibodies of p53 and bcl-2 was the appearance of a chestnut-brown staining in the cell cytoplasm. The presence of anti-Ki-67 primary antibody was taken to be the appearance of a dark brown stain in the nuclei. Slides of histological sections previously confirmed to be positive for these markers were used as positive controls. The same slide was used as a negative control by subtracting the primary antibody from the reaction.

The criteria used to evaluate the p53, bcl-2 and Ki-67 markers were based on the use of a categorical score, which predetermined the cutoff value for the percentage of stained cells in the tumor. Positive cases were considered to be those with a value greater than ten (10%) and the negative cases, with a value less than or equal to ten (10%). The site selection included the location of the area of highest marking (hot spot) and counting 200 cells in this area. To ensure the representativeness of each selected area in the donor block were collected at least two samples, and each of them represented in two different points of the same block receiver, forming mirror image in the samples. The three evaluators read the slides independently and did not have access to the patients' clinical data. The agreement between the results that they presented was greater than 90%, and the discordant cases were reevaluated by the pathologists jointly, reaching a decision by consensus.

The tissue immunohistochemical expressions of the tumor markers and the individual and group associations with the variables represented by the degree of cell differentiation, staging, recurrence and specific death, were analyzed using the chi-square test. For the tissue immunohistochemical expressions of the markers in the tumors and the individual and group associations with the disease-free interval and survival, the Kaplan-Meier survival table method was used. To test the significance of the differences between the curves of the disease-free interval and survival, the Logrank and Wilcoxon (Breslow version) tests were used. The limit of $P<0.05$ was used to reject the nullity hypothesis, and statistical values considered significant were highlighted.

RESULTS

The p53, bcl-2 and Ki-67 tumor expressions were analyzed in all of the 82 patients. The immunohistochemical expression of p53 was positive in 70 tumors (85.4%) and negative in 12 (14.6%). Bcl-2 was positive in 26 tumors (31.7%) and negative in 56 (68.3%). Ki-67 was positive in 62 tumors (75.6%) and negative in 20 (24.4%).

There was tumor recurrence in 18 patients. There was no statistically significant correlation between the immunohistochemical expressions and recurrence, with $P = 0.460$ for p53; $P = 0.479$ for bcl-2; and $P = 0.275$ for p53, bcl-2 and Ki-67 in association. The immunohistochemical expression of Ki-67 was positive in 10 recurrent tumors (55.6%) and negative in 8 (44.4%), with $P = 0.035$, which was a statistically significant result (Table 2).

TABLE 2. Distribution of the immunohistochemical expression of the p53, bcl-2 and Ki-67 proteins in relation to recurrence

	p53 n (%)	bcl-2 n (%)	Ki-67 n (%)	p53, bcl-2 and Ki-67 n (%)
Positive	16 (88.9)	5 (27.8)	10 (55.6)	3 (16.7)
Negative	2 (11.1)	13 (72.2)	8(44.4)	15 (83.3)
Total	18 (100.0)	18 (100.0)	18 (100.0)	18 (100.0)
Fisher's exact test	$P = 0.460$	$P = 0.479$	$P = 0.035$*	$P = 0.275$

In relation to mortality, among the 16 patients who died during the postoperative follow-up period, p53 was positive in 14 (87.5%) and negative in 2 (12.5%), with $P = 0.572$. bcl-2 was positive in 6 patients (37.5%) and negative in 10 (62.5%), with $P = 0.391$. Ki-67 was positive in 12 patients (75.0%) and negative in 4 (25.0%), with $P = 0.589$.

No significant correlation was found between the immunohistochemical expressions and the disease-free interval, with $P = 0.367$ for p53, $P = 0.746$ for bcl-2, $P = 0.108$ for Ki-67 and $P = 0.199$ for p53, bcl-2 and Ki-67 in association.

The survival analysis showed means of 54.0 months for p53 ($P = 0.966$); 45.9 months for bcl-2 ($P = 0.507$); 48.9 months for Ki-67 ($P = 0.723$) and 48.3 months for p53, bcl-2 and Ki-67 in association ($P = 0.728$). These results were not statistically significant (Figure 1 and Table 3).

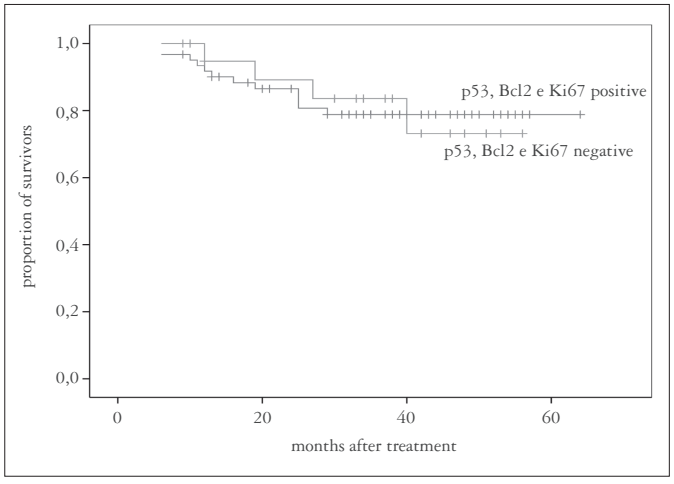

FIGURE 1. Curve of the immunohistochemical expression of p53, bcl-2 and Ki-67 in conjunction, in relation to survival

TABLE 3. Mean immunohistochemical expression of p53, bcl-2 and Ki-67 in conjunction, in relation to survival

bcl-2, p53 and Ki-67	Mean	95% confidence interval
Positive	48.3	41.6 – 55.0
Negative	54.0	49.9 – 59.0

$P = 0.728$ (Wilcoxon test – Breslow version)

Comparison of p53 immunohistochemical expression between tumors with low and high degrees of cell differentiation presented $P = 0.524$ (84.6% positive among the low-degree tumors and 100% positive among the high-degree tumors). Bcl-2 presented $P = 0.622$ (32.1% positive among the low-degree tumors and 67.9% positive among the high-degree tumors). Ki-67 presented $P = 0.319$ (74.4% among the low-degree tumors and 25.6% among the high-degree tumors). The association between the three markers presented $P = 0.702$ (25.6% among the low-degree tumors and 74.4% among the high-degree tumors). These results did not present significant correlations.

Regarding the staging, there was no statistical correlation between stages I, II, III and IV and the tumor markers, with $P = 0.120$ for p53; 0.542 for bcl-2, 0.523 for Ki-67 and 0.523 for p53, bcl-2 and Ki-67 in association.

DISCUSSION

The anatomopathological status of colorectal cancer based on the TNM system is the current basis for making prognoses and decisions on adjuvant treatment. It is accepted that the most important determining factor for curative resection is the absence of lymph nodes and distant metastases. Although individuals at an early stage of colorectal cancer are excluded from adjuvant chemotherapy because tumor resection is potentially curative, 20% to 30% of such cases present recurrence of the disease. This suggests that the anatomopathological staging in itself is incapable of guaranteeing the patient's prognosis[24]. Therefore, investigation of molecular markers is important for evaluating the prognosis, and for allocating individuals to appropriate treatment protocols. The identification of gene lesions that are responsible for carcinogenesis could create a new staging system based on molecular biology, which would increase the accuracy in predicting the prognosis for individuals with cancer. Likewise, identification of molecular markers that determine the behavior and aggressiveness of tumors is a necessary step towards improvement of cancer treatment[1]. However, few individual markers supplying prognostic information have been identified. Some have limited value because tumors frequently express multiple proto-oncogenes, suppressor genes and oncofetal antigens, each contributing towards tumor progression and metastasis. Thus, a better understanding of tumor behavior and its aggressiveness can be acquired by examining the immunohistochemical expression of multiple markers. Molecular markers may be the basis for decision-making of lower cost and greater precision, for prognoses and appropriate adjuvant therapies. Over recent years, the major focus within colorectal cancer research has been on searching for molecular markers that provide a prognosis and identify population types for specific treatment regimens[24].

The present study analyzed proteins that are directly involved in cell tumorigenesis, relating to cell apoptosis and proliferation. In the literature, the possibility of using these proteins as markers for colorectal cancer prognosis has been raised. In this study, the immunohistochemical expressions of p53, bcl-2 and Ki-67 was investigated separately and together in the colorectal tumors. The results were correlated with recurrence, mortality, disease-free interval, survival, degree of cell differentiation and staging.

There were no statistically significant findings from the distribution of the immunohistochemical expression of p53 and bcl-2 in relation to tumor recurrence, in the present study. However, contrary to these findings, Galizia et al.[8] showed that p53 was a strong molecular marker for recurrence. Among their 65 patients, they observed that the immunohistochemical expression of p53 presented high reactivity in all of them, with 92% tumor recurrence over 5 years. However, many studies have shown that, although p53 is the most frequently immunoexpressed molecular marker

in colorectal cancer, there is no correlation with recurrence of this tumor. On the other hand, bcl-2 has been correlated with recurrence by some authors, although other studies have shown the same result as in the present study[19, 20]. With regard to Ki-67, this study showed a statistically significant correlation between immunohistochemical expression of this protein and recurrence ($P = 0.035$). This result is in agreement with Ishida et al.[14], whose study showed that, even though the use of Ki-67 as a predictive factor for survival in cases of primary tumors is limited, its proliferative activity was significant in relation to the appearance of lymph node and hepatic metastases. In colorectal cancer, high levels of Ki-67 have been correlated with poor survival. Ki-67 staining is a simple and useful method for estimating proliferative activity. The importance of Ki-67 as an indicator of tumor behaviour is not clear. In colorectal cancer this index may be used as a marker of prognosis[21]. However, other studies have contested these findings through affirming that Ki-67 is unrelated to the prognosis for colorectal cancer[12]. When the three proteins were correlated jointly, there was no statistically significant difference in relation to tumor recurrence.

In this study, 16 patients died due to colorectal cancer. The specific mortality was not statistically significant when correlated with the immunohistochemical expressions separately and together. This was similar to the findings of Lustosa et al.[19], who also studied p53 and bcl-2 separately and together.

Regarding the disease-free interval, no statistically significant correlation was observed when these markers were correlated separately or together. This finding is in agreement with the results presented by Kim et al.[16], in a study on 542 patients. On the other hand, despite similar data for p53 and bcl-2, Garrity et al.[9] presented a different result for Ki-67 and concluded that the latter marker was strongly associated with longer disease-free intervals.

From analysis of the survival variable, there was no statistically significant correlation for these markers individually or together. Abnormalities of p53 are important in colorectal cancer cases, but the results have been conflicting. Such results may be due to many factors, including differences in antibody types, fixing methods, detection, marking and numbers of patients included[23]. In fact, many studies have shown inconsistency of results regarding p53, because some show a relationship between p53 and survival, while others do not. Since p53 is related to apoptosis, its super expression does not come as a surprise. This had already been described in a study that analyzed survival over 10 years of observation and showed that the prognosis was worse when the p53 expression was greater[22]. In relation to bcl-2, studies have also presented conflicting results relating to survival. The difficulty in interpreting the literature is because different staging systems or small groups in different stages have been used[30]. In colorectal cancer, bcl-2 has been found to be greater (76.9%) than in adenomas (59%), but bcl-2 expression in colorectal cancer and adenomas has not been correlated with some relevant clinical and pathological parameters, including dysplasia, differentiation and prognosis, in univariate and multivariate analyses. This suggests that bcl-2 plays a role in tumorigenesis during the early phases of the adenoma-carcinoma sequence[31]. Regarding Ki-67, studies have shown that its immunohistochemical expression is not correlated with survival[12]. However, in contrast, Garrity et al.[9] reported that this marker was significantly associated with increased survival.

When cell differentiation was correlated with p53, no statistical significance was found. This was also observed by Lima et al.[18]. Among the present study sample, the immunohistochemical expression of bcl-2 was not significantly correlated with the degree of cell differentiation, and this has also been shown in some other studies[2]. On the other hand, a study by Nehls et al.[20] showed a statistically significant correlation between bcl-2 and a high degree of cell differentiation. Despite this result, a clear difference was observed between the p53 and bcl-2 expressions, in comparison with tumor expression (85.4% and 31.7%) and degree of cell differentiation (84.6% and 32.1% when evaluating a low degree of differentiation). This shows that there is an inverse relationship with the marker expressions and suggests that the proteins can interact by means of complex opposing mechanisms. Studies have shown that bcl-2 expression in colorectal cancer is associated with a better clinical course, thus suggesting that neoplastic transformations relating to inhibition of apoptosis may result in less aggressive malignant tumors[23]. In relation to Ki-67, the present study did not show any statistically significant correlation with the degree of cell differentiation, which is in agreement with Ribeiro Jr. et al.[25]. However, Saleh et al.[27] showed that Ki-67 presented statistical significance in relation to poorly differentiated tumors.

No statistically significant correlation between the markers separately or together and the staging of the colorectal cancer was found in this study. This result is in agreement with the study by Kim et al.[16], which did not find significant correlation between p53 and staging, using the Dukes classification. However, the study by Allegra et al.[1] showed a significant association between p53 and colorectal cancer in Dukes stage C. In relation to bcl-2, and similarly to the present study, Bendardaf et al.[2] did not find any statistically significant correlation between the marker and the staging. Nevertheless, other studies have shown that this marker is associated with a better prognosis, especially among patients in Dukes stage B, thus showing an important decline in the immunohistochemical expression of bcl-2 during the progression of the tumor from adenoma to carcinoma. This emphasizes the ability of bcl-2 to inhibit cell apoptosis. Although the relationship between bcl-2 expression and the evolution of the normal colon epithelium to invasive cancer remains unknown, there is evidence to suggest that bcl-2 expression gradually diminishes during the evolution to colorectal cancer[30]. The immunohistochemical expression of Ki-67 did not present any statistically significant correlation with staging in the present study. This result is in agreement with the findings of Hashimoto et al.[12], who demonstrated that high immunohistochemical expression was correlated with staging. However, their analysis conflicts with that of Saleh et al.[27], whose study showed that this marker, despite presenting a clear tendency to increase with progression of the

disease, did not present a statistically significant correlation in relation to the Dukes classification. These results may be a reflection of the fact that Ki-67 also shows the status of cell proliferation and not exclusively the tumorigenic event.

In most cases, studies seeking biological markers for colorectal cancer prognosis have produced inconclusive results. This is partly due to non-homogeneous populations, tumors in different stages, follow-up, variations in statistical analyses, different sites for the symptoms and different treatment protocols, as well as primary factors regarding the immunohistochemical technique and the evaluation of the results. Although immunohistochemistry has been used in most studies, the results have been interpreted using a wide variety of protocol variables and scores, which makes it impossible to compare the results between studies. In fact, positive immunohistochemical expression of p53, for example, does not necessarily imply mutation. Likewise, absence of staining can occur in cases of gene deletion, transcription failure or unstable mutation[1]. In this respect, tissue microarray analysis, which allows simultaneous evaluation of multiple cases on a single slide, presents the advantage of being a method that eliminates variations that are found on each slide individually. This is an important factor for reducing methodological bias[17]. The present study used tissue microarrays, in line with the conclusions from several recent reviews of the literature, which have confirmed that the tissue microarray technique is the method of choice. It provides researchers with a powerful and relatively simple tool for investigating protein expression in tumor markers.

There is great variation in the criteria for dividing samples into groups categorized according to the intensity of the immunohistochemical reaction, among the main research groups[5]. In the present study, the classification chosen was "positive" or "negative" and two independent examiners were used, to ensure better accuracy. Marker positivity was evaluated using a scoring system in which the cutoff value is predetermined in terms of the percentage of stained cells due to the tumor. The immunohistochemical expression was considered negative when ≤10% of the cells were stained (neoplastic cells), and positive when >10% were stained. This interpretation model was validated by Zlobec et al.[32], who demonstrated a high degree of concordance between observers, and it has been used in other studies[10, 28]. This shows that this method can present greater reproducibility than the semi-quantitative method, in which a scale indicating the proportion of stained cells is analyzed, along with a score for the intensity and proportion of stained neoplastic cells.

In summary, no association was found between the markers and the factors, correlated individually or in conjunction, except for Ki-67, which showed a statistically significant result regarding tumor recurrence, between positive and negative immunohistochemical expression. However, the possibility that one tumor marker might contribute separately towards the prognostic evaluation of colorectal cancer is remote, considering the current knowledge available regarding the carcinogenesis of this tumor. The results from this study, compared with those in the literature, show with certainty that it is not possible to use tumor immunohistochemical expression in daily clinical practice among colorectal cancer patients. Tumor staging is still the main determining factor for survival among these patients.

Menezes HL, Jucá MJ, Gomes EGA, Nunes BLBBP, Costa HO, Matos D. Análise das expressões imunoistoquímicas da p53, bcl-2 e Ki-67 no adenocarcinoma colorretal e suas correlações com os fatores prognósticos. Arq Gastroenterol. 2010;47(2):141-7.

RESUMO – ***Contexto*** - Pesquisa de marcadores tumorais que permitam tratamento com maiores índices de sobrevida, além de indicarem a resposta ao tratamento e a recurrência da neoplasia. ***Objetivo*** - Analisar as expressões imunoistoquímicas das proteínas p53, bcl-2 e Ki-67 no adenocarcinoma colorretal, correlacionando-as com os fatores prognósticos clínico-patológicos. ***Método*** - Foram confeccionados blocos de parafina de TMA com tecido de adenocarcinoma colorretal ressecados cirurgicamente em 82 pacientes no Hospital São Paulo da Universidade Federal der São Paulo, São Paulo, SP, de 2002 a 2005, não submetidos a radio ou quimioterapia. Cortes de 4 μm foram submetidos a reação imunoistoquímica e obtidos escores de intensidade das imunoexpressões, que foram correlacionados com o grau de diferenciação celular, estádio, tempo livre de doença, recidiva, sobrevida e mortalidade específica. As variáveis do estudo foram analisadas pelos testes do qui ao quadrado e de Kaplan-Meier para verificar as associações com os marcadores. A significância das diferenças entre as curvas do tempo livre de doença e da sobrevida foi analisada pelos testes de Logrank e Wilcoxon. ***Resultados*** - A expressão imunoistoquímica da p53 foi positiva em 70 tumores (85,4%) e negativa em 12 (14,6%). A bcl-2 foi positiva em 26 tumores (31,7%) e negativa em 56 (68,3%). A expressão imunoistoquímica da Ki-67 foi positiva em 62 tumores (75,6%), sendo em 20 (24,4%) negativa. Não houve correlação estatisticamente significante entre as expressões imunoistoquímicas dos marcadores analisadas separadamente ou em conjunto, envolvendo o grau de diferenciação celular, estádio, tempo livre de doença, sobrevida e mortalidade específica. Com relação à recidiva, observou-se correlação estatisticamente significante com a expressão imunoistoquímica positiva da Ki-67 (P = 0,035). ***Conclusão*** - A expressão imunoistoquímica positiva da Ki-67 no câncer colorretal está relacionada com a incidência de recidiva da doença.

DESCRITORES - Neoplasias colorretais. Adenocarcinoma. Imunoistoquímica. Proteína proto-oncogênicas c-bcl-2. Proteína killer-antagonista homóloga a bcl-2.

REFERENCES

1. Allegra CJ, Paik S, Colangelo LH, Parr AL, Kirsch I, Kim G, Klein P, Johnston PG, Wolmark N, Wieand HS. Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol. 2003;21:241-50.
2. Bendardaf R, Ristamaki R, Kujari H, Laine J, Lamlum H, Collan Y, Pyrhonen S. Apoptotic index and bcl-2 expression as prognostic factors in colorectal carcinoma. Oncology. 2003;64:435-42.
3. Bishnupuri KS, Luo Q, Murmu N, Houchen CW, Anant S, Dieckgraefe BK. Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. Gastroenterology. 2006;130:137-49.
4. Compton C, Fenoglio-Preiser CM, Pettigrew N, Fielding LP. American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. Cancer. 2000;88:1739-57.
5. Costa HO. Imunoexpressão das proteínas da família bcl-2 em carcinomas colorretais e sua correlação com índice apoptótico, proliferação celular e sobrevida [tese]. São Paulo: Universidade Federal de São Paulo; 2008.
6. Fernandes LC, Matos D. Marcadores tumorais no câncer colorretal. Rev Col Bras Cir. 2002;29:106-11.
7. Forones NM, Oshima C, Nanogaki S, Tanaka M, Barbosa V. Determinação da atividade proliferativa pelo Ki-67 e p53 em doentes com câncer colorretal. Arq. gastroenterol. 1999;36:122-6.
8. Galizia G, Ferraraccio F, Lieto E, Orditura M, Castellano P, Imperatore V, Romano C, Vollaro M, Agostini B, Pignatelli C, De Vita F. Prognostic value of p27, p53, and vascular endothelial growth factor in Dukes A and B colon cancer patients undergoing potentially curative surgery. Dis Colon Rectum. 2004;47:1904-14.
9. Garrity MM, Burgart LJ, Mahoney MR, Windschitl HE, Salim M, Wiesenfeld M, Krook JE, Michalak JC, Goldberg RM, O'Connell MJ, Furth AF, Sargent DJ, Murphy LM, Hill E, Riehle DL, Meyers CH, Witzig TE. Prognostic value of proliferation, apoptosis, defective DNA mismatch repair, and p53 overexpression in patients with resected Dukes' B2 or C colon cancer: a North Central Cancer Treatment Group Study. J Clin Oncol. 2004;22:1572-82.
10. Gomes EGA. Análise das expressões imunohistoquímicas da metaloproteinase-1, metaloproteinase-7 e do fator de crescimento endotelial vascular no adenocarcinoma colorretal e suas correlações com os fatores prognósticos [tese]. São Paulo: Universidade Federal de São Paulo; 2008.
11. Grivicich I, Regner A, Rocha AB. Morte celular por apoptose. Rev Bras Cancerol. 2007;53:335-43.
12. Hashimoto Y, Skacel M, Lavery IC, Mukherjee AL, Casey G, Adams JC. Prognostic significance of fascin expression in advanced colorectal cancer: an immunohistochemical study of colorectal adenomas and adenocarcinomas. BMC Cancer. 2006;6:241.
13. Instituto Nacional do Câncer. Estimativa 2008 – incidência de câncer no Brasil. Rio de Janeiro; 2008 [citado 2008 aug 24]. Disponível em: http://www.inca.gov.br/estimativa/2008/topo.asp.
14. Ishidaa H, Miwab H, Tatsutaa M, Masutania S, Imamuraa H, Shimizua J, Ezumia K, Katoa H, Kawasakia T, Furukawaa H, Kawakami H. Ki-67 and CEA expression as prognostic markers in Dukes' C colorectal cancer. Cancer Lett. 2004;207:109-15.
15. Jucá, M, Nunes BLBBP, Menezes HL, Gomes EGA, Matos D. Metaloproteinases 1 e 7 e câncer colorretal. Rev Bras Coloproctol. 2008;28:353-62.
16. Kim GP, Colangelo LH, Wieand HS, Paik S, Kirsch IR, Wolmark N, Allegra CJ. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute–National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol. 2007;25:767-72.
17. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, Tam L, Munro AF, Dunne B, Bartlett JMS. Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology. 2006;48: 787-94.
18. Lima JM, Serafim PVP, Silva IDCG, Forones NM. Estudo do polimorfismo genético no gene p53 (códon 72) em câncer colorretal. Arq Gastroenterol. 2006;43: 8-13.
19. Lustosa SAS, Logullo A, Artigiani R, Saad SS, Goldenberg A, Matos D. Analysis of the correlation between p53 and bcl-2 expression with staging and prognosis of the colorectal adenocarcinoma. Acta Cir Bras. 2005;20:353-7.
20. Nehls O, Okech T, Hsieh CJ, Enzinger T, Sarbia M, Borchard F, Gruenagel HH, Gaco V, Hass HG, ArkenauHT, Hartmann JT, Porschen R, Grego M, Klump B. Studies on p53, BAX and Bcl-2 protein expression and microsatellite instability in stage III (UICC) colon cancer treated by adjuvant chemoterapy: major prognostic impact of proapoptotic BAX. Br J Cancer. 2007; 96:1409-18.
21. Oshima CT, Iriya K, Forones NM. Ki 67 as a prognostic marker in colorectal cancer but not in gastric cancer. Neoplasma. 2005;52:420-4.
22. Paluszkiewicz P, Berbec H, Wakowicz BP, Cybulski M, Paszkowska A. p53 protein accumulation in colorectal cancer tissue has prognostic value only in left-sided colon tumours. Cancer Detec Prev. 2004;28:252-9.
23. Pereira H, Silva S, Julião R, Garcia P, Perpétua F. Prognostic markers for colorectal cancer: expression of p53 and bcl-2. World J Surg. 1997;21:210-3.
24. Resnick MB, Routhier J, Konkin T, Sabo E, Pricolo VE. Epidermal growth factor receptor, c-MET, beta-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. Clin Cancer Res. 2004;10: 3069-75.
25. Ribeiro Jr U, Alves VA, Souza PMSB, Ribeiro AVS, Rawet V, Nonogaki S, Rodrigues JG, Habr-Gama A. Correlação das proteínas p53 e Ki67 com o prognóstico de pacientes com adenocarcinoma do reto distal. Rev Bras Coloproctol. 2000;20:248-56.
26. Rocha FTR, Lourenço LG, Jucá MJ, Leal AT, Paz AMDC. Expressão da proteína p53 no adenocarcinoma gástrico: correlação clínica, anatomopatológica e significância prognóstica. Rev Col Bras Cir. 2004;31:186-93.
27. Saleh HA, Jackson H, Banerjee M. Immunohistochemical expression of bcl-2 and p53 oncoproteins: correlation with Ki67 proliferation index and prognostic histopathologic parameters in colorectal neoplasia. Appl Immunohistochem Mol Morphol. 2000;8:175-82.
28. Schwandner O, Schlamp A, Broll R, Bruch HP. Clinicopathologic and prognostic significance of matrix metalloproteinases in rectal cancer. Int J Colorectal Dis. 2007;22:127-36.
29. Xi Y, Shalgi R, Fodstad O, Pilpel Y, Ju J. Differentially regulated micro-RNAs and actively translated messenger RNA transcripts by tumor suppressor p53 in colon cancer. Clin Cancer Res. 2006;12:2014-24.
30. Zavrides H, Zizi-Sermpetzoglou A, Elemenoglou I, Papatheofanis I, Peros G, Athanasas G, Panousopoulos D. Immunohistochemical expression of bcl-2 in UICC stage I and III colorectal carcinoma patients: correlation with c-erbB-2, p53, ki-67, CD44, laminin and collagen IV in evaluating prognostic significance. Pol J Pathol. 2006;57:149-59.
31. Zhao DP, Ding XW, Peng JP, Zheng YX, Zhang SZ. Prognostic significance of bcl-2 and p53 expression in colorectal carcinoma. J Zheijiang Univ Sci. 2005;6:1163-9.
32. Zlobec I, Steele R, Michel RP, Compton CC, Lugli A, Jass JR. Scoring of p53, VEGF, Bcl-2 and APAF-1 immunohistochemistry and interobserver reliability in colorectal cancer. Mod Pathol. 2006;19:1236-42.

Received 23/4/2009.
Accepted 27/8/2009.