

## Ki-67 and Bcl-2 Antigen Expression in Adenomatous Colorectal Polyps from Women with Breast Cancer

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### ABSTRACT

**Background.** The aim of this study was to evaluate Ki-67 and Bcl-2 antigen expression in colorectal polyps from women with breast cancer.

**Methods.** A randomized, controlled study was carried out in 35 women, either with or without breast cancer, who had adenomatous colorectal polyps. The patients were divided into two groups: group A (without breast cancer; control group;  $n = 17$ ) and group B (with breast cancer; study group;  $n = 18$ ). Immunohistochemistry was performed on the colorectal polyps to evaluate Ki-67 and Bcl-2 antigen expression. Student's  $t$ -test and the  $\chi^2$  test were used for the statistical analysis of Ki-67 and Bcl-2 expression, respectively. Statistical significance was established as  $P < 0.05$ .

**Results.** The mean percentage of Ki-67-stained nuclei in groups A and B was  $36.25 \pm 2.31$  and  $59.44 \pm 3.34$  ( $\pm$  SEM), respectively ( $P < 0.0001$ ), while the percentage of cases with cells expressing Bcl-2 in groups A and B was 23.5 and 77.8%, respectively ( $P < 0.001$ ).

**Conclusions.** In the present study, there was greater proliferative activity and greater expression of the antiapoptotic protein Bcl-2 in the colorectal polyps of women with breast cancer.

Breast cancer is the most common malignant disease in women in Western Europe and North America.<sup>1</sup> In recent decades, the survival of women with breast cancer has

increased considerably as a consequence of early diagnosis and the widespread use of adjuvant therapies.<sup>2,3</sup>

Despite controversy, studies have reported a potential increase in the risk of developing a second primary cancer in patients who survive breast cancer. Of these second primary tumors, with the exception of contralateral breast tumors, colorectal cancer is the most frequently found malignancy in this group of patients.<sup>4</sup> Population cohort studies suggest that the risk of colorectal cancer is also high in female relatives of women with breast cancer compared to the general population.<sup>5</sup>

Ochsenkühn et al. have also reported that women aged  $>65$  years with breast cancer have an increased risk of developing colorectal adenomas.<sup>6</sup> Adenomatous polyps are classically considered premalignant lesions and are part of the multistep process of colon carcinogenesis.<sup>7</sup> The sequence from adenoma to cancer in colorectal neoplasias has been well documented; however, various series of molecular mechanisms still remain to be clarified.<sup>8</sup> The imbalance between cell proliferation and programmed cell death in colon crypts of the adenomas is a key element in the development of colorectal cancer.<sup>7–9</sup> Expression of the Ki-67 protein and the antiapoptotic protein Bcl-2 have been the most frequently used biomolecular markers for the evaluation of cell proliferation and apoptosis, respectively.<sup>10</sup>

Only one study in the medical literature has assessed the relationship between breast cancer and colorectal cancer within the realm of cell kinetics, and these investigators reported greater proliferative activity in the rectal mucosa of women with breast cancer; nevertheless, this study failed to evaluate proliferative activity in lesions considered premalignant, such as adenomas.<sup>11</sup> Therefore, the present study was designed in view of the lack of studies evaluating proliferative and apoptotic activity, particularly Ki-67 and Bcl-2 protein activity, in colorectal polyps from women with breast cancer.

## PATIENTS AND METHODS

### Patients

This study was approved by the Internal Review Board of the Federal University of Piauí and all patients gave their signed informed consent before study initiation. Thirty-five women with or without breast cancer who had adenomatous colorectal polyps were included between October 2007 and December 2008. The patients who had breast cancer were receiving were recruited from the mastology clinic at Getúlio Vargas Hospital, Federal University of Piauí, whereas the patients without breast cancer were recruited from the proctology clinic of the General Surgery Department in the same institute and were volunteers for a colonoscopy. Those breast cancer patients who had a history of treatment for the disease were excluded from the study, while patients without breast cancer who had a history of any previous breast surgery or who were considered to have a high risk of developing breast cancer were also excluded. All the patients without breast cancer had been submitted to imaging tests (mammography and ultrasonography) with negative results for malignancy. None of the patients in the study had any history of colonoscopy or gastrointestinal surgery.

### Study Design

This is a cross-sectional, controlled study carried out in 35 women with and without breast cancer who had adenomatous colorectal polyps. The patients were divided into two groups: group A (no breast cancer, control group,  $n = 17$ ) and group B (breast cancer, study group,  $n = 18$ ). The body mass index (BMI) of groups A and B was 25 and 24, respectively ( $P = 0.318$ ). These groups were also considered homogenous with respect to age, age at menarche, parity, number of deliveries, and menopausal status; the only statistically significant difference between the two groups was the size of the colorectal polyps (Table 1).

### Colonoscopy

On the eve of videocolonoscopy, patients were given 40 mg of oral bisacodyl, fractioned into two doses, for colon cleansing. On the morning of the examination, the fasted patients ingested 500 mL of 20% Mannitol, and the examination was performed 4 hours later with an Olympus CF-100 colonoscope (Olympus Optical, Tokyo, Japan). In all cases, colonoscopy was performed under anesthetic sedation and by the same physician, who was blinded with respect to the identification of the cases. Sessile polyps <5 mm in size were removed with biopsy forceps, and those >5 mm in size or pediculated polyps were removed

**TABLE 1** Patient characteristics

Characteristic	Group A (control) ( $n = 17$ )	Group B (study) ( $n = 18$ )	<i>P</i> value
Age (y)			
Mean	53.10	54.70	0.453
Menarche age (y)			
Mean	13.92	13.76	0.381
Parity			
Yes	15 (88%)	15 (83%)	1.000
No	2 (12%)	3 (17%)	
Delivery number			
Mean	3.94	4.76	0.409
Menopausal status			
Yes	14 (82%)	14 (78%)	1.000
No	3 (18%)	4 (22%)	
Polyp size (cm)			
Mean	0.4	0.34	0.005

with a polypectomy loop. The surgical specimens were fixed in buffered formalin for 12 to 24 h and subsequently submitted to histopathological analysis using a standardized procedure to confirm diagnosis of an adenomatous polyp.

### Immunohistochemistry for Ki-67 and Bcl-2

For the immunohistochemical evaluation of Ki-67 and Bcl-2 protein expression, the polyp samples fixed in buffered formalin were cut into 3- $\mu$ m-thick sections. Next, the sections were deparaffinized in xylol for 5 min, dehydrated in absolute ethanol, and washed in buffered saline solution at pH 7.4 for 5 min. Subsequently, for blocking endogenous peroxide, the sections were treated for 5 min with 3% hydrogen peroxide ( $H_2O_2$ ) diluted in buffered solution. For antigen recovery, the slides were placed in racks containing 0.21% citric acid (pH 6.0) and heated in a microwave oven for 15 min at maximum power. Phosphate-buffered saline containing Tween (PBS-Tween) was added to the slides after they had been allowed to cool for 20 min. The tissue samples were incubated with primary mouse anti-Ki-67 monoclonal antibody (clone MIB1, Ref. M7240, Dako, Carpinteria, CA; 1:4800) and with mouse anti-Bcl-2 monoclonal antibody (clone 124, Ref. M0887, Dako; 1:2000) and incubated overnight at 4 to 8°C. The slides were then washed with PBS-Tween and instilled with secondary reagent (anti-mouse BA 2000, Vector Laboratories, Burlingame, CA), incubated for 60 min at room temperature, washed again in PBS-Tween, and instilled with the ABC Elite detection system (PK 6100, Vector Laboratories), incubated for 45 min at room temperature, washed once again with PBS-Tween, instilled with DAB

(3-3'-diaminobenzidine tetrahydrochloride, Ref. D5637, Sigma, St. Louis, MO), and incubated for 5 min. Finally, the slides were washed with distilled water, counterstained with hematoxylin, stained with ammoniacal solution, dehydrated with absolute ethanol, passed through Coplin jars containing xylol, and mounted in Permount resin. The cells that expressed the Ki-67 and Bcl-2 proteins were identified by the dark brown coloring of the nucleus and cytoplasm, respectively.

#### Quantitative Method

Quantification was carried out by two observers who were blinded with respect to the patients' identity and who had no previous knowledge of any of the cases. It was performed with a light microscope (Eclipse E-400, optical microscope, Nikon, Tokyo, Japan) connected to a color videocamera (digital camera CHC-370 N, Samsung, Seoul, Korea), which captured the image and transmitted it to a computer equipped with the Imagelab software program, version 2.3, developed by Softium Informática (São Paulo, Brazil) for image analysis.

For Ki-67 expression, a minimum of 500 cells were counted on each slide at a magnification of  $\times 400$ , irrespective of whether they had been stained by anti-Ki-67 or not. Only crypts that were visible along their entire length with the base of the crypt touching the muscularis mucosa were evaluated. In each case, 7 to 9 colon crypts (mean, 8) were evaluated. The percentage of stained cells for each case was obtained from the ratio between the number of cells with stained nuclei and unstained nuclei multiplied by 100 (label index).

Bcl-2 immunoreaction was evaluated semiquantitatively according to the criteria established by van Slooten et al. taking the following parameters into consideration: intensity of cell staining (I) and the fraction of stained neoplastic cells (F).<sup>12</sup> The intensity of cell staining was classified as follows: 0 (negative), 1 (weakly stained), 2 (moderately stained), or 3 (strongly stained). The fraction of stained cells was classified as follows: I (0 to 25%), II (25 to 75%), or III (75 to 100%). The final score was the result of the combination of the two parameters (I and F) and ranged from 0 to 6. Cases with a final score of  $\geq 3$  were classified as positive for Bcl-2. In all cases, brownish staining in the cytoplasm was adopted as the standard for positivity.<sup>12</sup>

#### Statistical Analysis

Student's *t*-test was used to verify homogeneity between the two groups with respect to body mass index, patient age, and age at menarche.<sup>13</sup> Fisher's exact test was used to evaluate parity and menopausal status between the two groups.<sup>14</sup> Mann-Whitney's nonparametric test was used to

evaluate the number of deliveries and the size of the polyps between the two groups.<sup>15</sup> Student's *t*-test was used to compare the means of the percentages of Ki-67-stained nuclei between the two groups. Comparison of the proportions of cases with cells expressing Bcl-2 in colorectal polyps in the two groups was performed by the  $\chi^2$  test.<sup>16</sup> Significance was established at  $P < 0.05$ .

## RESULTS

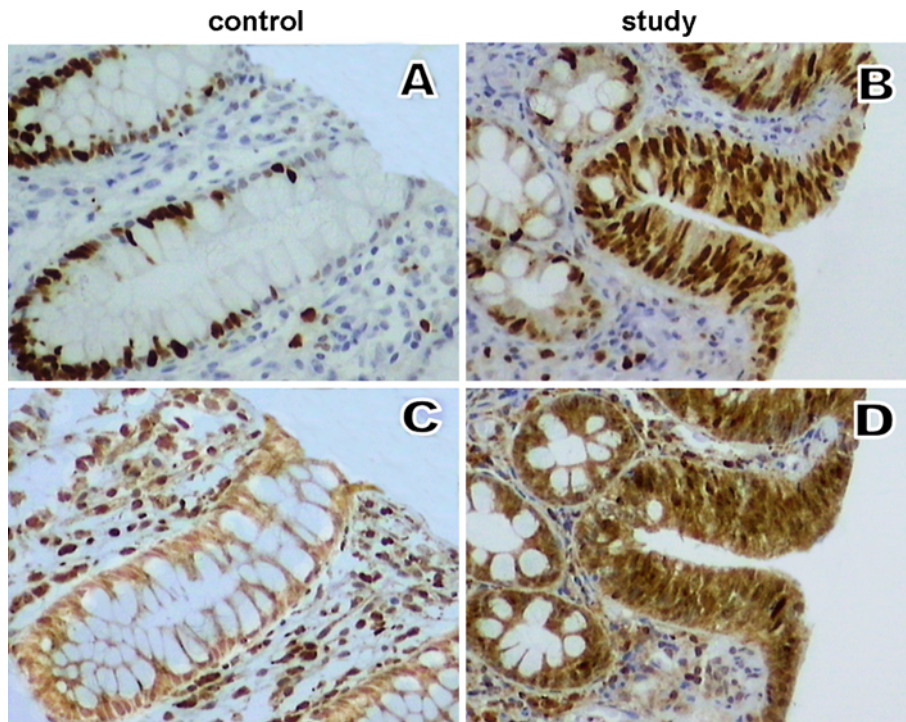
Only one colorectal polyp was found in each patient of both groups. Under light microscopy, the most common type of adenomatous polyp was tubular (77%), followed by tubulovillous in both groups. Villous adenoma was not found. Mild atypia was found in 50% of cases in each group. A greater concentration of Ki-67-stained nuclei was found in the cells of the colorectal polyps of patients in group B (the study group) compared to those of group A (the control group). The percentage of cells intensely stained for Bcl-2 was higher in the cells of the colorectal polyps from patients in group B compared to group A (Fig. 1). The mean percentage of Ki-67-stained nuclei was  $36.25 \pm 2.31$  and  $59.44 \pm 3.34$  ( $\pm$  SEM) in the colon crypts of the patients in groups A and B, respectively ( $P < 0.0001$ ) (Table 2, Fig. 2). With respect to Bcl-2 antigen expression, the number of cases with positive Bcl-2 expression in the cells of the colorectal polyps was 4 (23.5%) of 17 and 14 (77.8%) of 18 in groups A and B, respectively ( $P < 0.001$ ) (Table 3).

## DISCUSSION

Women with breast cancer have a higher risk for colorectal cancer, and adenomatous polyps constitute part of colon carcinogenesis; nonetheless, there is a lack of studies evaluating the cell kinetics of adenomatous colorectal polyps in women with breast cancer. In studies carried out with barium enema, before the advent of colonoscopy, some authors have shown that large polyps ( $>1$  cm) left in situ after diagnosis have a 5-, 10-, and 20-year cumulative risk of developing malignancy of 2.5, 8, and 24%, respectively.<sup>17</sup> It is probable that women with breast cancer and adenomatous colorectal polyps have an increased risk of developing malignancy in these lesions compared to controls, and this probably involves a change in the expression of proteins related to cell proliferation and apoptosis.

In the present study, the adenomatous colorectal polyps of patients with breast cancer were found to have greatly increased cell proliferative activity as evaluated by Ki-67 protein expression and a markedly increased expression of the antiapoptotic protein Bcl-2 compared to adenomatous

**FIG. 1** Photomicrographs of histological sections of adenomatous colorectal polyps. Note the lower concentration of Ki-67-stained nuclei in the polyp cells of a patient from the control group (a) compared to a patient with breast cancer (b). Observe a negative immunohistochemical reaction for the Bcl-2 protein in the polyp cells of a patient from the control group (c), and a positive immunohistochemical reaction, as expressed by numerous cells with cytoplasm intensely stained of a patient from the study group (d) (original magnification, ×200)

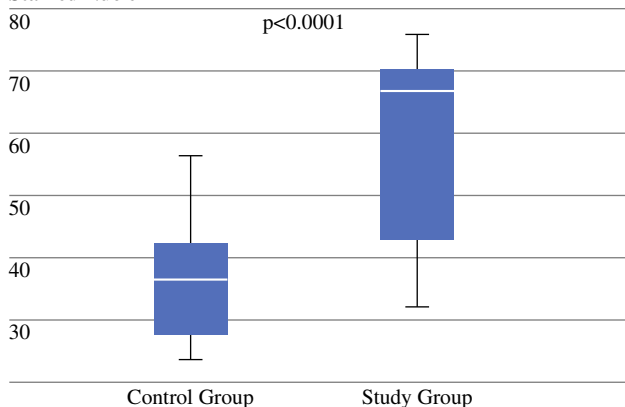


**TABLE 2** Mean percentage of Ki-67-stained nuclei in colorectal polyps

Group	n	Mean	SE mean	SD mean	Minimum	Median	Maximum
A (control)	17	36.25	2.31	9.53	23.38	36.42	56.38
B (study)	18	59.44*	3.34	14.18	31.97	66.56	75.90

\* The difference between the two groups was statistically significant ( $P < 0.0001$ )

**Percentage of Ki-67-Stained Nuclei**



**FIG. 2** Box plot of the percentage of cells with Ki-67-stained nuclei in the colorectal polyps of patients in the control and study groups

polyps from women in the control group. The control and study groups were homogenous with respect to age, age at menarche, parity, number of deliveries, menopausal status, and BMI; however, they differed with respect to the size of

**TABLE 3** Percentage of cases with cells expressing Bcl-2 in colorectal polyps<sup>a</sup>

Group	Negative, n (%)	Positive, n (%)	Total, n (%)
A (control)	13 (76.5)	4 (23.5)	17 (100.0)
B (study)	4 (22.2)	14 (77.8)*	18 (100.0)
Total	17 (48.6)	18 (51.4)	35 (100.0)

\* The percentage of cases with positive cells for Bcl-2 in group B was statistically significantly higher compared to group A ( $P < 0.001$ )

the polypoid lesion. Although some authors have shown that overweight and obesity, especially among men, are associated with increased risk of colon cancer, in the present study, both groups of women had normal BMI.<sup>18</sup> The size of polyps has been shown in some studies to represent an important risk factor for the development of dysplasia and cancer.<sup>19</sup> Nevertheless, some authors have failed to find any correlation between growth fraction and the size of the adenomas.<sup>20</sup> In this study, the polyps from the women with breast cancer were smaller; however, proliferative activity was greater, probably suggesting a more aggressive biological behavior.

Both breast and colorectal cancers are likely to involve interaction between genetic and environmental factors. DNA-based screening is available for hereditary breast ovarian cancer, hereditary nonpolyposis colonic cancer (HNPCC or Lynch syndrome), and Gardner syndrome (polyposis coli).<sup>21</sup> Lynch syndrome is characterized by the development of colorectal, endometrial, and other cancers; however, the inclusion of breast in this syndrome is controversial.<sup>21,22</sup> Likewise, *BRCA1* germ-line mutation is responsible for a large number of predispositions to breast and ovarian cancer. It has also been shown that *BRCA1* mutation carriers are at increased risk of colon cancer.<sup>23</sup>

The degree of atypia in adenomatous colorectal polyps may be classified as low, moderate, or intense.<sup>24</sup> In the present study, the degree of atypia in the adenomas was not taken into consideration in the evaluation of kinetic changes of the cells. This decision was taken on the basis of the findings of Vernillo et al., who examined 78 colon polyps in women without breast cancer and reported that 29 (56.9%) had low-grade dysplasia, while 22 (43.1%) had high-grade dysplasia.<sup>25</sup> Nevertheless, these authors found no difference in Ki-67 expression between these two degrees of atypia.

Some investigators have divided the colon crypt into two or more parts to evaluate immunoreactivity because it is in the lower third that proliferative activity is greatest and the germinative cells are found.<sup>26</sup> Nevertheless, in adenomas, the patterns of immunoreactivity may be divided into two principal types, the first characterized by high proliferative activity in the lower third and the second characterized by the highest labeling indexes in the upper third, which may correlate with the phase II lesion described by Lipkin in which a net accumulation of proliferating cells occurs at the luminal surface.<sup>27,28</sup> This concept is supported by studies on the sequence from adenoma to carcinoma that show a shift in the site of the proliferating cell population toward the surface. Therefore, taking these characteristics into consideration, evaluation of Ki-67 and Bcl-2 protein expression was performed along the entire length of the colon crypt in the present study.

These markers were selected for use in the present study in view of the fact that the Ki-67 protein is considered a sensitive marker of cell proliferation because it is expressed in all phases of the cell cycle except in the resting phase (G<sub>0</sub>), and its increased expression has been associated with more aggressive neoplasias.<sup>29-31</sup> Likewise, Bcl-2 is part of a family of proteins related to apoptosis and codified as proto-oncogene Bcl-2, considered antiapoptotic, the tumorigenic potential of which has been demonstrated in animal models and in some human tumors such as lymphomas.<sup>32-34</sup>

To our knowledge, this is the first study to evaluate Ki-67 and Bcl-2 expression in adenomatous colorectal polyps

of women with breast cancer. In conclusion, the present findings show not only an increase in Ki-67 and Bcl-2 protein expression in colorectal polyps of women with breast cancer, but also the importance of the use of routine colonoscopy in these patients to screen for and remove these lesions.

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